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(54) Titre : DELIVRANCE, FABRICATION ET OPTIMISATION DE SYSTEMES, DE PROCEDES ET DE COMPOSITIONS POUR LA MANIPULATION DE SEQUENCES ET APPLICATIONS THERAPEUTIQUES

(54) Title: DELIVERY, ENGINEERING AND OPTIMIZATION OF SYSTEMS, METHODS AND COMPOSITIONS FOR SEQUENCE MANIPULATION AND THERAPEUTIC APPLICATIONS

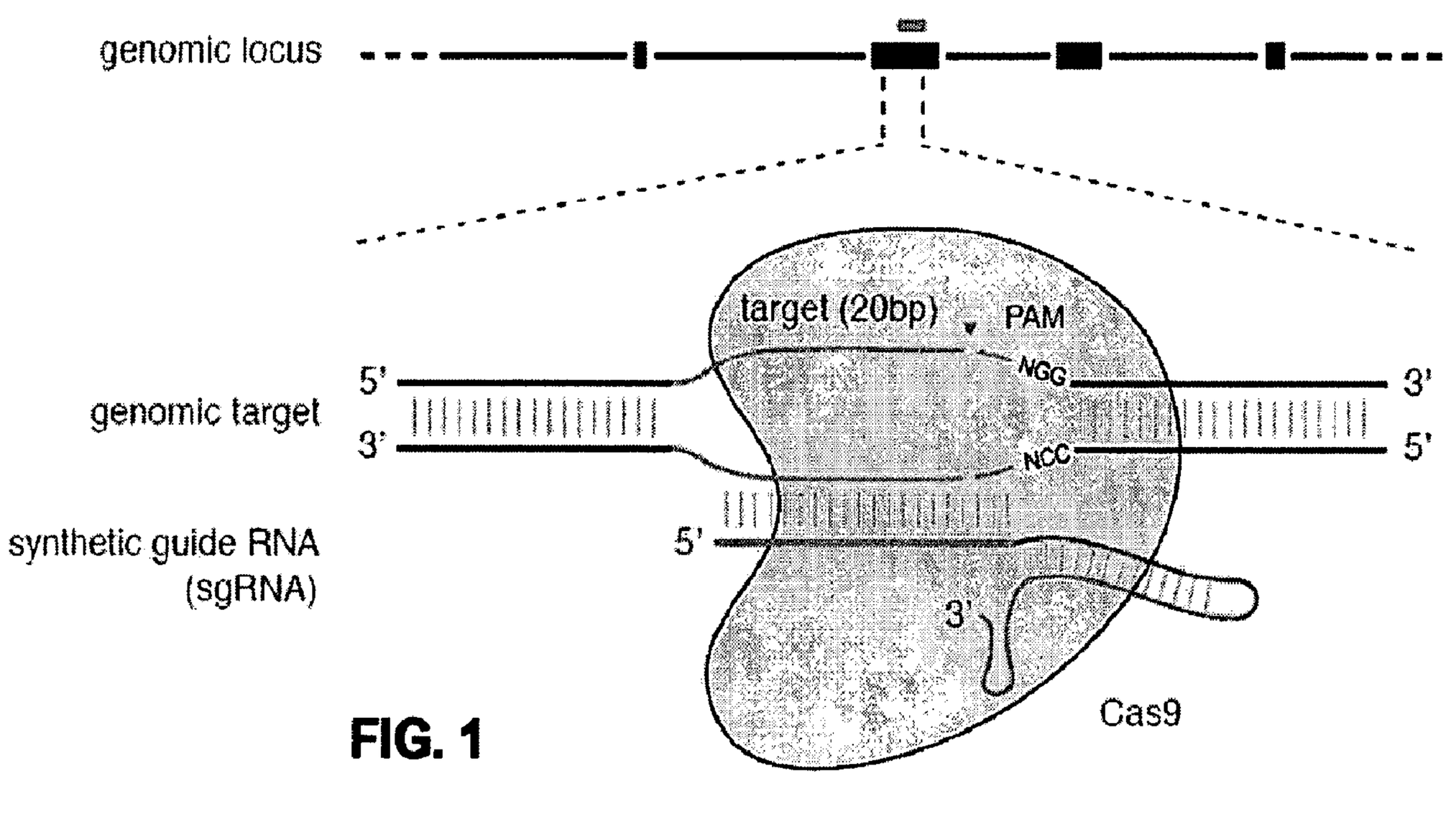

FIG. 1

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

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(54) Title: DELIVERY, ENGINEERING AND OPTIMIZATION OF SYSTEMS, METHODS AND COMPOSITIONS FOR SEQUENCE MANIPULATION AND THERAPEUTIC APPLICATIONS

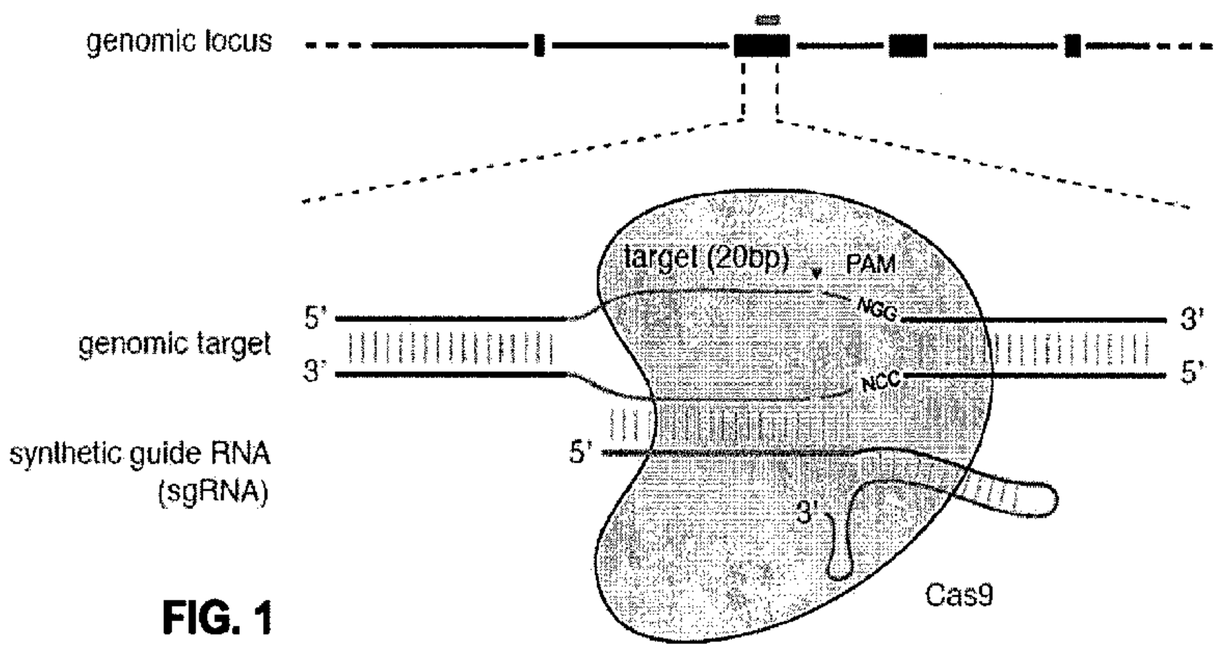

FIG. 1

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

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CECI EST LE TOME 1 DE 2
CONTENANT LES PAGES 1 À 179

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THIS SECTION OF THE APPLICATION/PATENT CONTAINS MORE THAN ONE VOLUME

THIS IS VOLUME 1 OF 2
CONTAINING PAGES 1 TO 179

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DELIVERY, ENGINEERING AND OPTIMIZATION OF SYSTEMS, METHODS AND COMPOSITIONS FOR SEQUENCE MANIPULATION AND THERAPEUTIC APPLICATIONS

RELATED APPLICATIONS AND INCORPORATION BY REFERENCE

[0001] This application claims benefit of and priority to US provisional patent application numbers 61/736,527 filed December 12, 2012; 61/748,427 filed January 2, 2013; 61/758,468 filed January 30, 2013, 61/769,046 filed February 25, 2013; 61/791,409 and 61/802,174 filed March 15, 2013, 61/806,375 filed March 28, 2013; 61/814,263 filed April 20, 2013; 61/819,803 filed May 6, 2013; 61/828,130 filed May 28, 2013; 61/835,931 and 61/836,123 filed June 17, 2013 and 61/847,537 filed July 17, 2013.

[0002] Reference is also made to US provisional patent application numbers 61/799,800 filed March 15, 2013; 61/835,931, 61/835,936, 61/836,127, 61/836,101, 61/836,080 and 61/835,973 filed June 17, 2013; 61/862,468 and 61/862,355 filed on August 5, 2013; 61/871,301 filed on August 28, 2013; 61/960,777 filed on September 25, 2013 and 61/961,980 filed on October 28, 2013.

[0003] The foregoing applications, and all documents cited therein or during their prosecution ("appln cited documents") and all documents cited or referenced in the appln cited documents, and all documents cited or referenced herein ("herein cited documents"), and all documents cited or referenced in herein cited documents, together with any manufacturer's instructions, descriptions, product specifications, and product sheets for any products mentioned herein or in any document incorporated by reference herein, are hereby incorporated herein by reference, and may be employed in the practice of the invention. More specifically, all referenced documents are incorporated by reference to the same extent as if each individual document was specifically and individually indicated to be incorporated by reference.

FIELD OF THE INVENTION

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

STATEMENT AS TO FEDERALLY SPONSORED RESEARCH

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

BACKGROUND OF THE INVENTION

[0006] Recent advances in genome sequencing techniques and analysis methods have significantly accelerated the ability to catalog and map genetic factors associated with a diverse range of biological functions and diseases. Precise genome targeting technologies are needed to enable systematic reverse engineering of causal genetic variations by allowing selective perturbation of individual genetic elements, as well as to advance synthetic biology, biotechnological, and medical applications. Although genome-editing techniques such as designer zinc fingers, transcription activator-like effectors (TALEs), or homing meganucleases are available for producing targeted genome perturbations, there remains a need for new genome engineering technologies that are affordable, easy to set up, scalable, and amenable to targeting multiple positions within the eukaryotic genome.

SUMMARY OF THE INVENTION

[0007] The CRISPR-Cas system does not require the generation of customized proteins to target specific sequences but rather a single Cas enzyme can be programmed by a short RNA molecule to recognize a specific DNA target. Adding the CRISPR-Cas system to the repertoire of genome sequencing techniques and analysis methods may significantly simplify the methodology and accelerate the ability to catalog and map genetic factors associated with a diverse range of biological functions and diseases. To utilize the CRISPR-Cas system effectively for genome editing without deleterious effects, it is critical to understand aspects of engineering, optimization and cell-type/tissue/organ specific delivery of these genome engineering tools, which are aspects of the claimed invention.

[0008] There exists a pressing need for alternative and robust systems and techniques for nucleic sequence targeting with a wide array of applications. Aspects of this invention address this need and provide related advantages. An exemplary CRISPR complex comprises a CRISPR enzyme complexed with a guide sequence hybridized to a target sequence within the target

polynucleotide. The guide sequence is linked to a tracr mate sequence, which in turn hybridizes to a tracr sequence.

[0009] In one aspect, the invention provides methods for using one or more elements of a CRISPR-Cas system. The CRISPR complex of the invention provides an effective means for modifying a target polynucleotide. The CRISPR complex of the invention has a wide variety of utilities including modifying (e.g., deleting, inserting, translocating, inactivating, activating) a target polynucleotide in a multiplicity of cell types in various tissues and organs. As such the CRISPR complex of the invention has a broad spectrum of applications in, e.g., gene or genome editing, gene therapy, drug discovery, drug screening, disease diagnosis, and prognosis.

[0010] Aspects of the invention relate to Cas9 enzymes having improved targeting specificity in a CRISPR-Cas9 system having guide RNAs having optimal activity, smaller in length than wild-type Cas9 enzymes and nucleic acid molecules coding therefor, and chimeric Cas9 enzymes, as well as methods of improving the target specificity of a Cas9 enzyme or of designing a CRISPR-Cas9 system comprising designing or preparing guide RNAs having optimal activity and/or selecting or preparing a Cas9 enzyme having a smaller size or length than wild-type Cas9 whereby packaging a nucleic acid coding therefor into a delivery vector is more advanced as there is less coding therefor in the delivery vector than for wild-type Cas9, and/or generating chimeric Cas9 enzymes.

[0011] Also provided are uses of the present sequences, vectors, enzymes or systems, in medicine. Also provided are uses of the same in gene or genome editing.

[0012] In an additional aspect of the invention, a Cas9 enzyme may comprise one or more mutations and may be used as a generic DNA binding protein with or without fusion to a functional domain. The mutations may be artificially introduced mutations or gain- or loss-of-function mutations. The mutations may include but are not limited to mutations in one of the catalytic domains (D10 and H840) in the RuvC and HNH catalytic domains, respectively. Further mutations have been characterized. In one aspect of the invention, the transcriptional activation domain may be VP64. In other aspects of the invention, the transcriptional repressor domain may be KRAB or SID4X. Other aspects of the invention relate to the mutated Cas 9 enzyme being fused to domains which include but are not limited to a transcriptional activator, repressor, a recombinase, a transposase, a histone remodeler, a demethylase, a DNA

methyltransferase, a cryptochrome, a light inducible/controllable domain or a chemically inducible/controllable domain.

[0013] In a further embodiment, the invention provides for methods to generate mutant tracrRNA and direct repeat sequences or mutant chimeric guide sequences that allow for enhancing performance of these RNAs in cells. Aspects of the invention also provide for selection of said sequences.

[0014] Aspects of the invention also provide for methods of simplifying the cloning and delivery of components of the CRISPR complex. In the preferred embodiment of the invention, a suitable promoter, such as the U6 promoter, is amplified with a DNA oligo and added onto the guide RNA. The resulting PCR product can then be transfected into cells to drive expression of the guide RNA. Aspects of the invention also relate to the guide RNA being transcribed *in vitro* or ordered from a synthesis company and directly transfected.

[0015] In one aspect, the invention provides for methods to improve activity by using a more active polymerase. In a preferred embodiment, the expression of guide RNAs under the control of the T7 promoter is driven by the expression of the T7 polymerase in the cell. In an advantageous embodiment, the cell is a eukaryotic cell. In a preferred embodiment the eukaryotic cell is a human cell. In a more preferred embodiment the human cell is a patient specific cell.

[0016] In one aspect, the invention provides for methods of reducing the toxicity of Cas enzymes. In certain aspects, the Cas enzyme is any Cas9 as described herein, for instance any naturally-occurring bacterial Cas9 as well as any chimaeras, mutants, homologs or orthologs. In a preferred embodiment, the Cas9 is delivered into the cell in the form of mRNA. This allows for the transient expression of the enzyme thereby reducing toxicity. In another preferred embodiment, the invention also provides for methods of expressing Cas9 under the control of an inducible promoter, and the constructs used therein.

[0017] In another aspect, the invention provides for methods of improving the *in vivo* applications of the CRISPR-Cas system. In the preferred embodiment, the Cas enzyme is wildtype Cas9 or any of the modified versions described herein, including any naturally-occurring bacterial Cas9 as well as any chimaeras, mutants, homologs or orthologs. An advantageous aspect of the invention provides for the selection of Cas9 homologs that are easily packaged into viral vectors for delivery. Cas9 orthologs typically share the general organization

of 3-4 RuvC domains and a HNH domain. The 5' most RuvC domain cleaves the non-complementary strand, and the HNH domain cleaves the complementary strand. All notations are in reference to the guide sequence.

[0018] The catalytic residue in the 5' RuvC domain is identified through homology comparison of the Cas9 of interest with other Cas9 orthologs (from S. pyogenes type II CRISPR locus, S. thermophilus CRISPR locus 1, S. thermophilus CRISPR locus 3, and Franciscilla novicida type II CRISPR locus), and the conserved Asp residue (D10) is mutated to alanine to convert Cas9 into a complementary-strand nicking enzyme. Similarly, the conserved His and Asn residues in the HNH domains are mutated to Alanine to convert Cas9 into a non-complementary-strand nicking enzyme. In some embodiments, both sets of mutations may be made, to convert Cas9 into a non-cutting enzyme.

[0019] In some embodiments, the CRISPR enzyme is a type I or III CRISPR enzyme, preferably a type II CRISPR enzyme. This type II CRISPR enzyme may be any Cas enzyme. A preferred Cas enzyme may be identified as Cas9 as this can refer to the general class of enzymes that share homology to the biggest nuclease with multiple nuclease domains from the type II CRISPR system. Most preferably, the Cas9 enzyme is from, or is derived from, spCas9 or saCas9. By derived, Applicants mean that the derived enzyme is largely based, in the sense of having a high degree of sequence homology with, a wildtype enzyme, but that it has been mutated (modified) in some way as described herein

[0020] It will be appreciated that the terms Cas and CRISPR enzyme are generally used herein interchangeably, unless otherwise apparent. As mentioned above, many of the residue numberings used herein refer to the Cas9 enzyme from the type II CRISPR locus in *Streptococcus pyogenes*. However, it will be appreciated that this invention includes many more Cas9s from other species of microbes, such as SpCas9, SaCas9, St1Cas9 and so forth. Further examples are provided herein. The skilled person will be able to determine appropriate corresponding residues in Cas9 enzymes other than SpCas9 by comparison of the relevant amino acid sequences. Thus, where a specific amino acid replacement is referred to using the SpCas9 numbering, then, unless the context makes it apparent this is not intended to refer to other Cas9 enzymes, the disclosure is intended to encompass corresponding modifications in other Cas9 enzymes.

[0021] An example of a codon optimized sequence, in this instance optimized for humans (i.e. being optimized for expression in humans) is provided herein, see the SaCas9 human codon optimized sequence. Whilst this is preferred, it will be appreciated that other examples are possible and codon optimization for a host species is known.

[0022] In further embodiments, the invention provides for methods of enhancing the function of Cas9 by generating chimeric Cas9 proteins. Chimeric Cas9 proteins chimeric Cas9s may be new Cas9 containing fragments from more than one naturally occurring Cas9. These methods may comprise fusing N-terminal fragments of one Cas9 homolog with C-terminal fragments of another Cas9 homolog. These methods also allow for the selection of new properties displayed by the chimeric Cas9 proteins.

[0023] It will be appreciated that in the present methods, where the organism is an animal or a plant, the modification may occur *ex vivo* or *in vitro*, for instance in a cell culture and in some instances not *in vivo*. In other embodiments, it may occur *in vivo*.

[0024] In one aspect, the invention provides a method of modifying an organism or a non-human organism by manipulation of a target sequence in a genomic locus of interest comprising: delivering a non-naturally occurring or engineered composition comprising :

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

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

(b) a tracr mate sequence, and

(c) a tracr sequence, and

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

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

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

or

(B) I. polynucleotides comprising:

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

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

II. a polynucleotide sequence encoding a CRISPR enzyme, and

III. a polynucleotide sequence comprising a tracr sequence,

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

[0025] Any or all of the polynucleotide sequence encoding a CRISPR enzyme, guide sequence, tracr mate sequence or tracr sequence, may be RNA. The polynucleotides encoding the sequence encoding a CRISPR enzyme, the guide sequence, tracr mate sequence or tracr sequence may be RNA and may be delivered via liposomes, nanoparticles, exosomes, microvesicles, or a gene-gun.

[0026] It will be appreciated that where reference is made to a polynucleotide, which is RNA and is said to 'comprise' a feature such a tracr mate sequence, the RNA sequence includes the feature. Where the polynucleotide is DNA and is said to comprise a feature such a tracr mate sequence, the DNA sequence is or can be transcribed into the RNA including the feature at issue. Where the feature is a protein, such as the CRISPR enzyme, the DNA or RNA sequence referred to is, or can be, translated (and in the case of DNA transcribed first).

[0027] Accordingly, in certain embodiments the invention provides a method of modifying an organism, e.g., mammal including human or a non-human mammal or organism by manipulation of a target sequence in a genomic locus of interest comprising delivering a non-naturally occurring or engineered composition comprising a viral or plasmid vector system comprising one or more viral or plasmid vectors operably encoding a composition for expression thereof, wherein the composition comprises: (A) a non-naturally occurring or engineered composition comprising a vector system comprising one or more vectors comprising I. a first regulatory element operably linked to a CRISPR-Cas system chimeric RNA (chiRNA) polynucleotide sequence, wherein the polynucleotide sequence comprises (a) a guide sequence

capable of hybridizing to a target sequence in a eukaryotic cell, (b) a tracr mate sequence, and (c) a tracr sequence, and II. a second regulatory element operably linked to an enzyme-coding sequence encoding a CRISPR enzyme comprising at least one or more nuclear localization sequences (or optionally at least one or more nuclear localization sequences as some embodiments can involve no NLS), wherein (a), (b) and (c) are arranged in a 5' to 3' orientation, wherein components I and II are located on the same or different vectors of the system, wherein when transcribed, the tracr mate sequence hybridizes to the tracr sequence and the guide sequence directs sequence-specific binding of a CRISPR complex to the target sequence, and wherein the CRISPR complex comprises the CRISPR enzyme complexed with (1) the guide sequence that is hybridized to the target sequence, and (2) the tracr mate sequence that is hybridized to the tracr sequence, or (B) a non-naturally occurring or engineered composition comprising a vector system comprising one or more vectors comprising I. a first regulatory element operably linked to (a) a guide sequence capable of hybridizing to a target sequence in a eukaryotic cell, and (b) at least one or more tracr mate sequences, II. a second regulatory element operably linked to an enzyme-coding sequence encoding a CRISPR enzyme, and III. a third regulatory element operably linked to a tracr sequence, wherein components I, II and III are located on the same or different vectors of the system, wherein when transcribed, the tracr mate sequence hybridizes to the tracr sequence and the guide sequence directs sequence-specific binding of a CRISPR complex to the target sequence, and wherein the CRISPR complex comprises the CRISPR enzyme complexed with (1) the guide sequence that is hybridized to the target sequence, and (2) the tracr mate sequence that is hybridized to the tracr sequence. In some embodiments, components I, II and III are located on the same vector. In other embodiments, components I and II are located on the same vector, while component III is located on another vector. In other embodiments, components I and III are located on the same vector, while component II is located on another vector. In other embodiments, components II and III are located on the same vector, while component I is located on another vector. In other embodiments, each of components I, II and III is located on different vectors. The invention also provides a viral or plasmid vector system as described herein.

[0028] Preferably, the vector is a viral vector, such as a lenti- or baculo- or preferably adeno-viral/adeno-associated viral vectors, but other means of delivery are known (such as yeast systems, microvesicles, gene guns/means of attaching vectors to gold nanoparticles) and are

provided. In some embodiments, one or more of the viral or plasmid vectors may be delivered via liposomes, nanoparticles, exosomes, microvesicles, or a gene-gun.

[0029] By manipulation of a target sequence, Applicants also mean the epigenetic manipulation of a target sequence. This may be of the chromatin state of a target sequence, such as by modification of the methylation state of the target sequence (i.e. addition or removal of methylation or methylation patterns or CpG islands), histone modification, increasing or reducing accessibility to the target sequence, or by promoting 3D folding.

[0030] It will be appreciated that where reference is made to a method of modifying an organism or mammal including human or a non-human mammal or organism by manipulation of a target sequence in a genomic locus of interest, this may apply to the organism (or mammal) as a whole or just a single cell or population of cells from that organism (if the organism is multicellular). In the case of humans, for instance, Applicants envisage, *inter alia*, a single cell or a population of cells and these may preferably be modified *ex vivo* and then re-introduced. In this case, a biopsy or other tissue or biological fluid sample may be necessary. Stem cells are also particularly preferred in this regard. But, of course, *in vivo* embodiments are also envisaged.

[0031] In certain embodiments the invention provides a method of treating or inhibiting a condition caused by a defect in a target sequence in a genomic locus of interest in a subject (e.g., mammal or human) or a non-human subject (e.g., mammal) in need thereof comprising modifying the subject or a non-human subject by manipulation of the target sequence and wherein the condition is susceptible to treatment or inhibition by manipulation of the target sequence comprising providing treatment comprising: delivering a non-naturally occurring or engineered composition comprising an AAV or lentivirus vector system comprising one or more AAV or lentivirus vectors operably encoding a composition for expression thereof, wherein the target sequence is manipulated by the composition when expressed, wherein the composition comprises: (A) a non-naturally occurring or engineered composition comprising a vector system comprising one or more vectors comprising I. a first regulatory element operably linked to a CRISPR-Cas system chimeric RNA (chiRNA) polynucleotide sequence, wherein the polynucleotide sequence comprises (a) a guide sequence capable of hybridizing to a target sequence in a eukaryotic cell, (b) a tracr mate sequence, and (c) a tracr sequence, and II. a second regulatory element operably linked to an enzyme-coding sequence encoding a CRISPR enzyme comprising at least one or more nuclear localization sequences (or optionally at least one

or more nuclear localization sequences as some embodiments can involve no NLS) wherein (a), (b) and (c) are arranged in a 5' to 3' orientation, wherein components I and II are located on the same or different vectors of the system, wherein when transcribed, the tracr mate sequence hybridizes to the tracr sequence and the guide sequence directs sequence-specific binding of a CRISPR complex to the target sequence, and wherein the CRISPR complex comprises the CRISPR enzyme complexed with (1) the guide sequence that is hybridized to the target sequence, and (2) the tracr mate sequence that is hybridized to the tracr sequence, or (B) a non-naturally occurring or engineered composition comprising a vector system comprising one or more vectors comprising I. a first regulatory element operably linked to (a) a guide sequence capable of hybridizing to a target sequence in a eukaryotic cell, and (b) at least one or more tracr mate sequences, II. a second regulatory element operably linked to an enzyme-coding sequence encoding a CRISPR enzyme, and III. a third regulatory element operably linked to a tracr sequence, wherein components I, II and III are located on the same or different vectors of the system, wherein when transcribed, the tracr mate sequence hybridizes to the tracr sequence and the guide sequence directs sequence-specific binding of a CRISPR complex to the target sequence, and wherein the CRISPR complex comprises the CRISPR enzyme complexed with (1) the guide sequence that is hybridized to the target sequence, and (2) the tracr mate sequence that is hybridized to the tracr sequence. In some embodiments, components I, II and III are located on the same vector. In other embodiments, components I and II are located on the same vector, while component III is located on another vector. In other embodiments, components I and III are located on the same vector, while component II is located on another vector. In other embodiments, components II and III are located on the same vector, while component I is located on another vector. In other embodiments, each of components I, II and III is located on different vectors. The invention also provides a viral (e.g. AAV or lentivirus) vector system as described herein. , and can be part of a vector system as described herein.

[0032] Some methods of the invention can include inducing expression. In some methods of the invention the organism or subject is a eukaryote (including mammal including human) or a non-human eukaryote or a non-human animal or a non-human mammal. In some embodiments, the organism or subject is a non-human animal, and may be an arthropod, for example, an insect, or may be a nematode. In some methods of the invention the organism or subject is a plant. In some methods of the invention the organism or subject is a mammal or a non-human mammal. A

non-human mammal may be for example a rodent (preferably a mouse or a rat), an ungulate, or a primate. In some methods of the invention the organism or subject is algae, including microalgae, or is a fungus. In some methods of the invention the viral vector is an AAV or a lentivirus, and can be part of a vector system as described herein. In some methods of the invention the CRISPR enzyme is a Cas9. In some methods of the invention the expression of the guide sequence is under the control of the T7 promoter and is driven by the expression of T7 polymerase.

[0033] The invention in some embodiments comprehends a method of delivering a CRISPR enzyme comprising delivering to a cell mRNA encoding the CRISPR enzyme. In some of these methods the CRISPR enzyme is a Cas9.

[0034] The invention also provides methods of preparing the vector systems of the invention, in particular the viral vector systems as described herein. The invention in some embodiments comprehends a method of preparing the AAV of the invention comprising transfecting plasmid(s) containing or consisting essentially of nucleic acid molecule(s) coding for the AAV into AAV-infected cells, and supplying AAV rep and/or cap obligatory for replication and packaging of the AAV. In some embodiments the AAV rep and/or cap obligatory for replication and packaging of the AAV are supplied by transfecting the cells with helper plasmid(s) or helper virus(es). In some embodiments the helper virus is a poxvirus, adenovirus, herpesvirus or baculovirus. In some embodiments the poxvirus is a vaccinia virus. In some embodiments the cells are mammalian cells. And in some embodiments the cells are insect cells and the helper virus is baculovirus. In other embodiments, the virus is a lentivirus.

[0035] In plants, pathogens are often host-specific. For example, *Fusarium oxysporum* f. sp. *lycopersici* causes tomato wilt but attacks only tomato, and *F. oxysporum f. dianthii Puccinia graminis* f. sp. *tritici* attacks only wheat. Plants have existing and induced defenses to resist most pathogens. Mutations and recombination events across plant generations lead to genetic variability that gives rise to susceptibility, especially as pathogens reproduce with more frequency than plants. In plants there can be non-host resistance, e.g., the host and pathogen are incompatible. There can also be Horizontal Resistance, e.g., partial resistance against all races of a pathogen, typically controlled by many genes and Vertical Resistance, e.g., complete resistance to some races of a pathogen but not to other races, typically controlled by a few genes. In a Gene-for-Gene level, plants and pathogens evolve together, and the genetic changes in one

balance changes in other. Accordingly, using Natural Variability, breeders combine most useful genes for Yield, Quality, Uniformity, Hardiness, Resistance. The sources of resistance genes include native or foreign Varieties, Heirloom Varieties, Wild Plant Relatives, and Induced Mutations, e.g., treating plant material with mutagenic agents. Using the present invention, plant breeders are provided with a new tool to induce mutations. Accordingly, one skilled in the art can analyze the genome of sources of resistance genes, and in Varieties having desired characteristics or traits employ the present invention to induce the rise of resistance genes, with more precision than previous mutagenic agents and hence accelerate and improve plant breeding programs.

[0036] The invention further comprehends a composition of the invention or a CRISPR enzyme thereof (including or alternatively mRNA encoding the CRISPR enzyme) for use in medicine or in therapy. In some embodiments the invention comprehends a composition according to the invention or a CRISPR enzyme thereof (including or alternatively mRNA encoding the CRISPR enzyme) for use in a method according to the invention. In some embodiments the invention provides for the use of a composition of the invention or a CRISPR enzyme thereof (including or alternatively mRNA encoding the CRISPR enzyme) in *ex vivo* gene or genome editing. In certain embodiments the invention comprehends use of a composition of the invention or a CRISPR enzyme thereof (including or alternatively mRNA encoding the CRISPR enzyme) in the manufacture of a medicament for *ex vivo* gene or genome editing or for use in a method according of the invention. The invention comprehends in some embodiments a composition of the invention or a CRISPR enzyme thereof (including or alternatively mRNA encoding the CRISPR enzyme), wherein the target sequence is flanked at its 3' end by a PAM (protospacer adjacent motif) sequence comprising 5'-motif, especially where the Cas9 is (or is derived from) *S. pyogenes* or *S. aureus* Cas9. For example, a suitable PAM is 5'-NRG or 5'-NNGRR (where N is any Nucleotide) for SpCas9 or SaCas9 enzymes (or derived enzymes), respectively, as mentioned below.

[0037] It will be appreciated that SpCas9 or SaCas9 are those from or derived from *S. pyogenes* or *S. aureus* Cas9.

[0038] Apects of the invention comprehend improving the specificity of a CRISPR enzyme, e.g. Cas9, mediated gene targeting and reducing the likelihood of off-target modification by the CRISPR enzyme, e.g. Cas9. The invention in some embodiments comprehends a method of

modifying an organism or a non-human organism by minimizing off-target modifications by manipulation of a first and a second target sequence on opposite strands of a DNA duplex in a genomic locus of interest in a cell comprising delivering a non-naturally occurring or engineered composition comprising :

[0039] I. a first CRISPR-Cas system chimeric RNA (chiRNA) polynucleotide sequence, wherein the first polynucleotide sequence comprises:

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

(b) a first tracr mate sequence, and

(c) a first tracr sequence,

[0040] II. a second CRISPR-Cas system chiRNA polynucleotide sequence, wherein the second polynucleotide sequence comprises:

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

(b) a second tracr mate sequence, and

(c) a second tracr sequence, and

[0041] III. a polynucleotide sequence encoding a CRISPR enzyme comprising at least one or more nuclear localization sequences and comprising one or more mutations, wherein (a), (b) and (c) are arranged in a 5' to 3' orientation, wherein when transcribed, the first and the second tracr mate sequence hybridize to the first and second tracr sequence respectively and the first and the second guide sequence directs sequence-specific binding of a first and a second CRISPR complex to the first and second target sequences respectively, wherein the first CRISPR complex comprises the CRISPR enzyme complexed with (1) the first guide sequence that is hybridized to the first target sequence, and (2) the first tracr mate sequence that is hybridized to the first tracr sequence, wherein the second CRISPR complex comprises the CRISPR enzyme complexed with (1) the second guide sequence that is hybridized to the second target sequence, and (2) the second tracr mate sequence that is hybridized to the second tracr sequence, wherein the polynucleotide sequence encoding a CRISPR enzyme is DNA or RNA, and wherein the first guide sequence directs cleavage of one strand of the DNA duplex near the first target sequence and the second guide sequence directs cleavage of the other strand near the second target sequence inducing a double strand break, thereby modifying the organism or the non-human organism by minimizing off-target modifications.

[0042] In some methods of the invention any or all of the polynucleotide sequence encoding the CRISPR enzyme, the first and the second guide sequence, the first and the second tracr mate sequence or the first and the second tracr sequence, is/are RNA. In further embodiments of the invention the polynucleotides encoding the sequence encoding the CRISPR enzyme, the first and the second guide sequence, the first and the second tracr mate sequence or the first and the second tracr sequence, is/are RNA and are delivered via liposomes, nanoparticles, exosomes, microvesicles, or a gene-gun. In certain embodiments of the invention, the first and second tracr mate sequence share 100% identity and/or the first and second tracr sequence share 100% identity. In some embodiments, the polynucleotides may be comprised within a vector system comprising one or more vectors. In preferred embodiments of the invention the CRISPR enzyme is a Cas9 enzyme, e.g. SpCas9. In an aspect of the invention the CRISPR enzyme comprises one or more mutations in a catalytic domain, wherein the one or more mutations are selected from the group consisting of D10A, E762A, H840A, N854A, N863A and D986A. In a highly preferred embodiment the CRISPR enzyme has the D10A mutation. In preferred embodiments, the first CRISPR enzyme has one or more mutations such that the enzyme is a complementary strand nicking enzyme, and the second CRISPR enzyme has one or more mutations such that the enzyme is a non-complementary strand nicking enzyme. Alternatively the first enzyme may be a non-complementary strand nicking enzyme, and the second enzyme may be a complementary strand nicking enzyme.

[0043] In preferred methods of the invention the first guide sequence directing cleavage of one strand of the DNA duplex near the first target sequence and the second guide sequence directing cleavage of the other strand near the second target sequence results in a 5' overhang. In embodiments of the invention the 5' overhang is at most 200 base pairs, preferably at most 100 base pairs, or more preferably at most 50 base pairs. In embodiments of the invention the 5' overhang is at least 26 base pairs, preferably at least 30 base pairs or more preferably 34-50 base pairs.

[0044] The invention in some embodiments comprehends a method of modifying an organism or a non-human organism by minimizing off-target modifications by manipulation of a first and a second target sequence on opposite strands of a DNA duplex in a genomic locus of interest in a cell comprising delivering a non-naturally occurring or engineered composition comprising a vector system comprising one or more vectors comprising

[0045] I. a first regulatory element operably linked to

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

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

[0046] II. a second regulatory element operably linked to

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

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

[0047] III. a third regulatory element operably linked to an enzyme-coding sequence encoding a CRISPR enzyme, and

[0048] IV. a fourth regulatory element operably linked to a tracr sequence,

[0049] wherein components I, II, III and IV are located on the same or different vectors of the system, when transcribed, the tracr mate sequence hybridizes to the tracr sequence and the first and the second guide sequence direct sequence-specific binding of a first and a second CRISPR complex to the first and second target sequences respectively, wherein the first CRISPR complex comprises the CRISPR enzyme complexed with (1) the first guide sequence that is hybridized to the first target sequence, and (2) the tracr mate sequence that is hybridized to the tracr sequence, wherein the second CRISPR complex comprises the CRISPR enzyme complexed with (1) the second guide sequence that is hybridized to the second target sequence, and (2) the tracr mate sequence that is hybridized to the tracr sequence, wherein the polynucleotide sequence encoding a CRISPR enzyme is DNA or RNA, and wherein the first guide sequence directs cleavage of one strand of the DNA duplex near the first target sequence and the second guide sequence directs cleavage of the other strand near the second target sequence inducing a double strand break, thereby modifying the organism or the non-human organism by minimizing off-target modifications.

[0050] The invention also provides a vector system as described herein. The system may comprise one, two, three or four different vectors. Components I, II, III and IV may thus be located on one, two, three or four different vectors, and all combinations for possible locations of the components are herein envisaged, for example: components I, II, III and IV can be located on the same vector; components I, II, III and IV can each be located on different vectors; components I, II, II I and IV may be located on a total of two or three different vectors, with all combinations of locations envisaged, etc.

[0051] In some methods of the invention any or all of the polynucleotide sequence encoding the CRISPR enzyme, the first and the second guide sequence, the first and the second tracr mate sequence or the first and the second tracr sequence, is/are RNA. In further embodiments of the invention the first and second tracr mate sequence share 100% identity and/or the first and second tracr sequence share 100% identity. In preferred embodiments of the invention the CRISPR enzyme is a Cas9 enzyme, e.g. SpCas9. In an aspect of the invention the CRISPR enzyme comprises one or more mutations in a catalytic domain, wherein the one or more mutations are selected from the group consisting of D10A, E762A, H840A, N854A, N863A and D986A. In a highly preferred embodiment the CRISPR enzyme has the D10A mutation. In preferred embodiments, the first CRISPR enzyme has one or more mutations such that the enzyme is a complementary strand nicking enzyme, and the second CRISPR enzyme has one or more mutations such that the enzyme is a non-complementary strand nicking enzyme. Alternatively the first enzyme may be a non-complementary strand nicking enzyme, and the second enzyme may be a complementary strand nicking enzyme. In a further embodiment of the invention, one or more of the viral vectors are delivered via liposomes, nanoparticles, exosomes, microvesicles, or a gene-gun.

[0052] In preferred methods of the invention the first guide sequence directing cleavage of one strand of the DNA duplex near the first target sequence and the second guide sequence directing cleavage of other strand near the second target sequence results in a 5' overhang. In embodiments of the invention the 5' overhang is at most 200 base pairs, preferably at most 100 base pairs, or more preferably at most 50 base pairs. In embodiments of the invention the 5' overhang is at least 26 base pairs, preferably at least 30 base pairs or more preferably 34-50 base pairs.

[0053] The invention in some embodiments comprehends a method of modifying a genomic locus of interest by minimizing off-target modifications by introducing into a cell containing and expressing a double stranded DNA molecule encoding a gene product of interest an engineered, non-naturally occurring CRISPR-Cas system comprising a Cas protein having one or more mutations and two guide RNAs that target a first strand and a second strand of the DNA molecule respectively, whereby the guide RNAs target the DNA molecule encoding the gene product and the Cas protein nicks each of the first strand and the second strand of the DNA

molecule encoding the gene product, whereby expression of the gene product is altered; and, wherein the Cas protein and the two guide RNAs do not naturally occur together.

[0054] In preferred methods of the invention the Cas protein nicking each of the first strand and the second strand of the DNA molecule encoding the gene product results in a 5' overhang. In embodiments of the invention the 5' overhang is at most 200 base pairs, preferably at most 100 base pairs, or more preferably at most 50 base pairs. In embodiments of the invention the 5' overhang is at least 26 base pairs, preferably at least 30 base pairs or more preferably 34-50 base pairs.

[0055] Embodiments of the invention also comprehend the guide RNAs comprising a guide sequence fused to a tracr mate sequence and a tracr sequence. In an aspect of the invention the Cas protein is codon optimized for expression in a eukaryotic cell, preferably a mammalian cell or a human cell. In further embodiments of the invention the Cas protein is a type II CRISPR-Cas protein, e.g. a Cas 9 protein. In a highly preferred embodiment the Cas protein is a Cas9 protein, e.g. SpCas9. In aspects of the invention the Cas protein has one or more mutations selected from the group consisting of D10A, E762A, H840A, N854A, N863A and D986A. In a highly preferred embodiment the Cas protein has the D10A mutation.

[0056] Aspects of the invention relate to the expression of the gene product being decreased or a template polynucleotide being further introduced into the DNA molecule encoding the gene product or an intervening sequence being excised precisely by allowing the two 5' overhangs to reanneal and ligate or the activity or function of the gene product being altered or the expression of the gene product being increased. In an embodiment of the invention, the gene product is a protein.

[0057] The invention also comprehends an engineered, non-naturally occurring CRISPR-Cas system comprising a Cas protein having one or more mutations and two guide RNAs that target a first strand and a second strand respectively of a double stranded DNA molecule encoding a gene product in a cell, whereby the guide RNAs target the DNA molecule encoding the gene product and the Cas protein nicks each of the first strand and the second strand of the DNA molecule encoding the gene product, whereby expression of the gene product is altered; and, wherein the Cas protein and the two guide RNAs do not naturally occur together.

[0058] In aspects of the invention the guide RNAs may comprise a guide sequence fused to a tracr mate sequence and a tracr sequence. In an embodiment of the invention the Cas protein is a

type II CRISPR-Cas protein. In an aspect of the invention the Cas protein is codon optimized for expression in a eukaryotic cell, preferably a mammalian cell or a human cell. In further embodiments of the invention the Cas protein is a type II CRISPR-Cas protein, e.g. a Cas 9 protein. In a highly preferred embodiment the Cas protein is a Cas9 protein, e.g. SpCas9. In aspects of the invention the Cas protein has one or more mutations selected from the group consisting of D10A, E762A, H840A, N854A, N863A and D986A. In a highly preferred embodiment the Cas protein has the D10A mutation.

[0059] Aspects of the invention relate to the expression of the gene product being decreased or a template polynucleotide being further introduced into the DNA molecule encoding the gene product or an intervening sequence being excised precisely by allowing the two 5' overhangs to reanneal and ligate or the activity or function of the gene product being altered or the expression of the gene product being increased. In an embodiment of the invention, the gene product is a protein.

[0060] The invention also comprehends an engineered, non-naturally occurring vector system comprising one or more vectors comprising:

a) a first regulatory element operably linked to each of two CRISPR-Cas system guide RNAs that target a first strand and a second strand respectively of a double stranded DNA molecule encoding a gene product,

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

wherein components (a) and (b) are located on same or different vectors of the system, whereby the guide RNAs target the DNA molecule encoding the gene product and the Cas protein nicks each of the first strand and the second strand of the DNA molecule encoding the gene product, whereby expression of the gene product is altered; and, wherein the Cas protein and the two guide RNAs do not naturally occur together.

[0061] In aspects of the invention the guide RNAs may comprise a guide sequence fused to a tracr mate sequence and a tracr sequence. In an embodiment of the invention the Cas protein is a type II CRISPR-Cas protein. In an aspect of the invention the Cas protein is codon optimized for expression in a eukaryotic cell, preferably a mammalian cell or a human cell. In further embodiments of the invention the Cas protein is a type II CRISPR-Cas protein, e.g. a Cas 9 protein. In a highly preferred embodiment the Cas protein is a Cas9 protein, e.g. SpCas9. In aspects of the invention the Cas protein has one or more mutations selected from the group

consisting of D10A, E762A, H840A, N854A, N863A and D986A. In a highly preferred embodiment the Cas protein has the D10A mutation.

[0062] Aspects of the invention relate to the expression of the gene product being decreased or a template polynucleotide being further introduced into the DNA molecule encoding the gene product or an intervening sequence being excised precisely by allowing the two 5' overhangs to reanneal and ligate or the activity or function of the gene product being altered or the expression of the gene product being increased. In an embodiment of the invention, the gene product is a protein. In preferred embodiments of the invention the vectors of the system are viral vectors. In a further embodiment, the vectors of the system are delivered via liposomes, nanoparticles, exosomes, microvesicles, or a gene-gun.

[0063] In one aspect, the invention provides a method of modifying a target polynucleotide in a eukaryotic cell. In some embodiments, the method comprises allowing a CRISPR complex to bind to the target polynucleotide to effect cleavage of said target polynucleotide thereby modifying the target polynucleotide, wherein the CRISPR complex comprises a CRISPR enzyme complexed with a guide sequence hybridized to a target sequence within said target polynucleotide, wherein said guide sequence is linked to a tracr mate sequence which in turn hybridizes to a tracr sequence. In some embodiments, said cleavage comprises cleaving one or two strands at the location of the target sequence by said CRISPR enzyme. In some embodiments, said cleavage results in decreased transcription of a target gene. In some embodiments, the method further comprises repairing said cleaved target polynucleotide by homologous recombination with an exogenous template polynucleotide, wherein said repair results in a mutation comprising an insertion, deletion, or substitution of one or more nucleotides of said target polynucleotide. In some embodiments, said mutation results in one or more amino acid changes in a protein expressed from a gene comprising the target sequence. In some embodiments, the method further comprises delivering one or more vectors to said eukaryotic cell, wherein the one or more vectors drive expression of one or more of: the CRISPR enzyme, the guide sequence linked to the tracr mate sequence, and the tracr sequence. In some embodiments, said vectors are delivered to the eukaryotic cell in a subject. In some embodiments, said modifying takes place in said eukaryotic cell in a cell culture. In some embodiments, the method further comprises isolating said eukaryotic cell from a subject prior to

said modifying. In some embodiments, the method further comprises returning said eukaryotic cell and/or cells derived therefrom to said subject.

[0064] In one aspect, the invention provides a method of modifying expression of a polynucleotide in a eukaryotic cell. In some embodiments, the method comprises allowing a CRISPR complex to bind to the polynucleotide such that said binding results in increased or decreased expression of said polynucleotide; wherein the CRISPR complex comprises a CRISPR enzyme complexed with a guide sequence hybridized to a target sequence within said polynucleotide, wherein said guide sequence is linked to a tracr mate sequence which in turn hybridizes to a tracr sequence. In some embodiments, the method further comprises delivering one or more vectors to said eukaryotic cells, wherein the one or more vectors drive expression of one or more of: the CRISPR enzyme, the guide sequence linked to the tracr mate sequence, and the tracr sequence.

[0065] In one aspect, the invention provides a method of generating a model eukaryotic cell comprising a mutated disease gene. In some embodiments, a disease gene is any gene associated with an increase in the risk of having or developing a disease. In some embodiments, the method comprises (a) introducing one or more vectors into a eukaryotic cell, wherein the one or more vectors drive expression of one or more of: a CRISPR enzyme, a guide sequence linked to a tracr mate sequence, and a tracr sequence; and (b) allowing a CRISPR complex to bind to a target polynucleotide to effect cleavage of the target polynucleotide within said disease gene, wherein the CRISPR complex comprises the CRISPR enzyme complexed with (1) the guide sequence that is hybridized to the target sequence within the target polynucleotide, and (2) the tracr mate sequence that is hybridized to the tracr sequence, thereby generating a model eukaryotic cell comprising a mutated disease gene. In some embodiments, said cleavage comprises cleaving one or two strands at the location of the target sequence by said CRISPR enzyme. In some embodiments, said cleavage results in decreased transcription of a target gene. In some embodiments, the method further comprises repairing said cleaved target polynucleotide by homologous recombination with an exogenous template polynucleotide, wherein said repair results in a mutation comprising an insertion, deletion, or substitution of one or more nucleotides of said target polynucleotide. In some embodiments, said mutation results in one or more amino acid changes in a protein expression from a gene comprising the target sequence.

[0066] In one aspect the invention provides for a method of selecting one or more prokaryotic cell(s) by introducing one or more mutations in a gene in the one or more prokaryotic cell (s), the method comprising: introducing one or more vectors into the prokaryotic cell (s), wherein the one or more vectors drive expression of one or more of: a CRISPR enzyme, a guide sequence linked to a tracr mate sequence, a tracr sequence, and an editing template; wherein the editing template comprises the one or more mutations that abolish CRISPR enzyme cleavage; allowing homologous recombination of the editing template with the target polynucleotide in the cell(s) to be selected; allowing a CRISPR complex to bind to a target polynucleotide to effect cleavage of the target polynucleotide within said gene, wherein the CRISPR complex comprises the CRISPR enzyme complexed with (1) the guide sequence that is hybridized to the target sequence within the target polynucleotide, and (2) the tracr mate sequence that is hybridized to the tracr sequence, wherein binding of the CRISPR complex to the target polynucleotide induces cell death, thereby allowing one or more prokaryotic cell(s) in which one or more mutations have been introduced to be selected. In a preferred embodiment, the CRISPR enzyme is Cas9. In another aspect of the invention the cell to be selected may be a eukaryotic cell. Aspects of the invention allow for selection of specific cells without requiring a selection marker or a two-step process that may include a counter-selection system.

[0067] In one aspect, the invention provides for methods of modifying a target polynucleotide in a eukaryotic cell. In some embodiments, the method comprises allowing a CRISPR complex to bind to the target polynucleotide to effect cleavage of said target polynucleotide thereby modifying the target polynucleotide, wherein the CRISPR complex comprises a CRISPR enzyme complexed with a guide sequence hybridized to a target sequence within said target polynucleotide, wherein said guide sequence is linked to a tracr mate sequence which in turn hybridizes to a tracr sequence.

[0068] In other embodiments, this invention provides a method of modifying expression of a polynucleotide in a eukaryotic cell. The method comprises increasing or decreasing expression of a target polynucleotide by using a CRISPR complex that binds to the polynucleotide.

[0069] Where desired, to effect the modification of the expression in a cell, one or more vectors comprising a tracr sequence, a guide sequence linked to the tracr mate sequence, a sequence encoding a CRISPR enzyme is delivered to a cell. In some methods, the one or more vectors comprises a regulatory element operably linked to an enzyme-coding sequence encoding

said CRISPR enzyme comprising a nuclear localization sequence; and a regulatory element operably linked to a tracr mate sequence and one or more insertion sites for inserting a guide sequence upstream of the tracr mate sequence. When expressed, the guide sequence directs sequence-specific binding of a CRISPR complex to a target sequence in a cell. Typically, 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.

[0070] In some methods, a target polynucleotide can be inactivated to effect the modification of the expression in a cell. For example, upon the binding of a CRISPR complex to a target sequence in a cell, the target polynucleotide is inactivated such that the sequence is not transcribed, the coded protein is not produced, or the sequence does not function as the wild-type sequence does. For example, a protein or microRNA coding sequence may be inactivated such that the protein is not produced.

[0071] In certain embodiments, the CRISPR enzyme comprises one or more mutations selected from the group consisting of D10A, E762A, H840A, N854A, N863A or D986A and/or the one or more mutations is in a RuvC1 or HNH domain of the CRISPR enzyme or is a mutation as otherwise as discussed herein. In some embodiments, the CRISPR enzyme has one or more mutations in a catalytic domain, wherein when transcribed, the tracr mate sequence hybridizes to the tracr sequence and the guide sequence directs sequence-specific binding of a CRISPR complex to the target sequence, and wherein the enzyme further comprises a functional domain. In some embodiments, the functional domain is a transcriptional activation domain, preferably VP64. In some embodiments, the functional domain is a transcription repression domain, preferably KRAB. In some embodiments, the transcription repression domain is SID, or concatemers of SID (eg SID4X). In some embodiments, the functional domain is an epigenetic modifying domain, such that an epigenetic modifying enzyme is provided. In some embodiments, the functional domain is an activation domain, which may be the P65 activation domain.

[0072] In some embodiments, the CRISPR enzyme is a type I or III CRISPR enzyme, but is preferably a type II CRISPR enzyme. This type II CRISPR enzyme may be any Cas enzyme. A Cas enzyme may be identified as Cas9 as this can refer to the general class of enzymes that share homology to the biggest nuclease with multiple nuclease domains from the type II CRISPR

system. Most preferably, the Cas9 enzyme is from, or is derived from, spCas9 or saCas9. By derived, Applicants mean that the derived enzyme is largely based, in the sense of having a high degree of sequence homology with, a wildtype enzyme, but that it has been mutated (modified) in some way as described herein.

[0073] It will be appreciated that the terms Cas and CRISPR enzyme are generally used herein interchangeably, unless otherwise apparent. As mentioned above, many of the residue numberings used herein refer to the Cas9 enzyme from the type II CRISPR locus in *Streptococcus pyogenes*. However, it will be appreciated that this invention includes many more Cas9s from other species of microbes, such as SpCas9, SaCa9, St1Cas9 and so forth.

[0074] An example of a codon optimized sequence, in this instance optimized for humans (i.e. being optimized for expression in humans) is provided herein, see the SaCas9 human codon optimized sequence. Whilst this is preferred, it will be appreciated that other examples are possible and codon optimization for a host species is known.

[0075] Preferably, delivery is in the form of a vector which may be a viral vector, such as a lenti- or baculo- or preferably adeno-viral/adeno-associated viral vectors, but other means of delivery are known (such as yeast systems, microvesicles, gene guns/means of attaching vectors to gold nanoparticles) and are provided. A vector may mean not only a viral or yeast system (for instance, where the nucleic acids of interest may be operably linked to and under the control of (in terms of expression, such as to ultimately provide a processed RNA) a promoter), but also direct delivery of nucleic acids into a host cell. While in herein methods the vector may be a viral vector and this is advantageously an AAV, other viral vectors as herein discussed can be employed, such as lentivirus. For example, baculoviruses may be used for expression in insect cells. These insect cells may, in turn be useful for producing large quantities of further vectors, such as AAV or lentivirus vectors adapted for delivery of the present invention. Also envisaged is a method of delivering the present CRISPR enzyme comprising delivering to a cell mRNA encoding the CRISPR enzyme. It will be appreciated that in certain embodiments the CRISPR enzyme is truncated, and/or comprised of less than one thousand amino acids or less than four thousand amino acids, and/or is a nuclease or nickase, and/or is codon-optimized, and/or comprises one or more mutations, and/or comprises a chimeric CRISPR enzyme, and/or the other options as herein discussed. AAV and lentiviral vectors are preferred.

[0076] In certain embodiments, the target sequence is flanked or followed, at its 3' end, by a PAM suitable for the CRISPR enzyme, typically a Cas and in particular a Cas9.

[0077] For example, a suitable PAM is 5'-NRG or 5'-NNGRR for SpCas9 or SaCas9 enzymes (or derived enzymes), respectively.

[0078] It will be appreciated that SpCas9 or SaCas9 are those from or derived from *S. pyogenes* or *S. aureus* Cas9.

[0079] Accordingly, it is an object of the invention to not encompass within the invention any previously known product, process of making the product, or method of using the product such that Applicants reserve the right and hereby disclose a disclaimer of any previously known product, process, or method. It is further noted that the invention does not intend to encompass within the scope of the invention any product, process, or making of the product or method of using the product, which does not meet the written description and enablement requirements of the USPTO (35 U.S.C. §112, first paragraph) or the EPO (Article 83 of the EPC), such that Applicants reserve the right and hereby disclose a disclaimer of any previously described product, process of making the product, or method of using the product.

[0080] It is noted that in this disclosure and particularly in the claims and/or paragraphs, terms such as "comprises", "comprised", "comprising" and the like can have the meaning attributed to it in U.S. Patent law; e.g., they can mean "includes", "included", "including", and the like; and that terms such as "consisting essentially of" and "consists essentially of" have the meaning ascribed to them in U.S. Patent law, e.g., they allow for elements not explicitly recited, but exclude elements that are found in the prior art or that affect a basic or novel characteristic of the invention.

[0081] These and other embodiments are disclosed or are obvious from and encompassed by, the following Detailed Description.

BRIEF DESCRIPTION OF THE DRAWINGS

[0082] The novel features of the invention are set forth with particularity in the appended claims. A better understanding of the features and advantages of the present invention will be obtained by reference to the following detailed description that sets forth illustrative embodiments, in which the principles of the invention are utilized, and the accompanying drawings of which:

[0083] Figure 1 shows a schematic model of the CRISPR system. The Cas9 nuclease from Streptococcus pyogenes (yellow) is targeted to genomic DNA by a synthetic guide RNA (sgRNA) consisting of a 20-nt guide sequence (blue) and a scaffold (red). The guide sequence base-pairs with the DNA target (blue), directly upstream of a requisite 5'-NGG protospacer adjacent motif (PAM; magenta), and Cas9 mediates a double-stranded break (DSB) ~3 bp upstream of the PAM (red triangle).

[0084] Figure 2A-F shows an exemplary CRISPR system, a possible mechanism of action, an example adaptation for expression in eukaryotic cells, and results of tests assessing nuclear localization and CRISPR activity.

[0085] Figure 3A-D shows results of an evaluation of SpCas9 specificity for an example target.

[0086] Figure 4A-G show an exemplary vector system and results for its use in directing homologous recombination in eukaryotic cells.

[0087] Figure 5 provides a table of protospacer sequences and summarizes modification efficiency results for protospacer targets designed based on exemplary S. pyogenes and S. thermophilus CRISPR systems with corresponding PAMs against loci in human and mouse genomes. Cells were transfected with Cas9 and either pre-crRNA/tracrRNA or chimeric RNA, and analyzed 72 hours after transfection. Percent indels are calculated based on Surveyor assay results from indicated cell lines (N=3 for all protospacer targets, errors are S.E.M., N.D. indicates not detectable using the Surveyor assay, and N.T. indicates not tested in this study).

[0088] Figure 6A-C shows a comparison of different tracrRNA transcripts for Cas9-mediated gene targeting.

[0089] Figure 7 shows a schematic of a surveyor nuclease assay for detection of double strand break-induced micro-insertions and –deletions.

[0090] Figure 8A-B shows exemplary bicistronic expression vectors for expression of CRISPR system elements in eukaryotic cells.

[0091] Figure 9A-C shows histograms of distances between adjacent S. pyogenes SF370 locus 1 PAM (NGG) (Figure 9A) and S. thermophilus LMD9 locus 2 PAM (NNAGAAW) (Figure 9B) in the human genome; and distances for each PAM by chromosome (Chr) (Figure 9C).

[0092] Figure 10A-D shows an exemplary CRISPR system, an example adaptation for expression in eukaryotic cells, and results of tests assessing CRISPR activity.

[0093] Figure 11A-C shows exemplary manipulations of a CRISPR system for targeting of genomic loci in mammalian cells.

[0094] Figure 12A-B shows the results of a Northern blot analysis of crRNA processing in mammalian cells.

[0095] Figure 13A-B shows an exemplary selection of protospacers in the human PVALB and mouse Th loci.

[0096] Figure 14 shows example protospacer and corresponding PAM sequence targets of the S. thermophilus CRISPR system in the human EMX1 locus.

[0097] Figure 15 provides a table of sequences for primers and probes used for Surveyor, RFLP, genomic sequencing, and Northern blot assays.

[0098] Figure 16A-C shows exemplary manipulation of a CRISPR system with chimeric RNAs and results of SURVEYOR assays for system activity in eukaryotic cells.

[0099] Figure 17A-B shows a graphical representation of the results of SURVEYOR assays for CRISPR system activity in eukaryotic cells.

[00100] Figure 18 shows an exemplary visualization of some S. pyogenes Cas9 target sites in the human genome using the UCSC genome browser.

[00101] Figure 19A-D shows a circular depiction of the phylogenetic analysis revealing five families of Cas9s, including three groups of large Cas9s (~1400 amino acids) and two of small Cas9s (~1100 amino acids).

[00102] Figure 20A-F shows the linear depiction of the phylogenetic analysis revealing five families of Cas9s, including three groups of large Cas9s (~1400 amino acids) and two of small Cas9s (~1100 amino acids).

[00103] Figure 21A-D shows genome editing via homologous recombination. (a) Schematic of SpCas9 nickase, with D10A mutation in the RuvC I catalytic domain. (b) Schematic representing homologous recombination (HR) at the human EMX1 locus using either sense or antisense single stranded oligonucleotides as repair templates. Red arrow above indicates sgRNA cleavage site; PCR primers for genotyping (Tables J and K) are indicated as arrows in right panel. (c) Sequence of region modified by HR. d, SURVEYOR assay for wildtype (wt) and

nickase (D10A) SpCas9-mediated indels at the EMX1 target 1 locus (n=3). Arrows indicate positions of expected fragment sizes.

[00104] Figure 22A-B shows single vector designs for SpCas9.

[00105] Figure 23 shows a graph representing the length distribution of Cas9 orthologs.

[00106] Figure 24A-M shows sequences where the mutation points are located within the SpCas9 gene.

[00107] Figure 25A shows the Conditional Cas9, Rosa26 targeting vector map.

[00108] Figure 25B shows the Constitutive Cas9, Rosa26 targeting vector map.

[00109] Figure 26 shows a schematic of the important elements in the Constitutive and Conditional Cas9 constructs.

[00110] Figure 27 shows delivery and *in vivo* mouse brain Cas9 expression data.

[00111] Figure 28 shows RNA delivery of Cas9 and chimeric RNA into cells (A) Delivery of a GFP reporter as either DNA or mRNA into Neuro-2A cells. (B) Delivery of Cas9 and chimeric RNA against the Icam2 gene as RNA results in cutting for one of two spacers tested. (C) Delivery of Cas9 and chimeric RNA against the F7 gene as RNA results in cutting for one of two spacers tested.

[00112] Figure 29 shows how DNA double-strand break (DSB) repair promotes gene editing. In the error-prone non-homologous end joining (NHEJ) pathway, the ends of a DSB are processed by endogenous DNA repair machineries and rejoined together, which can result in random insertion/deletion (indel) mutations at the site of junction. Indel mutations occurring within the coding region of a gene can result in frame-shift and a premature stop codon, leading to gene knockout. Alternatively, a repair template in the form of a plasmid or single-stranded oligodeoxynucleotides (ssODN) can be supplied to leverage the homology-directed repair (HDR) pathway, which allows high fidelity and precise editing.

[00113] Figure 30A-C shows anticipated results for HDR in HEK and HUES9 cells. (a) Either a targeting plasmid or an ssODN (sense or antisense) with homology arms can be used to edit the sequence at a target genomic locus cleaved by Cas9 (red triangle). To assay the efficiency of HDR, we introduced a HindIII site (red bar) into the target locus, which was PCR-amplified with primers that anneal outside of the region of homology. Digestion of the PCR product with HindIII reveals the occurrence of HDR events. (b) ssODNs, oriented in either the sense or the antisense (s or a) direction relative to the locus of interest, can be used in combination with Cas9

to achieve efficient HDR-mediated editing at the target locus. A minimal homology region of 40 bp, and preferably 90 bp, is recommended on either side of the modification (red bar). (c) Example of the effect of ssODNs on HDR in the EMX1 locus is shown using both wild-type Cas9 and Cas9 nickase (D10A). Each ssODN contains homology arms of 90 bp flanking a 12-bp insertion of two restriction sites

[00114] Figure 31A-C shows the repair strategy for Cystic Fibrosis delta F508 mutation.

[00115] Figure 32A-B (a) shows a schematic of the GAA repeat expansion in FXN intron 1 and (b) shows a schematic of the strategy adopted to excise the GAA expansion region using the CRISPR/Cas system.

[00116] Figure 33 shows a screen for efficient SpCas9 mediated targeting of Tet1-3 and Dnmt1, 3a and 3b gene loci. Surveyor assay on DNA from transfected N2A cells demonstrates efficient DNA cleavage by using different gRNAs.

[00117] Figure 34 shows a strategy of multiplex genome targeting using a 2-vector system in an AAV1/2 delivery system. Tet1-3 and Dnmt1, 3a and 3b gRNA under the control of the U6 promoter. GFP-KASH under the control of the human synapsin promoter. Restriction sides shows simple gRNA replacement strategy by subcloning. HA-tagged SpCas9 flanked by two nuclear localization signals (NLS) is shown. Both vectors are delivered into the brain by AAV1/2 virus in a 1:1 ratio.

[00118] Figure 35 shows verification of multiplex DNMT targeting vector #1 functionality using Surveyor assay. N2A cells were co-transfected with the DNMT targeting vector #1 (+) and the SpCas9 encoding vector for testing SpCas9 mediated cleavage of DNMTs genes family loci. gRNA only (-) is negative control. Cells were harvested for DNA purification and downstream processing 48 h after transfection.

[00119] Figure 36 shows verification of multiplex DNMT targeting vector #2 functionality using Surveyor assay. N2A cells were co-transfected with the DNMT targeting vector #1 (+) and the SpCas9 encoding vector for testing SpCas9 mediated cleavage of DNMTs genes family loci. gRNA only (-) is negative control. Cells were harvested for DNA purification and downstream processing 48 h after transfection.

[00120] Figure 37 shows schematic overview of short promoters and short polyA versions used for HA-SpCas9 expression in vivo. Sizes of the encoding region from L-ITR to R-ITR are shown on the right.

[00121] Figure 38 shows schematic overview of short promoters and short polyA versions used for HA-SaCas9 expression in vivo. Sizes of the encoding region from L-ITR to R-ITR are shown on the right.

[00122] Figure 39 shows expression of SpCas9 and SaCas9 in N2A cells. Representative Western blot of HA-tagged SpCas9 and SaCas9 versions under the control of different short promoters and with or short polyA (spA) sequences. Tubulin is loading control. mCherry (mCh) is a transfection control. Cells were harvested and further processed for Western blotting 48 h after transfection.

[00123] Figure 40 shows screen for efficient SaCas9 mediated targeting of Tet3 gene locus. Surveyor assay on DNA from transfected N2A cells demonstrates efficient DNA cleavage by using different gRNAs with NNGGGT PUM sequence. GFP transfected cells and cells expressing only SaCas9 are controls.

[00124] Figure 41 shows expression of HA-SaCas9 in the mouse brain. Animals were injected into dentate gyri with virus driving expression of HA-SaCas9 under the control of human Synapsin promoter. Animals were sacrificed 2 weeks after surgery. HA tag was detected using rabbit monoclonal antibody C29F4 (Cell Signaling). Cell nuclei stained in blue with DAPI stain.

[00125] Figure 42 shows expression of SpCas9 and SaCas9 in cortical primary neurons in culture 7 days after transduction. Representative Western blot of HA-tagged SpCas9 and SaCas9 versions under the control of different promoters and with bgh or short polyA (spA) sequences. Tubulin is loading control.

[00126] Figure 43 shows LIVE/DEAD stain of primary cortical neurons 7 days after transduction with AAV1 particles carrying SpCas9 with different promoters and multiplex gRNAs constructs (example shown on the last panel for DNMTs). Neurons after AAV transduction were compared with control untransduced neurons. Red nuclei indicate permeabilized, dead cells (second line of panels). Live cells are marked in green color (third line of panels).

[00127] Figure 44 shows LIVE/DEAD stain of primary cortical neurons 7 days after transduction with AAV1 particles carrying SaCas9 with different promoters. Red nuclei indicate permeabilized, dead cells (second line of panels). Live cells are marked in green color (third line of panels).

[00128] Figure 45 shows comparison of morphology of neurons after transduction with AAV1 virus carrying SpCas9 and gRNA multiplexes for TETs and DNMTs genes loci. Neurons without transduction are shown as a control.

[00129] Figure 46 shows verification of multiplex DNMT targeting vector #1 functionality using Surveyor assay in primary cortical neurons. Cells were co-transduced with the DNMT targeting vector #1 and the SpCas9 viruses with different promoters for testing SpCas9 mediated cleavage of DNMTs genes family loci.

[00130] Figure 47 shows in vivo efficiency of SpCas9 cleavage in the brain. Mice were injected with AAV1/2 virus carrying gRNA multiplex targeting DNMT family genes loci together with SpCas9 viruses under control of 2 different promoters: mouse Mecp2 and rat Map1b. Two weeks after injection brain tissue was extracted and nuclei were prepped and sorted using FACS, based on the GFP expression driven by Synapsin promoter from gRNA multiplex construct. After gDNA extraction Surveyor assay was run. + indicates GFP positive nuclei and – control, GFP-negative nuclei from the same animal. Numbers on the gel indicate assessed SpCas9 efficiency.

[00131] Figure 48 shows purification of GFP-KASH labeled cell nuclei from hippocampal neurons. The outer nuclear membrane (ONM) of the cell nuclear membrane is tagged with a fusion of GFP and the KASH protein transmembrane domain. Strong GFP expression in the brain after one week of stereotactic surgery and AAV1/2 injection. Density gradient centrifugation step to purify cell nuclei from intact brain. Purified nuclei are shown. Chromatin stain by Vybrant® DyeCycle™ Ruby Stain is shown in red, GFP labeled nuclei are green. Representative FACS profile of GFP+ and GFP- cell nuclei (Magenta: Vybrant® DyeCycle™ Ruby Stain, Green: GFP).

[00132] Figure 49 shows efficiency of SpCas9 cleavage in the mouse brain. Mice were injected with AAV1/2 virus carrying gRNA multiplex targeting TET family genes loci together with SpCas9 viruses under control of 2 different promoters: mouse Mecp2 and rat Map1b. Three weeks after injection brain tissue was extracted, nuclei were prepped and sorted using FACS, based on the GFP expression driven by Synapsin promoter from gRNA multiplex construct. After gDNA extraction Surveyor assay was run. + indicates GFP positive nuclei and – control, GFP-negative nuclei from the same animal. Numbers on the gel indicate assessed SpCas9 efficiency.

[00133] Figure 50 shows GFP-KASH expression in cortical neurons in culture. Neurons were transduced with AAV1 virus carrying gRNA multiplex constructs targeting TET genes loci. The strongest signal localize around cells nuclei due to KASH domain localization.

[00134] Figure 51 shows (top) a list of spacing (as indicated by the pattern of arrangement for two PAM sequences) between pairs of guide RNAs. Only guide RNA pairs satisfying patterns 1, 2, 3, 4 exhibited indels when used with SpCas9(D10A) nickase. (bottom) Gel images showing that combination of SpCas9(D10A) with pairs of guide RNA satisfying patterns 1, 2, 3, 4 led to the formation of indels in the target site.

[00135] Figure 52 shows a list of U6 reverse primer sequences used to generate U6-guide RNA expression casssettes. Each primer needs to be paired with the U6 forward primer "gcactgagggcctatttcccatgattc" to generate amplicons containing U6 and the desired guide RNA.

[00136] Figure 53 shows a Genomic sequence map from the human Emx1 locus showing the locations of the 24 patterns listed in Figure 33.

[00137] Figure 54 shows on (right) a gel image indicating the formation of indels at the target site when variable 5' overhangs are present after cleavage by the Cas9 nickase targeted by different pairs of guide RNAs. on (left) a table indicating the lane numbers of the gel on the right and various parameters including identifying the guide RNA pairs used and the length of the 5' overhang present following cleavage by the Cas9 nickase.

[00138] Figure 55 shows a Genomic sequence map from the human Emx1 locus showing the locations of the different pairs of guide RNAs that result in the gel patterns of Fig. 54 (right) and which are further described in Example 35.

[00139] Figure 56 shows staining of HA-SpCas9 in dorsal and ventral hippocampus 8 weeks after injection of viruses encoding Mecp2-HA-SpCas9 and 3xgRNA-TETS with Syn-KASH-GFP.

[00140] Figure 57 shows Syn_GFP-KASH expression 8 weeks after 3xgRNA virus injection is specific for neurons (NeuN positive cells) and not for glia cells (GFAP positive).

[00141] Figure 58 shows behavior tests conducted 5 weeks after CRISPR-mediated KD of TETs and DNMTs in dentate gyrus (ventral and dorsal part) showed increased level of anxiety and learning deficits. A) time spend in the open arm during elevated plus maze test. B) open field test, time spent in the center of arena vs time in the corners was measured. C) Novel object recognition test, results were measured 3h after familiarization phase. D) Barnes maze;

efficiency in finding escape within 3 days of training. E) Barnes maze results. F) Freezing behavior during contextual fear conditioning. G) Latency to first freezing episode during contextual fear conditioning. H) Trace fear conditioning results for TETs KD and DNMTs KD (I). Control – animals injected with SpCas9 virus and GFP-KASH construct without gRNAs. TETs – animals injected with both SpCas9 and construct encoding gRNAs against Tet1, Tet2 and Tet3. DNMTs - animals injected with both SpCas9 and construct encoding gRNAs against Dnmt1, Dnmt3a and Dnmt3b.

[00142] Figure 59 shows cutting efficiency of Tet loci in the brain, 8 weeks after Mecp_SpCas9 virus injection in compare to control animals injected with Mecp2_SpCas9 virus only.

[00143] Figure 60 shows cutting efficiency of Dnmt loci in the brain, 8 weeks after Mecp_SpCas9 virus injection in compare to control animals injected with Mecp2_SpCas9 virus only.

[00144] Figure 61 shows Dnmt3a staining in the brain, 8 weeks after stereotaxic injection of virus encoding Mecp2_SpCas9 and gRNAs targeting Dnmt loci. Bottom panel shows magnification of ROI indicated on the upper panel.

[00145] Figure 62 shows staining of Syn_HA-SaCas9 in the dorsal hippocampus, 4 weeks after injection of virus. First column shows animal injected with Sa-Cas9 only, middle column animal injected with both SaCas9 and gRNAs against TETs loci and the right column represents animal injected with only gRNAs encoding virus. SaCas9 nuclear localization depends on the presence of gRNA.

[00146] Figure 63 shows SpCas9 in N2a cells. A) Targeting- and SpCas9 expression vector. B) Western Blot analysis of N2a cells expressing HA-tagged SpCas9 under the control of different promoters. C) Cutting efficiency of Dnmt loci. D) Western blot analysis demonstrating efficient knock down of Dnmt3a. e) Cutting efficiency of Tet loci.

[00147] Figure 64 shows SpCas9 in primary neurons. A) Schematic overview of SpCas9 cloning strategies used in this study. Short promoters and short polyA for efficient packaging into AAV delivering system. B) Schematic overview of combined multiplex targeting and nuclear envelope labeling strategy. C) Western blot analysis showing expression of HA-tagged SpCas9 under the control of rMap1b and mMecp2 promoter and bGH and spA signal. D) Immunocytochemistry demonstrating co-expression of SpCas9 and GFP-KASH in primary

neurons. SpCas9 under the control of the mMecp2 promoter is expressed in neurons (Map1b, NeuN) but not in astroglia cells (GFAP).

[00148] Figure 65 shows knock down of Dnmt3a in primary neurons. A) Immunocytochemistry demonstrating efficient knock down of Dnmt3a after targeting with multiplex targeting vector and mMecp2-SpCas9. B) Quantification of Dnmt3a antibody staining in control and targeted neurons. C) Western blot analysis demonstrating reduced Dnmt3a protein level. D) Quantification of Western Blot analysis demonstrating a total knock down of Dnmt3a protein level of approx. 75% in a mixed primary neuron culture (neurons and astroglia).

[00149] Figure 66 shows knock down of Dnmt3a in vivo. A) Cutting efficiency of Dnmt loci in the brain, 8 weeks after Mecp_SpCas9 virus injection in compare to control animals injected with Mecp2_SpCas9 virus only. B) Western blot analysis showing reduced Dnmt3a protein level in targeted neuronal nuclei (KASH-GFP positive) compared to control nuclei (RubyDye positive) after sorting cell nuclei using FACS.

[00150] Figure 67 shows expression of SaCas9 in primary neurons. A) Size of SaCas9 expression vector using hSynapsin promoter and bGH signal. B) Expression of SaCas9 in primary neurons (NeuN) but not in astroglia (GFAP). C) Extranuclear localization of SaCas9 in absence of gRNA. C' Higher magnification of SaCas9 positive neurons shown in C). D) Nuclear localization of SaCas9 in presence of gRNA . D') Higher magnification of SaCas9 positive neurons shown in D). E) Western blot analysis demonstrating expression of HA-tagged SaCas9 and GFP-KASH. F) Cutting efficiency of Dnmt loci 1 week after AAV infection.

[00151] Figure 68 shows gRNA dependent nuclear localization of SaCas9. A) Confocal imaging analysis demonstrating extranuclear localization of SaCas9 in absence of gRNA in primary neurons. B) Nuclear localization of SaCas9 in presence of gRNA. C) Line Scan analysis of confocal picture A) showing extranuclear localization of SaCas9 in absence of gRNA (red, SaCas9 signal; blue, DAPI signal; green, GFP-KASH signal . D) Line Scan analysis of confocal picture B) showing nuclear localization of SaCas9 in presence of gRNA (red, SaCas9 signal; blue, DAPI signal; green, GFP-KASH signal). E) Subcellular localization of SaCas9 and SpCas9 under conditions without (-) and with (+) gRNA in N2a cells. SaCas9 signal at 250 kDa in the cytoplasm fraction (Tubulin positive) indicating dimerization of SaCas9 in the cytoplasm. In the presence of gRNA a shift of SaCas9 protein into the nuclear fraction (Sun2 positive) is visible. SaCas9 signal at 100 kDa indicates a gRNA dependent formation of SaCas9 homomers and

transport into the cell nucleus. In contrast, SpCas9 is mainly present as homomer and its nuclear localization is independent of gRNA.

[00152] Figure 69 shows an AAV-Sa-Cas9 vector, a liver-specific AAV-Sa-Cas9 vector and an alternate AAV-Sa-Cas9 vector.

[00153] Figure 70 shows data on optimized CMV-SaCas9-NLS-U6-sgRNA vector (submitted vector design last time); new data compares N'-term vs C'-term tagged SaCas9 and shows enhanced cleavage efficiency using C'-term NLS tagging.

[00154] Figure 71 shows SURVEYOR image showing indels generated by new Pcsk9 targets.

[00155] Figure 72 shows SaCas9 specificity: genome-wide off target sites (GWOTs) are predicted based on 2 criteria: they contain 4 or fewer mismatched bases to intended SaCas9 target and bear the least restrictive PAM for SaCas9, NNGRR. HEK 293FT cells are transfected with either SpCas9 or SaCas9 with their corresponding sgRNAs at a target site (EMX1: TAGGGTTAGGGGCCCCAGGC) that has CGGGGT as a PAM so that it can be cut by either SpCas9 (CGG) or SaCas9 (CGGGGT). DNAs from cells are harvested and analyzed for indels by Illumina sequencing at on-target and 41 predicted off-target loci (following protocols from Hsu et al. Nature Biotech 2013 and data analysis pipeline developed by David Scott and Josh Weinstein).

[00156] Figure 73 shows that that SaCas9 may have a higher level of off-target activity than SpCas9 at certain loci.

[00157] The figures herein are for illustrative purposes only and are not necessarily drawn to scale.

DETAILED DESCRIPTION OF THE INVENTION

[00158] The invention relates to the engineering and optimization of systems, methods and compositions used for the control of gene expression involving sequence targeting, such as genome perturbation or gene-editing, that relate to the CRISPR-Cas system and components thereof. In advantageous embodiments, the Cas enzyme is Cas9.

[00159] An advantage of the present methods is that the CRISPR system avoids off-target binding and its resulting side effects. This is achieved using systems arranged to have a high degree of sequence specificity for the target DNA.

Cas9

[00160] Cas9 optimization may be used to enhance function or to develop new functions, one can generate chimeric Cas9 proteins. Examples that the Applicants have generated are provided in Example 6. Chimeric Cas9 proteins can be made by combining fragments from different Cas9 homologs. For example, two example chimeric Cas9 proteins from the Cas9s described herein. For example, Applicants fused the N-term of St1Cas9 (fragment from this protein is in bold) with C-term of SpCas9. The benefit of making chimeric Cas9s include any or all of: reduced toxicity; improved expression in eukaryotic cells; enhanced specificity; reduced molecular weight of protein, for example, making the protein smaller by combining the smallest domains from different Cas9 homologs; and/or altering the PAM sequence requirement.

[00161] The Cas9 may be used as a generic DNA binding protein. For example, and as shown in Example 7, Applicants used Cas9 as a generic DNA binding protein by mutating the two catalytic domains (D10 and H840) responsible for cleaving both strands of the DNA target. In order to upregulate gene transcription at a target locus Applicants fused a transcriptional activation domain (VP64) to Cas9. Other transcriptional activation domains are known. As shown in Example 17, transcriptional activation is possible. As also shown in Example 17, gene repression (in this case of the beta-catenin gene) is possible using a Cas9 repressor (DNA-binding domain) that binds to the target gene sequence, thus repressing its activity.

[00162] Cas9 and one or more guide RNA can be delivered using adeno associated virus (AAV), lentivirus, adenovirus or other plasmid or viral vector types, in particular, using formulations and doses from, for example, US Patents Nos. 8,454,972 (formulations, doses for adenovirus), 8,404,658 (formulations, doses for AAV) and 5,846,946 (formulations, doses for DNA plasmids) and from clinical trials and publications regarding the clinical trials involving lentivirus, AAV and adenovirus. For examples, for AAV, the route of administration, formulation and dose can be as in US Patent No. 8,454,972 and as in clinical trials involving AAV. For Adenovirus, the route of administration, formulation and dose can be as in US Patent No. 8,404,658 and as in clinical trials involving adenovirus. For plasmid delivery, the route of administration, formulation and dose can be as in US Patent No 5,846,946 and as in clinical studies involving plasmids. Doses may be based on or extrapolated to an average 70 kg individual, and can be adjusted for patients, subjects, mammals of different weight and species. Frequency of administration is within the ambit of the medical or veterinary practitioner (e.g.,

physician, veterinarian), depending on usual factors including the age, sex, general health, other conditions of the patient or subject and the particular condition or symptoms being addressed.

[00163] The viral vectors can be injected into the tissue of interest. For cell-type specific genome modification, the expression of Cas9 can be driven by a cell-type specific promoter. For example, liver-specific expression might use the Albumin promoter and neuron-specific expression might use the Synapsin I promoter.

Transgenic animals and plants

[00164] Transgenic animals are also provided. Preferred examples include animals comprising Cas9, in terms of polynucleotides encoding Cas9 or the protein itself. Mice, rats and rabbits are preferred. To generate transgenic mice with the constructs, as exemplified herein one may inject pure, linear DNA into the pronucleus of a zygote from a pseudo pregnant female, e.g. a CB56 female. Founders may then be identified, genotyped, and backcrossed to CB57 mice. The constructs may then be cloned and optionally verified, for instance by Sanger sequencing. Knock outs are envisaged where for instance one or more genes are knocked out in a model. However, are knockins are also envisaged (alone or in combination). An example knockin Cas9 mouse was generated and this is exemplified, but Cas9 knockins are preferred. To generate a Cas9 knock in mice one may target the same constitutive and conditional constructs to the Rosa26 locus, as described herein (Figs. 25A-B and 26). 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. In another embodiment, the 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.

[00165] Utility of the conditional Cas9 mouse: Applicants have shown in 293 cells that the Cas9 conditional expression construct can be activated by co-expression with Cre. Applicants also show that the correctly targeted R1 mESCs can have active Cas9 when Cre is expressed. Because Cas9 is followed by the P2A peptide cleavage sequence and then EGFP Applicants identify successful expression by observing EGFP. Applicants have shown Cas9 activation in mESCs. This same concept is what makes the conditional Cas9 mouse so useful. Applicants may cross their conditional Cas9 mouse with a mouse that ubiquitously expresses Cre (ACTB-Cre line) and may arrive at a mouse that expresses Cas9 in every cell. It should only take the delivery

of chimeric RNA to induce genome editing in embryonic or adult mice. Interestingly, if the conditional Cas9 mouse is crossed with a mouse expressing Cre under a tissue specific promoter, there should only be Cas9 in the tissues that also express Cre. This approach may be used to edit the genome in only precise tissues by delivering chimeric RNA to the same tissue.

[00166] As mentioned above, transgenic animals are also provided, as are transgenic plants, especially crops and algae. The transgenic plants may be useful in applications outside of providing a disease model. These may include food or feed production through expression of, for instance, higher protein, carbohydrate, nutrient or vitamin levels than would normally be seen in the wildtype. In this regard, transgenic plants, especially pulses and tubers, and animals, especially mammals such as livestock (cows, sheep, goats and pigs), but also poultry and edible insects, are preferred.

[00167] 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.

Adeno associated virus (AAV)

[00168] In terms of *in vivo* delivery, AAV is advantageous over other viral vectors for a couple of reasons:

[00169] Low toxicity (this may be due to the purification method not requiring ultra centrifugation of cell particles that can activate the immune response)

[00170] Low probability of causing insertional mutagenesis because it doesn't integrate into the host genome.

[00171] AAV has a packaging limit of 4.5 or 4.75 Kb. This means that Cas9 as well as a promoter and transcription terminator have to be all fit into the same viral vector. Constructs larger than 4.5 or 4.75 Kb will lead to significantly reduced virus production. SpCas9 is quite large, the gene itself is over 4.1 Kb, which makes it difficult for packing into AAV. Therefore embodiments of the invention include utilizing homologs of Cas9 that are shorter. For example:

Species	Cas9 Size
Corynebacter diphtheriae	3252
Eubacterium ventriosum	3321
Streptococcus pasteurianus	3390
Lactobacillus farciminis	3378
Sphaerochaeta globus	3537

Azospirillum B510	3504
Gluconacetobacter diazotrophicus	3150
Neisseria cinerea	3246
Roseburia intestinalis	3420
Parvibaculum lavamentivorans	3111
Staphylococcus aureus	3159
Nitratifractor salsuginis DSM 16511	3396
Campylobacter lari CF89-12	3009
Streptococcus thermophilus LMD-9	3396

[00172] These species are therefore, in general, preferred Cas9 species. Applicants have shown delivery and *in vivo* mouse brain Cas9 expression data.

[00173] Two ways to package Cas9 coding nucleic acid molecules, e.g., DNA, into viral vectors to mediate genome modification *in vivo* are preferred:

[00174] To achieve NHEJ-mediated gene knockout:

[00175] Single virus vector:

Vector containing two or more expression cassettes:

Promoter-Cas9 coding nucleic acid molecule -terminator

Promoter-gRNA1-terminator

Promoter-gRNA2-terminator

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

[00176] Double virus vector:

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

Promoter-Cas9 coding nucleic acid molecule-terminator

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

Promoter-gRNA1-terminator

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

[00177] To mediate homology-directed repair. In addition to the single and double virus vector approaches described above, an additional vector is used to deliver a homology-direct repair template.

[00178] Promoter used to drive Cas9 coding nucleic acid molecule expression can include:

[00179] AAV ITR can serve as a promoter: this is advantageous for eliminating the need for an additional promoter element (which can take up space in the vector). The additional space

freed up can be used to drive the expression of additional elements (gRNA, etc.). Also, ITR activity is relatively weaker, so can be used to reduce toxicity due to over expression of Cas9.

[00180] For ubiquitous expression, can use promoters: CMV, CAG, CBh, PGK, SV40, Ferritin heavy or light chains, etc.

[00181] For brain expression, can use promoters: SynapsinI for all neurons, CaMKIIalpha for excitatory neurons, GAD67 or GAD65 or VGAT for GABAergic neurons, etc.

[00182] For liver expression, can use Albumin promoter.

[00183] For lung expression, can use SP-B.

[00184] For endothelial cells, can use ICAM.

[00185] For hematopoietic cells can use IFNbeta or CD45.

[00186] For Osteoblasts can use OG-2.

[00187] Promoter used to drive guide RNA can include:

Pol III promoters such as U6 or H1

Use of Pol II promoter and intronic cassettes to express gRNA

[00188] As to AAV, the AAV can be AAV1, AAV2, AAV5 or any combination thereof. One can select the AAV of the AAV with regard to the cells to be targeted; e.g., one can select AAV serotypes 1, 2, 5 or a hybrid or capsid AAV1, AAV2, AAV5 or any combination thereof for targeting brain or neuronal cells; and one can select AAV4 for targeting cardiac tissue. AAV8 is useful for delivery to the liver. The above promoters and vectors are preferred individually.

[00189] RNA delivery is also a useful method of *in vivo* delivery. Fig. 27 shows delivery and *in vivo* mouse brain Cas9 expression data. It is possible to deliver Cas9 and gRNA (and, for instance, HR repair template) into cells using liposomes or nanoparticles. Thus delivery of the CRISPR enzyme, such as a Cas9 and/or delivery of the RNAs of the invention may be in RNA form and via microvesicles, liposomes or nanoparticles. For example, Cas9 mRNA and gRNA can be packaged into liposomal particles for delivery *in vivo*. Liposomal transfection reagents such as lipofectamine from Life Technologies and other reagents on the market can effectively deliver RNA molecules into the liver.

[00190] Enhancing NHEJ or HR efficiency is also helpful for delivery. It is preferred that NHEJ efficiency is enhanced by co-expressing end-processing enzymes such as Trex2 (Dumitrache et al. Genetics. 2011 August; 188(4): 787–797). It is preferred that HR efficiency is increased by transiently inhibiting NHEJ machineries such as Ku70 and Ku86. HR efficiency can

also be increased by co-expressing prokaryotic or eukaryotic homologous recombination enzymes such as RecBCD, RecA.

[00191] Various means of delivery are described herein, and further discussed in this section.

[00192] Viral delivery: The CRISPR enzyme, for instance a Cas9, and/or any of the present RNAs, for instance a guide RNA, can be delivered using adeno associated virus (AAV), lentivirus, adenovirus or other viral vector types, or combinations thereof. Cas9 and one or more guide RNAs can be packaged into one or more viral vectors. In some embodiments, the viral vector is delivered to the tissue of interest by, for example, an intramuscular injection, while other times the viral delivery is via intravenous, transdermal, intranasal, oral, mucosal, or other delivery methods. Such delivery may be either via a single dose, or multiple doses. One skilled in the art understands that the actual dosage to be delivered herein may vary greatly depending upon a variety of factors, such as the vector chose, the target cell, organism, or tissue, the general condition of the subject to be treated, the degree of transformation/modification sought, the administration route, the administration mode, the type of transformation/modification sought, etc.

[00193] Such a dosage may further contain, for example, a carrier (water, saline, ethanol, glycerol, lactose, sucrose, calcium phosphate, gelatin, dextran, agar, pectin, peanut oil, sesame oil, etc.), a diluent, a pharmaceutically-acceptable carrier (e.g., phosphate-buffered saline), a pharmaceutically-acceptable excipient, an adjuvant to enhance antigenicity, an immunostimulatory compound or molecule, and/or other compounds known in the art. The adjuvant herein may contain a suspension of minerals (alum, aluminum hydroxide, aluminum phosphate) on which antigen is adsorbed; or water-in-oil emulsion in which antigen solution is emulsified in oil (MF-59, Freund's incomplete adjuvant), sometimes with the inclusion of killed mycobacteria (Freund's complete adjuvant) to further enhance antigenicity (inhibits degradation of antigen and/or causes influx of macrophages). Adjuvants also include immunostimulatory molecules, such as cytokines, costimulatory molecules, and for example, immunostimulatory DNA or RNA molecules, such as CpG oligonucleotides. Such a dosage formulation is readily ascertainable by one skilled in the art. The dosage may further contain one or more pharmaceutically acceptable salts such as, for example, a mineral acid salt such as a hydrochloride, a hydrobromide, a phosphate, a sulfate, etc.; and the salts of organic acids such as acetates, propionates, malonates, benzoates, etc. Additionally, auxiliary substances, such as

wetting or emulsifying agents, pH buffering substances, gels or gelling materials, flavorings, colorants, microspheres, polymers, suspension agents, etc. may also be present herein. In addition, one or more other conventional pharmaceutical ingredients, such as preservatives, humectants, suspending agents, surfactants, antioxidants, anticaking agents, fillers, chelating agents, coating agents, chemical stabilizers, etc. may also be present, especially if the dosage form is a reconstitutable form. Suitable exemplary ingredients include microcrystalline cellulose, carboxymethylcellulose sodium, polysorbate 80, phenylethyl alcohol, chlorobutanol, potassium sorbate, sorbic acid, sulfur dioxide, propyl gallate, the parabens, ethyl vanillin, glycerin, phenol, parachlorophenol, gelatin, albumin and a combination thereof. A thorough discussion of pharmaceutically acceptable excipients is available in REMINGTON'S PHARMACEUTICAL SCIENCES (Mack Pub. Co., N.J. 1991) which is incorporated by reference herein.

[00194] In an embodiment herein the delivery is via an adenovirus, which may be at a single booster dose containing at least 1 x 10^5 particles (also referred to as particle units, pu) of adenoviral vector. In an embodiment herein, the dose preferably is at least about 1 x 10^6 particles (for example, about 1 x 10^6-1 x 10^{12} particles), more preferably at least about 1 x 10^7 particles, more preferably at least about 1 x 10^8 particles (e.g., about 1 x 10^8-1 x 10^{11} particles or about 1 x 10^8-1 x 10^{12} particles), and most preferably at least about 1 x 10^0 particles (e.g., about 1* x 10^9-1 x 10^{10} particles or about 1 x 10^9-1 x 10^{12} particles), or even at least about 1 x 10^{10} particles (e.g., about 1 x 10^{10}-1 x 10^{12} particles) of the adenoviral vector. Alternatively, the dose comprises no more than about 1 x 10^{14} particles, preferably no more than about 1 x 10^{13} particles, even more preferably no more than about 1 x 10^{12} particles, even more preferably no more than about 1 x 10^{11} particles, and most preferably no more than about 1 x 10^{10} particles (e.g., no more than about 1 x 10^9 articles). Thus, the dose may contain a single dose of adenoviral vector with, for example, about 1 x 10^6 particle units (pu), about 2 x 10^6 pu, about 4 x 10^6 pu, about 1 x 10^7 pu, about 2 x 10^7 pu, about 4 x 10^7 pu, about 1 x 10^8 pu, about 2 x 10^8 pu, about 4 x 10^8 pu, about 1 x 10^9 pu, about 2 x 10^9 pu, about 4 x 10^9 pu, about 1 x 10^{10} pu, about 2 x 10^{10} pu, about 4 x 10^{10} pu, about 1 x 10^{11} pu, about 2 x 10^{11} pu, about 4 x 10^{11} pu, about 1 x 10^{12} pu, about 2 x 10^{12} pu, or about 4 x 10^{12} pu of adenoviral vector. See, for example, the adenoviral vectors in U.S. Patent No. 8,454,972 B2 to Nabel, et. al., granted on June 4, 2013; incorporated by reference herein, and the dosages at col 29, lines 36-58 thereof. In an embodiment herein, the adenovirus is delivered via multiple doses.

[00195] In an embodiment herein, the delivery is via an AAV. A therapeutically effective dosage for in vivo delivery of the AAV to a human is believed to be in the range of from about 20 to about 50 ml of saline solution containing from about 1×10^{10} to about 1×10^{10} functional AAV/ml solution. The dosage may be adjusted to balance the therapeutic benefit against any side effects. In an embodiment herein, the AAV dose is generally in the range of concentrations of from about 1×10^{5} to 1×10^{50} genomes AAV, from about 1×10^{8} to 1×10^{20} genomes AAV, from about 1×10^{10} to about 1×10^{16} genomes, or about 1×10^{11} to about 1×10^{16} genomes AAV. A human dosage may be about 1×10^{13} genomes AAV. Such concentrations may be delivered in from about 0.001 ml to about 100 ml, about 0.05 to about 50 ml, or about 10 to about 25 ml of a carrier solution. Other effective dosages can be readily established by one of ordinary skill in the art through routine trials establishing dose response curves. See, for example, U.S. Patent No. 8,404,658 B2 to Hajjar, et al., granted on March 26, 2013, at col. 27, lines 45-60.

[00196] In an embodiment herein the delivery is via a plasmid. In such plasmid compositions, the dosage should be a sufficient amount of plasmid to elicit a response. For instance, suitable quantities of plasmid DNA in plasmid compositions can be from about 0.1 to about 2 mg, or from about 1 μg to about 10 μg.

[00197] The doses herein are based on an average 70 kg individual. The frequency of administration is within the ambit of the medical or veterinary practitioner (e.g., physician, veterinarian), or scientist skilled in the art.

Lentivirus

[00198] Lentiviruses are complex retroviruses that have the ability to infect and express their genes in both mitotic and post-mitotic cells. The most commonly known lentivirus is the human immunodeficiency virus (HIV), which uses the envelope glycoproteins of other viruses to target a broad range of cell types.

[00199] Lentiviruses may be prepared as follows. After cloning pCasES10 (which contains a lentiviral transfer plasmid backbone), HEK293FT at low passage (p=5) were seeded in a T-75 flask to 50% confluence the day before transfection in DMEM with 10% fetal bovine serum and without antibiotics. After 20 hours, media was changed to OptiMEM (serum-free) media and transfection was done 4 hours later. Cells were transfected with 10 μg of lentiviral transfer plasmid (pCasES10) and the following packaging plasmids: 5 μg of pMD2.G (VSV-g

pseudotype), and 7.5ug of psPAX2 (gag/pol/rev/tat). Transfection was done in 4mL OptiMEM with a cationic lipid delivery agent (50uL Lipofectamine 2000 and 100ul Plus reagent). After 6 hours, the media was changed to antibiotic-free DMEM with 10% fetal bovine serum.

[00200] Lentivirus may be purified as follows. Viral supernatants were harvested after 48 hours. Supernatants were first cleared of debris and filtered through a 0.45um low protein binding (PVDF) filter. They were then spun in a ultracentrifuge for 2 hours at 24,000 rpm. Viral pellets were resuspended in 50ul of DMEM overnight at 4C. They were then aliquotted and immediately frozen at -80C.

[00201] In another embodiment, minimal non-primate lentiviral vectors based on the equine infectious anemia virus (EIAV) are also contemplated, especially for ocular gene therapy (see, e.g., Balagaan, J Gene Med 2006; 8: 275 – 285, Published online 21 November 2005 in Wiley InterScience (www.interscience.wiley.com). DOI: 10.1002/jgm.845). In another embodiment, RetinoStat®, an equine infectious anemia virus-based lentiviral gene therapy vector that expresses angiostatic proteins endostain and angiostatin that is delivered via a subretinal injection for the treatment of the web form of age-related macular degeneration is also contemplated (see, e.g., Binley et al., HUMAN GENE THERAPY 23:980–991 (September 2012)) may be modified for the CRISPR-Cas system of the present invention.

[00202] In another embodiment, self-inactivating lentiviral vectors with an siRNA targeting a common exon shared by HIV tat/rev, a nucleolar-localizing TAR decoy, and an anti–CCR5-specific hammerhead ribozyme (see, e.g., DiGiusto et al. (2010) Sci Transl Med 2:36ra43) may be used/and or adapted to the CRISPR-Cas system of the present invention. A minimum of 2.5 × 10^6 CD34+ cells per kilogram patient weight may be collected and prestimulated for 16 to 20 hours in X-VIVO 15 medium (Lonza) containing 2mML-glutamine, stem cell factor (100 ng/ml), Flt-3 ligand (Flt-3L) (100 ng/ml), and thrombopoietin (10 ng/ml) (CellGenix) at a density of 2 × 10^6 cells/ml. Prestimulated cells may be transduced with lentiviral at a multiplicity of infection of 5 for 16 to 24 hours in 75-cm^2 tissue culture flasks coated with fibronectin (25 mg/cm^2) (RetroNectin,Takara Bio Inc.).

[00203] Lentiviral vectors have been disclosed as in the treatment for Parkinson's Disease, see, e.g., US Patent Publication No. 20120295960 and US Patent Nos. 7303910 and 7351585. Lentiviral vectors have also been disclosed for the treatment of ocular diseases, see e.g., US Patent Publication Nos. 20060281180, 20090007284, US20110117189; US20090017543;

US20070054961, US20100317109. Lentiviral vectors have also been disclosed for delivery to the train, see, e.g., US Patent Publication Nos. US20110293571; US20110293571, US20040013648, US20070025970, US20090111106 and US Patent No. US7259015.

RNA delivery

[00204] RNA delivery: The CRISPR enzyme, for instance a Cas9, and/or any of the present RNAs, for instance a guide RNA, can also be delivered in the form of RNA. Cas9 mRNA can be generated using *in vitro* transcription. For example, Cas9 mRNA can be synthesized using a PCR cassette containing the following elements: T7_promoter-kozak sequence (GCCACC)-Cas9-3' UTR from beta globin-polyA tail (a string of 120 or more adenines). The cassette can be used for transcription by T7 polymerase. Guide RNAs can also be transcribed using in vitro transcription from a cassette containing T7_promoter-GG-guide RNA sequence.

[00205] To enhance expression and reduce toxicity, the CRISPR enzyme and/or guide RNA can be modified using pseudo-U or 5-Methyl-C.

[00206] mRNA delivery methods are especially promising for liver delivery currently. In particular, for AAV8 is particularly preferred for delivery to the liver.

Nanoparticles

[00207] CRISPR enzyme mRNA and guide RNA may be delivered simultaneously using nanoparticles or lipid envelopes.

[00208] For example, Su X, Fricke J, Kavanagh DG, Irvine DJ ("In vitro and in vivo mRNA delivery using lipid-enveloped pH-responsive polymer nanoparticles" Mol Pharm. 2011 Jun 6;8(3):774-87. doi: 10.1021/mp100390w. Epub 2011 Apr 1) describes biodegradable core-shell structured nanoparticles with a poly(β-amino ester) (PBAE) core enveloped by a phospholipid bilayer shell. These were developed for in vivo mRNA delivery. The pH-responsive PBAE component was chosen to promote endosome disruption, while the lipid surface layer was selected to minimize toxicity of the polycation core. Such are, therefore, preferred for delivering RNA of the present invention.

[00209] In one embodiment, nanoparticles based on self assembling bioadhesive polymers are contemplated, which may be applied to oral delivery of peptides, intravenous delivery of peptides and nasal delivery of peptides, all to the brain. Other embodiments, such as oral absorption and ocular deliver of hydrophobic drugs are also contemplated. The molecular envelope technology involves an engineered polymer envelope which is protected and delivered

to the site of the disease (see, e.g., Mazza, M. et al. ACSNano, 2013. 7(2): 1016-1026; Siew, A., et al. Mol Pharm, 2012. 9(1):14-28; Lalatsa, A., et al. J Contr Rel, 2012. 161(2):523-36; Lalatsa, A., et al., Mol Pharm, 2012. 9(6):1665-80; Lalatsa, A., et al. Mol Pharm, 2012. 9(6):1764-74; Garrett, N.L., et al. J Biophotonics, 2012. 5(5-6):458-68; Garrett, N.L., et al. J Raman Spect, 2012. 43(5):681-688; Ahmad, S., et al. J Royal Soc Interface 2010. 7:S423-33; Uchegbu, I.F. Expert Opin Drug Deliv, 2006. 3(5):629-40; Qu, X.,et al. Biomacromolecules, 2006. 7(12):3452-9 and Uchegbu, I.F., et al. Int J Pharm, 2001. 224:185-199). Doses of about 5 mg/kg are contemplated, with single or multiple doses, depending on the target tissue.

[00210] In one embodiment, nanoparticles that can deliver RNA to a cancer cell to stop tumor growth developed by Dan Anderson's lab at MIT may be used/and or adapted to the CRISPR Cas system of the present invention. In particular, the Anderson lab developed fully automated, combinatorial systems for the synthesis, purification, characterization, and formulation of new biomaterials and nanoformulations. See, e.g., Alabi et al., Proc Natl Acad Sci U S A. 2013 Aug 6;110(32):12881-6; Zhang et al., Adv Mater. 2013 Sep 6;25(33):4641-5; Jiang et al., Nano Lett. 2013 Mar 13;13(3):1059-64; Karagiannis et al., ACS Nano. 2012 Oct 23;6(10):8484-7; Whitehead et al., ACS Nano. 2012 Aug 28;6(8):6922-9 and Lee et al., Nat Nanotechnol. 2012 Jun 3;7(6):389-93.

[00211] US patent application 20110293703 relates to lipidoid compounds are also particularly useful in the administration of polynucleotides, which may be applied to deliver the CRISPR Cas system of the present invention. In one aspect, the aminoalcohol lipidoid compounds are combined with an agent to be delivered to a cell or a subject to form microparticles, nanoparticles, liposomes, or micelles. The agent to be delivered by the particles, liposomes, or micelles may be in the form of a gas, liquid, or solid, and the agent may be a polynucleotide, protein, peptide, or small molecule. The minoalcohol lipidoid compounds may be combined with other aminoalcohol lipidoid compounds, polymers (synthetic or natural), surfactants, cholesterol, carbohydrates, proteins, lipids, etc. to form the particles. These particles may then optionally be combined with a pharmaceutical excipient to form a pharmaceutical composition.

[00212] US Patent Publication No. 0110293703 also provides methods of preparing the aminoalcohol lipidoid compounds. One or more equivalents of an amine are allowed to react with one or more equivalents of an epoxide-terminated compound under suitable conditions to

form an aminoalcohol lipidoid compound of the present invention. In certain embodiments, all the amino groups of the amine are fully reacted with the epoxide-terminated compound to form tertiary amines. In other embodiments, all the amino groups of the amine are not fully reacted with the epoxide-terminated compound to form tertiary amines thereby resulting in primary or secondary amines in the aminoalcohol lipidoid compound. These primary or secondary amines are left as is or may be reacted with another electrophile such as a different epoxide-terminated compound. As will be appreciated by one skilled in the art, reacting an amine with less than excess of epoxide-terminated compound will result in a plurality of different aminoalcohol lipidoid compounds with various numbers of tails. Certain amines may be fully functionalized with two epoxide-derived compound tails while other molecules will not be completely functionalized with epoxide-derived compound tails. For example, a diamine or polyamine may include one, two, three, or four epoxide-derived compound tails off the various amino moieties of the molecule resulting in primary, secondary, and tertiary amines. In certain embodiments, all the amino groups are not fully functionalized. In certain embodiments, two of the same types of epoxide-terminated compounds are used. In other embodiments, two or more different epoxide-terminated compounds are used. The synthesis of the aminoalcohol lipidoid compounds is performed with or without solvent, and the synthesis may be performed at higher temperatures ranging from 30.-100 C., preferably at approximately 50.-90 C. The prepared aminoalcohol lipidoid compounds may be optionally purified. For example, the mixture of aminoalcohol lipidoid compounds may be purified to yield an aminoalcohol lipidoid compound with a particular number of epoxide-derived compound tails. Or the mixture may be purified to yield a particular stereo- or regioisomer. The aminoalcohol lipidoid compounds may also be alkylated using an alkyl halide (e.g., methyl iodide) or other alkylating agent, and/or they may be acylated.

[00213] US Patent Publication No. 0110293703 also provides libraries of aminoalcohol lipidoid compounds prepared by the inventive methods. These aminoalcohol lipidoid compounds may be prepared and/or screened using high-throughput techniques involving liquid handlers, robots, microtiter plates, computers, etc. In certain embodiments, the aminoalcohol lipidoid compounds are screened for their ability to transfect polynucleotides or other agents (e.g., proteins, peptides, small molecules) into the cell.

[00214] US Patent Publication No. 20130302401 relates to a class of poly(beta-amino alcohols) (PBAAs) has been prepared using combinatorial polymerization. The inventive PBAAs

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

[00215] In another embodiment, lipid nanoparticles (LNPs) are contemplated. In particular, an antitransthyretin small interfering RNA encapsulated in lipid nanoparticles (see, e.g., Coelho et al., N Engl J Med 2013;369:819-29) may be applied to the CRISPR Cas system of the present invention. Doses of about 0.01 to about 1 mg per kg of body weight administered intravenously are contemplated. Medications to reduce the risk of infusion-related reactions are contemplated, such as dexamethasone, acetampinophen, diphenhydramine or cetirizine, and ranitidine are contemplated. Multiple doses of about 0.3 mg per kilogram every 4 weeks for five doses are also contemplated.

[00216] LNPs have been shown to be highly effective in delivering siRNAs to the liver (see, e.g., Tabernero et al., Cancer Discovery, April 2013, Vol. 3, No. 4, pages 363-470) and are therefore contemplated for delivering CRISPR Cas to the liver. A dosage of about four doses of 6 mg/kg of the LNP every two weeks may be contemplated. Tabernero et al. demonstrated that tumor regression was observed after the first 2 cycles of LNPs dosed at 0.7 mg/kg, and by the end of 6 cycles the patient had achieved a partial response with complete regression of the lymph node metastasis and substantial shrinkage of the liver tumors. A complete response was obtained after 40 doses in this patient, who has remained in remission and completed treatment after receiving doses over 26 months. Two patients with RCC and extrahepatic sites of disease including kidney, lung, and lymph nodes that were progressing following prior therapy with VEGF pathway inhibitors had stable disease at all sites for approximately 8 to 12 months, and a

patient with PNET and liver metastases continued on the extension study for 18 months (36 doses) with stable disease.

[00217] However, the charge of the LNP must be taken into consideration. As cationic lipids combined with negatively charged lipids to induce nonbilayer structures that facilitate intracellular delivery. Because charged LNPs are rapidly cleared from circulation following intravenous injection, ionizable cationic lipids with pKa values below 7 were developed (see, e.g., Rosin et al, Molecular Therapy, vol. 19, no. 12, pages 1286-2200, Dec. 2011). Negatively charged polymers such as siRNA oligonucleotides may be loaded into LNPs at low pH values (e.g., pH 4) where the ionizable lipids display a positive charge. However, at physiological pH values, the LNPs exhibit a low surface charge compatible with longer circulation times. Four species of ionizable cationic lipids have been focused upon, namely 1,2-dilineoyl-3-dimethylammonium-propane (DLinDAP), 1,2-dilinoleyloxy-3-N,N-dimethylaminopropane (DLinDMA), 1,2-dilinoleyloxy-keto-N,N-dimethyl-3-aminopropane (DLinKDMA), and 1,2-dilinoleyl-4-(2-dimethylaminoethyl)-[1,3]-dioxolane (DLinKC2-DMA). It has been shown that LNP siRNA systems containing these lipids exhibit remarkably different gene silencing properties in hepatocytes in vivo, with potencies varying according to the series DLinKC2-DMA>DLinKDMA>DLinDMA>>DLinDAP employing a Factor VII gene silencing model (see, e.g., Rosin et al, Molecular Therapy, vol. 19, no. 12, pages 1286-2200, Dec. 2011). A dosage of 1 μg/ml levels may be contemplated, especially for a formulation containing DLinKC2-DMA.

[00218] Preparation of LNPs and CRISPR Cas encapsulation may be used/and or adapted from Rosin et al, Molecular Therapy, vol. 19, no. 12, pages 1286-2200, Dec. 2011). The cationic lipids 1,2-dilineoyl-3-dimethylammonium-propane (DLinDAP), 1,2-dilinoleyloxy-3-N,N-dimethylaminopropane (DLinDMA), 1,2-dilinoleyloxyketo-N,N-dimethyl-3-aminopropane (DLinK-DMA), 1,2-dilinoleyl-4-(2-dimethylaminoethyl)-[1,3]-dioxolane (DLinKC2-DMA), (3-o-[2″ -(methoxypolyethyleneglycol 2000) succinoyl]-1,2-dimyristoyl-sn-glycol (PEG-S-DMG), and R-3-[(ω-methoxy-poly(ethylene glycol)2000) carbamoyl]-1,2-dimyristyloxlpropyl-3-amine (PEG-C-DOMG) may be provided by Tekmira Pharmaceuticals (Vancouver, Canada) or synthesized. Cholesterol may be purchased from Sigma (St Louis, MO). The specific CRISPR Cas RNA may be encapsulated in LNPs containing DLinDAP, DLinDMA, DLinK-DMA, and DLinKC2-DMA (cationic lipid:DSPC:CHOL: PEGS-DMG or PEG-C-DOMG at 40:10:40:10 molar ratios). When required, 0.2% SP-DiOC18 (Invitrogen, Burlington, Canada) may be

incorporated to assess cellular uptake, intracellular delivery, and biodistribution. Encapsulation may be performed by dissolving lipid mixtures comprised of cationic lipid:DSPC:cholesterol:PEG-c-DOMG (40:10:40:10 molar ratio) in ethanol to a final lipid concentration of 10 mmol/l. This ethanol solution of lipid may be added drop-wise to 50 mmol/l citrate, pH 4.0 to form multilamellar vesicles to produce a final concentration of 30% ethanol vol/vol. Large unilamellar vesicles may be formed following extrusion of multilamellar vesicles through two stacked 80 nm Nuclepore polycarbonate filters using the Extruder (Northern Lipids, Vancouver, Canada). Encapsulation may be achieved by adding RNA dissolved at 2 mg/ml in 50 mmol/l citrate, pH 4.0 containing 30% ethanol vol/vol drop-wise to extruded preformed large unilamellar vesicles and incubation at 31 °C for 30 minutes with constant mixing to a final RNA/lipid weight ratio of 0.06/1 wt/wt. Removal of ethanol and neutralization of formulation buffer were performed by dialysis against phosphate-buffered saline (PBS), pH 7.4 for 16 hours using Spectra/Por 2 regenerated cellulose dialysis membranes. Nanoparticle size distribution may be determined by dynamic light scattering using a NICOMP 370 particle sizer, the vesicle/intensity modes, and Gaussian fitting (Nicomp Particle Sizing, Santa Barbara, CA). The particle size for all three LNP systems may be ~70 nm in diameter. siRNA encapsulation efficiency may be determined by removal of free siRNA using VivaPureD MiniH columns (Sartorius Stedim Biotech) from samples collected before and after dialysis. The encapsulated RNA may be extracted from the eluted nanoparticles and quantified at 260 nm. siRNA to lipid ratio was determined by measurement of cholesterol content in vesicles using the Cholesterol E enzymatic assay from Wako Chemicals USA (Richmond, VA).

[00219] Preparation of large LNPs may be used/and or adapted from Rosin et al, Molecular Therapy, vol. 19, no. 12, pages 1286-2200, Dec. 2011. A lipid premix solution (20.4 mg/ml total lipid concentration) may be prepared in ethanol containing DLinKC2-DMA, DSPC, and cholesterol at 50:10:38.5 molar ratios. Sodium acetate may be added to the lipid premix at a molar ratio of 0.75:1 (sodium acetate:DLinKC2-DMA). The lipids may be subsequently hydrated by combining the mixture with 1.85 volumes of citrate buffer (10 mmol/l, pH 3.0) with vigorous stirring, resulting in spontaneous liposome formation in aqueous buffer containing 35% ethanol. The liposome solution may be incubated at 37 °C to allow for time-dependent increase in particle size. Aliquots may be removed at various times during incubation to investigate changes in liposome size by dynamic light scattering (Zetasizer Nano ZS, Malvern Instruments,

Worcestershire, UK). Once the desired particle size is achieved, an aqueous PEG lipid solution (stock = 10 mg/ml PEG-DMG in 35% (vol/vol) ethanol) may be added to the liposome mixture to yield a final PEG molar concentration of 3.5% of total lipid. Upon addition of PEG-lipids, the liposomes should their size, effectively quenching further growth. RNA may then be added to the empty liposomes at an siRNA to total lipid ratio of approximately 1:10 (wt:wt), followed by incubation for 30 minutes at 37 °C to form loaded LNPs. The mixture may be subsequently dialyzed overnight in PBS and filtered with a 0.45-μm syringe filter.

[00220] Spherical Nucleic Acid (SNA™) constructs and other nanoparticles (particularly gold nanoparticles) are also contemplate as a means to delivery CRISPR/Cas system to intended targets. Significant data show that AuraSense Therapeutics' Spherical Nucleic Acid (SNA™) constructs, based upon nucleic acid-functionalized gold nanoparticles, are superior to alternative platforms based on multiple key success factors, such as:

[00221] High in vivo stability. Due to their dense loading, a majority of cargo (DNA or siRNA) remains bound to the constructs inside cells, conferring nucleic acid stability and resistance to enzymatic degradation.

[00222] Deliverability. For all cell types studied (e.g., neurons, tumor cell lines, etc.) the constructs demonstrate a transfection efficiency of 99% with no need for carriers or transfection agents.

[00223] Therapeutic targeting. The unique target binding affinity and specificity of the constructs allow exquisite specificity for matched target sequences (i.e., limited off-target effects).

[00224] Superior efficacy. The constructs significantly outperform leading conventional transfection reagents (Lipofectamine 2000 and Cytofectin).

[00225] Low toxicity. The constructs can enter a variety of cultured cells, primary cells, and tissues with no apparent toxicity.

[00226] No significant immune response. The constructs elicit minimal changes in global gene expression as measured by whole-genome microarray studies and cytokine-specific protein assays.

[00227] Chemical tailorability. Any number of single or combinatorial agents (e.g., proteins, peptides, small molecules) can be used to tailor the surface of the constructs.

[00228] This platform for nucleic acid-based therapeutics may be applicable to numerous disease states, including inflammation and infectious disease, cancer, skin disorders and cardiovascular disease.

[00229] Citable literature includes: Cutler et al.,. J. Am. Chem. Soc. 2011 133:9254-9257, Hao et al., Small. 2011 7:3158-3162, Zhang et al., ACS Nano. 2011 5:6962-6970, Cutler et al., J. Am. Chem. Soc. 2012 134:1376-1391, Young et al.,. Nano Lett. 2012 12:3867-71, Zheng et al., Proc. Natl. Acad. Sci. USA. 2012 109:11975-80, Mirkin, Nanomedicine 2012 7:635-638 Zhang et al., J. Am. Chem. Soc. 2012 134:16488-1691, Weintraub, Nature 2013 495:S14-S16, Choi et al., Proc. Natl. Acad. Sci. USA. 2013 110(19):7625-7630, Jensen et al., Sci. Transl. Med. 5, 209ra152 (2013) and Mirkin, et al., Small, doi.org/10.1002/smll.201302143.

[00230] Self-assembling nanoparticles with siRNA may be constructed with polyethyleneimine (PEI) that is PEGylated with an Arg-Gly-Asp (RGD) peptide ligand attached at the distal end of the polyethylene glycol (PEG), for example, as a means to target tumor neovasculature expressing integrins and used to deliver siRNA inhibiting vascular endothelial growth factor receptor-2 (VEGF R2) expression and thereby tumor angiogenesis (see, e.g., Schiffelers et al., Nucleic Acids Research, 2004, Vol. 32, No. 19). Nanoplexes may be prepared by mixing equal volumes of aqueous solutions of cationic polymer and nucleic acid to give a net molar excess of ionizable nitrogen (polymer) to phosphate (nucleic acid) over the range of 2 to 6. The electrostatic interactions between cationic polymers and nucleic acid resulted in the formation of polyplexes with average particle size distribution of about 100 nm, hence referred to here as nanoplexes. A dosage of about 100 to 200 mg of CRISPR Cas is envisioned for delivery in the self-assembling nanoparticles of Schiffelers et al.

[00231] The nanoplexes of Bartlett et al. (PNAS, September 25, 2007,vol. 104, no. 39) may also be applied to the present invention. The nanoplexes of Bartlett et al. are prepared by mixing equal volumes of aqueous solutions of cationic polymer and nucleic acid to give a net molar excess of ionizable nitrogen (polymer) to phosphate (nucleic acid) over the range of 2 to 6. The electrostatic interactions between cationic polymers and nucleic acid resulted in the formation of polyplexes with average particle size distribution of about 100 nm, hence referred to here as nanoplexes. The DOTA-siRNA of Bartlett et al. was synthesized as follows: 1,4,7,10-tetraazacyclododecane-1,4,7,10-tetraacetic acid mono(N-hydroxysuccinimide ester) (DOTA-NHSester) was ordered from Macrocyclics (Dallas, TX). The amine modified RNA sense strand

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

[00232] Davis et al. (Nature, Vol 464, 15 April 2010) conducts a siRNA clinical trial that uses a targeted nanoparticle-delivery system (clinical trial registration number NCT00689065). Patients with solid cancers refractory to standard-of-care therapies are administered doses of targeted nanoparticles on days 1, 3, 8 and 10 of a 21-day cycle by a 30-min intravenous infusion. The nanoparticles consist of a synthetic delivery system containing: (1) a linear, cyclodextrin-based polymer (CDP), (2) a human transferrin protein (TF) targeting ligand displayed on the exterior of the nanoparticle to engage TF receptors (TFR) on the surface of the cancer cells, (3) a hydrophilic polymer (polyethylene glycol (PEG) used to promote nanoparticle stability in biological fluids), and (4) siRNA designed to reduce the expression of the RRM2 (sequence used in the clinic was previously denoted siR2B+5). The TFR has long been known to be upregulated in malignant cells, and RRM2 is an established anti-cancer target. These nanoparticles (clinical version denoted as CALAA-01) have been shown to be well tolerated in multi-dosing studies in non-human primates. Although a single patient with chronic myeloid leukaemia has been administered siRNAby liposomal delivery, Davis et al.'s clinical trial is the initial human trial to systemically deliver siRNA with a targeted delivery system and to treat patients with solid cancer. To ascertain whether the targeted delivery system can provide effective delivery of functional siRNA to human tumours, Davis et al. investigated biopsies from three patients from three different dosing cohorts; patients A, B and C, all of whom had metastatic melanoma and received CALAA-01 doses of 18, 24 and 30 mg m^{-2} siRNA, respectively. Similar doses may also be contemplated for the CRISPR Cas system of the present invention. The delivery of the invention may be achieved with nanoparticles containing a linear, cyclodextrin-based polymer

(CDP), a human transferrin protein (TF) targeting ligand displayed on the exterior of the nanoparticle to engage TF receptors (TFR) on the surface of the cancer cells and/or a hydrophilic polymer (for example, polyethylene glycol (PEG) used to promote nanoparticle stability in biological fluids).

Exosomes

[00233] Exosomes are endogenous nano-vesicles that transport RNAs and proteins which can deliver short interfering (si)RNA to the brain in mice. To reduce immunogenicity, Alvarez-Erviti et al. (2011, Nat Biotechnol 29: 341) used self-derived dendritic cells for exosome production. Targeting was achieved by engineering the dendritic cells to express Lamp2b, an exosomal membrane protein, fused to the neuron-specific RVG peptide3. Purified exosomes were loaded with exogenous siRNA by electroporation. Intravenously injected RVG-targeted exosomes delivered GAPDH siRNA specifically to neurons, microglia, oligodendrocytes in the brain, resulting in a specific gene knockdown. Pre-exposure to RVG exosomes did not attenuate knockdown, and non-specific uptake in other tissues was not observed. The therapeutic potential of exosome-mediated siRNA delivery was demonstrated by the strong mRNA (60%) and protein (62%) knockdown of BACE1, a therapeutic target in Alzheimer's disease.

[00234] To obtain a pool of immunologically inert exosomes, Alvarez-Erviti et al. harvested bone marrow from inbred C57BL/6 mice with a homogenous major histocompatibility complex (MHC) haplotype. As immature dendritic cells produce large quantities of exosomes devoid of T-cell activators such as MHC-II and CD86, Alvarez-Erviti et al. selected for dendritic cells with granulocyte/macrophage-colony stimulating factor (GM-CSF) for 7 d. Exosomes were purified from the culture supernatant the following day using well-established ultracentrifugation protocols. The exosomes produced were physically homogenous, with a size distribution peaking at 80 nm in diameter as determined by nanoparticle tracking analysis (NTA) and electron microscopy. Alvarez-Erviti et al. obtained 6-12 μg of exosomes (measured based on protein concentration) per 10^6 cells.

[00235] Next, Alvarez-Erviti et al. investigated the possibility of loading modified exosomes with exogenous cargoes using electroporation protocols adapted for nanoscale applications. As electroporation for membrane particles at the nanometer scale is not well-characterized, nonspecific Cy5-labeled siRNA was used for the empirical optimization of the electroporation protocol. The amount of encapsulated siRNA was assayed after ultracentrifugation and lysis of

exosomes. Electroporation at 400 V and 125 μF resulted in the greatest retention of siRNA and was used for all subsequent experiments.

[00236] Alvarez-Erviti et al. administered 150 μg of each BACE1 siRNA encapsulated in 150 μg of RVG exosomes to normal C57BL/6 mice and compared the knockdown efficiency to four controls: untreated mice, mice injected with RVG exosomes only, mice injected with BACE1 siRNA complexed to an in vivo cationic liposome reagent and mice injected with BACE1 siRNA complexed to RVG-9R, the RVG pep tide conjugated to 9 D-arginines that electrostatically binds to the siRNA. Cortical tissue samples were analyzed 3 d after administration and a significant protein knockdown (45%, $P < 0.05$, versus 62%, $P < 0.01$) in both siRNA-RVG-9R-treated and siRNARVG exosome-treated mice was observed, resulting from a significant decrease in BACE1 mRNA levels (66% [+ or -] 15%, $P < 0.001$ and 61% [+ or -] 13% respectively, $P < 0.01$). Moreover, Applicants demonstrated a significant decrease (55%, $P < 0.05$) in the total [beta]-amyloid 1-42 levels, a main component of the amyloid plaques in Alzheimer's pathology, in the RVG-exosome-treated animals. The decrease observed was greater than the β-amyloid 1-40 decrease demonstrated in normal mice after intraventricular injection of BACE1 inhibitors. Alvarez-Erviti et al. carried out 5'-rapid amplification of cDNA ends (RACE) on BACE1 cleavage product, which provided evidence of RNAi-mediated knockdown by the siRNA.

[00237] Finally, Alvarez-Erviti et al. investigated whether siRNA-RVG exosomes induced immune responses in vivo by assessing IL-6, IP-10, TNFα and IFN-α serum concentrations. Following siRNA-RVG exosome treatment, nonsignificant changes in all cytokines were registered similar to siRNA-transfection reagent treatment in contrast to siRNA-RVG-9R, which potently stimulated IL-6 secretion, confirming the immunologically inert profile of the exosome treatment. Given that exosomes encapsulate only 20% of siRNA, delivery with RVG-exosome appears to be more efficient than RVG-9R delivery as comparable mRNA knockdown and greater protein knockdown was achieved with fivefold less siRNA without the corresponding level of immune stimulation. This experiment demonstrated the therapeutic potential of RVG-exosome technology, which is potentially suited for long-term silencing of genes related to neurodegenerative diseases. The exosome delivery system of Alvarez-Erviti et al. may be applied to deliver the CRISPR-Cas system of the present invention to therapeutic targets, especially neurodegenerative diseases. A dosage of about 100 to 1000 mg of CRISPR Cas encapsulated in about 100 to 1000 mg of RVG exosomes may be contemplated for the present invention.

[00238] El-Andaloussi et al. (Nature Protocols 7,2112–2126(2012)) discloses how exosomes derived from cultured cells can be harnessed for delivery of siRNA in vitro and in vivo. This protocol first describes the generation of targeted exosomes through transfection of an expression vector, comprising an exosomal protein fused with a peptide ligand. Next, El-Andaloussi et al. explain how to purify and characterize exosomes from transfected cell supernatant. Next, El-Andaloussi et al. detail crucial steps for loading siRNA into exosomes. Finally, El-Andaloussi et al. outline how to use exosomes to efficiently deliver siRNA in vitro and in vivo in mouse brain. Examples of anticipated results in which exosome-mediated siRNA delivery is evaluated by functional assays and imaging are also provided. The entire protocol takes ~3 weeks. Delivery or administration according to the invention may be performed using exosomes produced from self-derived dendritic cells.

[00239] In another embodiment, the plasma exosomes of Wahlgren et al. (Nucleic Acids Research, 2012, Vol. 40, No. 17 e130) are contemplated. Exosomes are nano-sized vesicles (30–90nm in size) produced by many cell types, including dendritic cells (DC), B cells, T cells, mast cells, epithelial cells and tumor cells. These vesicles are formed by inward budding of late endosomes and are then released to the extracellular environment upon fusion with the plasma membrane. Because exosomes naturally carry RNA between cells, this property might be useful in gene therapy.

[00240] Exosomes from plasma are prepared by centrifugation of buffy coat at 900g for 20 min to isolate the plasma followed by harvesting cell supernatants, centrifuging at 300g for 10 min to eliminate cells and at 16 500g for 30 min followed by filtration through a 0.22 mm filter. Exosomes are pelleted by ultracentrifugation at 120 000g for70 min. Chemical transfection of siRNA into exosomes is carried out according to the manufacturer's instructions in RNAi Human/Mouse Starter Kit (Quiagen, Hilden, Germany). siRNA is added to 100 ml PBS at a final concentration of 2 mmol/ml. After adding HiPerFect transfection reagent, the mixture is incubated for 10 min at RT. In order to remove the excess of micelles, the exosomes are re-isolated using aldehyde/sulfate latex beads. The chemical transfection of CRISPR Cas into exosomes may be conducted similarly to siRNA. The exosomes may be co-cultured with monocytes and lymphocytes isolated from the peripheral blood of healthy donors. Therefore, it may be contemplated that exosomes containing CRISPR Cas may be introduced to monocytes

and lymphocytes of and autologously reintroduced into a human. Accordingly, delivery or administration according to the invention may beperformed using plasma exosomes.

Liposomes

[00241] Delivery or administration according to the invention can be performed with liposomes. Liposomes are spherical vesicle structures composed of a uni- or multilamellar lipid bilayer surrounding internal aqueous compartments and a relatively impermeable outer lipophilic phospholipid bilayer. Liposomes have gained considerable attention as drug delivery carriers because they are biocompatible, nontoxic, can deliver both hydrophilic and lipophilic drug molecules, protect their cargo from degradation by plasma enzymes, and transport their load across biological membranes and the blood brain barrier (BBB) (see, e.g., Spuch and Navarro, Journal of Drug Delivery, vol. 2011, Article ID 469679, 12 pages, 2011. doi:10.1155/2011/469679 for review).

[00242] Liposomes can be made from several different types of lipids; however, phospholipids are most commonly used to generate liposomes as drug carriers. Although liposome formation is spontaneous when a lipid film is mixed with an aqueous solution, it can also be expedited by applying force in the form of shaking by using a homogenizer, sonicator, or an extrusion apparatus (see, e.g., Spuch and Navarro, Journal of Drug Delivery, vol. 2011, Article ID 469679, 12 pages, 2011. doi:10.1155/2011/469679 for review).

[00243] Several other additives may be added to liposomes in order to modify their structure and properties. For instance, either cholesterol or sphingomyelin may be added to the liposomal mixture in order to help stabilize the liposomal structure and to prevent the leakage of the liposomal inner cargo. Further, liposomes are prepared from hydrogenated egg phosphatidylcholine or egg phosphatidylcholine, cholesterol, and dicetyl phosphate, and their mean vesicle sizes were adjusted to about 50 and 100 nm. (see, e.g., Spuch and Navarro, Journal of Drug Delivery, vol. 2011, Article ID 469679, 12 pages, 2011. doi:10.1155/2011/469679 for review).

[00244] Conventional liposome formulation is mainly comprised of natural phospholipids and lipids such as 1,2-distearoryl-sn-glycero-3-phosphatidyl choline (DSPC), sphingomyelin, egg phosphatidylcholines and monosialoganglioside. Since this formulation is made up of phospholipids only, liposomal formulations have encountered many challenges, one of the ones being the instability in plasma. Several attempts to overcome these challenges have been made,

specifically in the manipulation of the lipid membrane. One of these attempts focused on the manipulation of cholesterol. Addition of cholesterol to conventional formulations reduces rapid release of the encapsulated bioactive compound into the plasma or 1,2-dioleoyl-sn-glycero-3-phosphoethanolamine (DOPE) increases the stability (see, e.g., Spuch and Navarro, Journal of Drug Delivery, vol. 2011, Article ID 469679, 12 pages, 2011. doi:10.1155/2011/469679 for review).

[00245] In a particularly advantageous embodiment, Trojan Horse liposomes (also known as Molecular Trojan Horses) are desirable and protocols may be found at http://cshprotocols.cshlp.org/content/2010/4/pdb.prot5407.long. These particles allow delivery of a transgene to the entire brain after an intravascular injection. Without being bound by limitation, it is believed that neutral lipid particles with specific antibodies conjugated to surface allow crossing of the blood brain barrier via endocytosis. Applicant postulates utilizing Trojan Horse Liposomes to deliver the CRISPR family of nucleases to the brain via an intravascular injection, which would allow whole brain transgenic animals without the need for embryonic manipulation. About 1-5 g of DNA may be contemplated for in vivo administration in liposomes.

[00246] In another embodiment, the CRISPR Cas system may be administered in liposomes, such as a stable nucleic-acid-lipid particle (SNALP) (see, e.g., Morrissey et al., Nature Biotechnology, Vol. 23, No. 8, August 2005). Daily intravenous injections of about 1, 3 or 5 mg/kg/day of a specific CRISPR Cas targeted in a SNALP are contemplated. The daily treatment may be over about three days and then weekly for about five weeks. In another embodiment, a specific CRISPR Cas encapsulated SNALP) administered by intravenous injection to at doses of abpit 1 or 2.5 mg/kg are also contemplated (see, e.g., Zimmerman et al., Nature Letters, Vol. 441, 4 May 2006). The SNALP formulation may contain the lipids 3-N-[(wmethoxypoly(ethylene glycol) 2000) carbamoyl] -! ,2-dimyristyloxy-propylamine (PEG-C-DMA), 1,2-dilinoleyloxy-N,N-dimethyl-3-aminopropane (DLinDMA), 1,2-distearoyl-sn-glycero-3-phosphocholine (DSPC) and cholesterol, in a 2:40:10:48 molar per cent ratio (see, e.g., Zimmerman et al., Nature Letters, Vol. 441, 4 May 2006).

[00247] In another embodiment, stable nucleic-acid-lipid particles (SNALPs) have proven to be effective delivery molecules to highly vascularized HepG2-derived liver tumors but not in poorly vascularized HCT-116 derived liver tumors (see, e.g., Li, Gene Therapy (2012) 19, 775–780). The SNALP liposomes may be prepared by formulating D-Lin-DMA and PEG-C-DMA

with distearoylphosphatidylcholine (DSPC), Cholesterol and siRNA using a 25:1 lipid/siRNA ratio and a 48/40/10/2 molar ratio of Cholesterol/D-Lin-DMA/DSPC/PEG-C-DMA. The resulted SNALP liposomes are about 80–100 nm in size.

[00248] In yet another embodiment, a SNALP may comprise synthetic cholesterol (Sigma-Aldrich, St Louis, MO, USA), dipalmitoylphosphatidylcholine (Avanti Polar Lipids, Alabaster, AL, USA), 3-N-[(w-methoxy poly(ethylene glycol)2000)carbamoyl]-1,2-dimyrestyloxypropylamine, and cationic 1,2-dilinoleyloxy-3-N,Ndimethylaminopropane (see, e.g., Geisbert et al., Lancet 2010; 375: 1896-905). A dosage of about 2 mg/kg total CRISPR Cas per dose administered as, for example, a bolus intravenous infusion may be contemplated.

[00249] In yet another embodiment, a SNALP may comprise synthetic cholesterol (Sigma-Aldrich), 1,2-distearoyl-sn-glycero-3-phosphocholine (DSPC; Avanti Polar Lipids Inc.), PEG-cDMA, and 1,2-dilinoleyloxy-3-(N;N-dimethyl)aminopropane (DLinDMA) (see, e.g., Judge, J. Clin. Invest. 119:661-673 (2009)). Formulations used for in vivo studies may comprise a final lipid/RNA mass ratio of about 9:1.

[00250] The safety profile of RNAi nanomedicines has been reviewed by Barros and Gollob of Alnylam Pharmaceuticals (see, e.g., Advanced Drug Delivery Reviews 64 (2012) 1730–1737). The stable nucleic acid lipid particle (SNALP) is comprised of four different lipids — an ionizable lipid (DLinDMA) that is cationic at low pH, a neutral helper lipid, cholesterol, and a diffusible polyethylene glycol (PEG)-lipid. The particle is approximately 80 nm in diameter and is charge-neutral at physiologic pH. During formulation, the ionizable lipid serves to condense lipid with the anionic siRNA during particle formation. When positively charged under increasingly acidic endosomal conditions, the ionizable lipid also mediates the fusion of SNALP with the endosomal membrane enabling release of siRNA into the cytoplasm. The PEG-lipid stabilizes the particle and reduces aggregation during formulation, and subsequently provides a neutral hydrophilic exterior that improves pharmacokinetic properties.

[00251] To date, two clinical programs have been initiated using SNALPsiRNA formulations. Tekmira Pharmaceuticals recently completed a phase I single-dose study of SNALP-ApoB in adult volunteers with elevated LDL cholesterol. ApoB is predominantly expressed in the liver and jejunum and is essential for the assembly and secretion of VLDL and LDL. Seventeen subjects received a single dose of SNALP-ApoB (dose escalation across 7 dose levels). There was no evidence of liver toxicity (anticipated as the potential dose-limiting toxicity based on

preclinical studies). One (of two) subjects at the highest dose experienced flu-like symptoms consistent with immune system stimulation, and the decision was made to conclude the trial.

[00252] Alnylam Pharmaceuticals has similarly advanced ALN-TTR01, which employs the SNALP technology described above and targets hepatocyte production of both mutant and wild-type TTR to treat TTR amyloidosis (ATTR). Three ATTR syndromes have been described: familial amyloidotic polyneuropathy (FAP) and familial amyloidotic cardiomyopathy (FAC) —— both caused by autosomal dominant mutations in TTR; and senile systemic amyloidosis (SSA) cause by wildtype TTR. A placebo-controlled, single dose-escalation phase I trial of ALN-TTR01 was recently completed in patients with ATTR. ALN-TTR01 was administered as a 15-minute IV infusion to 31 patients (23 with study drug and 8 with placebo) within a dose range of 0.01 to 1.0 mg/kg (based on siRNA). Treatmentwaswell tolerated with no significant increases in liver function tests. Infusion-related reactions were noted in 3 of 23 patients at≥0.4 mg/kg; all responded to slowing of the infusion rate and all continued on study. Minimal and transient elevations of serum cytokines IL-6, IP-10 and IL-1ra were noted in two patients at the highest dose of 1 mg/kg (as anticipated from preclinical and NHP studies). Lowering of serum TTR, the expected pharmacodynamics effect of ALN-TTR01, was observed at 1 mg/kg.

[00253] In yet another embodiment, a SNALP may be made by solubilizing a cationic lipid, DSPC, cholesterol and PEG-lipid were solubilized in ethanol at a molar ratio of 40:10:40:10, respectively (see, Semple et al., Nature Niotechnology, Volume 28 Number 2 February 2010, pp. 172-177). The lipid mixture was added to an aqueous buffer (50 mM citrate, pH 4) with mixing to a final ethanol and lipid concentration of 30% (vol/vol) and 6.1 mg/ml, respectively, and allowed to equilibrate at 22 °C for 2 min before extrusion. The hydrated lipids were extruded through two stacked 80 nm pore-sized filters (Nuclepore) at 22 °C using a Lipex Extruder (Northern Lipids) until a vesicle diameter of 70–90 nm, as determined by dynamic light scattering analysis, was obtained. This generally required 1–3 passes. The siRNA (solubilized in a 50 mM citrate, pH 4 aqueous solution containing 30% ethanol) was added to the pre-equilibrated (35 °C) vesicles at a rate of ~5 ml/min with mixing. After a final target siRNA/lipid ratio of 0.06 (wt/wt) was reached, the mixture was incubated for a further 30 min at 35 °C to allow vesicle reorganization and encapsulation of the siRNA. The ethanol was then removed and the external buffer replaced with PBS (155 mM NaCl, 3 mM Na2HPO4, 1 mM KH2PO4, pH 7.5) by either dialysis or tangential flow diafiltration. siRNA were encapsulated in SNALP using

a controlled step-wise dilution method process. The lipid constituents of KC2-SNALP were DLin-KC2-DMA (cationic lipid), dipalmitoylphosphatidylcholine (DPPC; Avanti Polar Lipids), synthetic cholesterol (Sigma) and PEG-C-DMA used at a molar ratio of 57.1:7.1:34.3:1.4. Upon formation of the loaded particles, SNALP were dialyzed against PBS and filter sterilized through a 0.2 μm filter before use. Mean particle sizes were 75–85 nm and 90–95% of the siRNA was encapsulated within the lipid particles. The final siRNA/lipid ratio in formulations used for in vivo testing was ~0.15 (wt/wt). LNP-siRNA systems containing Factor VII siRNA were diluted to the appropriate concentrations in sterile PBS immediately before use and the formulations were administered intravenously through the lateral tail vein in a total volume of 10 ml/kg. This method may be extrapolated to the CRISPR Cas system of the present invention.

Other Lipids

[00254] Other cationic lipids, such as amino lipid 2,2-dilinoleyl-4-dimethylaminoethyl-[1,3]-dioxolane (DLin-KC2-DMA) may be utilized to encapsulate CRISPR Cas similar to SiRNA (see, e.g., Jayaraman, Angew. Chem. Int. Ed. 2012, 51, 8529 –8533). A preformed vesicle with the following lipid composition may be contemplated: amino lipid, distearoylphosphatidylcholine (DSPC), cholesterol and (R)-2,3-bis(octadecyloxy) propyl-1-(methoxy poly(ethylene glycol)2000)propylcarbamate (PEG-lipid) in the molar ratio 40/10/40/10, respectively, and a FVII siRNA/total lipid ratio of approximately 0.05 (w/w). To ensure a narrow particle size distribution in the range of 70–90 nm and a low polydispersity index of 0.11_0.04 (n=56), the particles may be extruded up to three times through 80 nm membranes prior to adding the CRISPR Cas RNA. Particles containing the highly potent amino lipid 16 may be used, in which the molar ratio of the four lipid components 16, DSPC, cholesterol and PEG-lipid (50/10/38.5/1.5) which may be further optimized to enhance in vivo activity.

[00255] Michael S D Kormann et al. ("Expression of therapeutic proteins after delivery of chemically modified mRNA in mice: Nature Biotechnology, Volume:29, Pages: 154–157 (2011) Published online 09 January 2011) describes the use of lipid envelopes to deliver RNA. Use of lipid envelopes is also preferred in the present invention.

[00256] In another embodiment, lipids may be formulated with the CRISPR Cas system of the present invention to form lipid nanoparticles (LNPs). Lipids include, but are not limited to, DLin-KC2-DMA4, C12-200 and colipids disteroylphosphatidyl choline, cholesterol, and PEG-

DMG may be formulated with CRISPR Cas instead of siRNA (see, e.g., Novobrantseva, Molecular Therapy--Nucleic Acids (2012) 1, e4; doi:10.1038/mtna.2011.3) using a spontaneous vesicle formation procedure. The component molar ratio may be about 50/10/38.5/1.5 (DLin-KC2-DMA or C12-200/disteroylphosphatidyl choline/cholesterol/PEG-DMG). The final lipid:siRNA weight ratio may be ~12:1 and 9:1 in the case of DLin-KC2-DMA and C12-200 lipid nanoparticles (LNPs), respectively. The formulations may have mean particle diameters of ~80 nm with >90% entrapment efficiency. A 3 mg/kg dose may be contemplated.

[00257] Tekmira has a portfolio of approximately 95 patent families, in the U.S. and abroad, that are directed to various aspects of LNPs and LNP formulations (see, e.g., U.S. Pat. Nos. 7,982,027; 7,799,565; 8,058,069; 8,283,333; 7,901,708; 7,745,651; 7,803,397; 8,101,741; 8,188,263; 7,915,399; 8,236,943 and 7,838,658 and European Pat. Nos .1766035; 1519714; 1781593 and 1664316), all of which may be used/and or adapted to the present invention.

[00258] The CRISPR Cas system may be delivered encapsulated in PLGA Microspheres such as that further described in US published applications 20130252281 and 20130245107 and 20130244279 (assigned to Moderna Therapeutics) which relate to aspects of formulation of compositions comprising modified nucleic acid molecules which may encode a protein, a protein precursor, or a partially or fully processed form of the protein or a protein precursor. The formulation may have a molar ratio 50:10:38.5:1.5-3.0 (cationic lipid:fusogenic lipid:cholesterol:PEG lipid). The PEG lipid may be selected from, but is not limited to PEG-c-DOMG, PEG-DMG. The fusogenic lipid may be DSPC. See also, Schrum et al., Delivery and Formulation of Engineered Nucleic Acids, US published application 20120251618.

[00259] Nanomerics' technology addresses bioavailability challenges for a broad range of therapeutics, including low molecular weight hydrophobic drugs, peptides, and nucleic acid based therapeutics (plasmid, siRNA, miRNA). Specific administration routes for which the technology has demonstrated clear advantages include the oral route, transport across the blood-brain-barrier, delivery to solid tumours, as well as to the eye. See, e.g., Mazza et al., 2013, ACS Nano. 2013 Feb 26;7(2):1016-26; Uchegbu and Siew, 2013, J Pharm Sci. 102(2):305-10 and Lalatsa et al., 2012, J Control Release. 2012 Jul 20;161(2):523-36.

[00260] US Patent Publication No. 20050019923 describes cationic dendrimers for delivering bioactive molecules, such as polynucleotide molecules, peptides and polypeptides and/or pharmaceutical agents, to a mammalian body. The dendrimers are suitable for targeting the

delivery of the bioactive molecules to, for example, the liver, spleen, lung, kidney or heart. Dendrimers are synthetic 3-dimensional macromolecules that are prepared in a step-wise fashion from simple branched monomer units, the nature and functionality of which can be easily controlled and varied. Dendrimers are synthesised from the repeated addition of building blocks to a multifunctional core (divergent approach to synthesis), or towards a multifunctional core (convergent approach to synthesis) and each addition of a 3-dimensional shell of building blocks leads to the formation of a higher generation of the dendrimers. Polypropylenimine dendrimers start from a diaminobutane core to which is added twice the number of amino groups by a double Michael addition of acrylonitrile to the primary amines followed by the hydrogenation of the nitriles. This results in a doubling of the amino groups. Polypropylenimine dendrimers contain 100% protonable nitrogens and up to 64 terminal amino groups (generation 5, DAB 64). Protonable groups are usually amine groups which are able to accept protons at neutral pH. The use of dendrimers as gene delivery agents has largely focused on the use of the polyamidoamine. and phosphorous containing compounds with a mixture of amine/amide or N--P(O_2)S as the conjugating units respectively with no work being reported on the use of the lower generation polypropylenimine dendrimers for gene delivery. Polypropylenimine dendrimers have also been studied as pH sensitive controlled release systems for drug delivery and for their encapsulation of guest molecules when chemically modified by peripheral amino acid groups. The cytotoxicity and interaction of polypropylenimine dendrimers with DNA as well as the transfection efficacy of DAB 64 has also been studied.

[00261] US Patent Publication No. 20050019923 is based upon the observation that, contrary to earlier reports, cationic dendrimers, such as polypropylenimine dendrimers, display suitable properties, such as specific targeting and low toxicity, for use in the targeted delivery of bioactive molecules, such as genetic material. In addition, derivatives of the cationic dendrimer also display suitable properties for the targeted delivery of bioactive molecules. See also, Bioactive Polymers, US published application 20080267903, which discloses "Various polymers, including cationic polyamine polymers and dendrimeric polymers, are shown to possess anti-proliferative activity, and may therefore be useful for treatment of disorders characterised by undesirable cellular proliferation such as neoplasms and tumours, inflammatory disorders (including autoimmune disorders), psoriasis and atherosclerosis. The polymers may be used alone as active agents, or as delivery vehicles for other therapeutic agents, such as drug

molecules or nucleic acids for gene therapy. In such cases, the polymers' own intrinsic anti-tumour activity may complement the activity of the agent to be delivered."

Supercharged proteins

[00262] Supercharged proteins are a class of engineered or naturally occurring proteins with unusually high positive or negative net theoretical charge. Both supernegatively and superpositively charged proteins exhibit a remarkable ability to withstand thermally or chemically induced aggregation. Superpositively charged proteins are also able to penetrate mammalian cells. Associating cargo with these proteins, such as plasmid DNA, siRNA, or other proteins, can enable the functional delivery of these macromolecules into mammalian cells both in vitro and in vivo. David Liu's lab reported the creation and characterization of supercharged proteins in 2007 (Lawrence et al., 2007, Journal of the American Chemical Society 129, 10110–10112).

[00263] The nonviral delivery of siRNA and plasmid DNA into mammalian cells are valuable both for research and therapeutic applications (Akinc et al., 2010, Nat. Biotech. 26, 561–569). Purified +36 GFP protein (or other superpositively charged protein) is mixed with siRNAs in the appropriate serum-free media and allowed to complex prior addition to cells. Inclusion of serum at this stage inhibits formation of the supercharged protein-siRNA complexes and reduces the effectiveness of the treatment. The following protocol has been found to be effective for a variety of cell lines (McNaughton et al., 2009, Proc. Natl. Acad. Sci. USA 106, 6111–6116). However, pilot experiments varying the dose of protein and siRNA should be performed to optimize the procedure for specific cell lines.

(1) One day before treatment, plate 1 x 10^5 cells per well in a 48-well plate.

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

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

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

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

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

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

[00264] David Liu's lab has further found +36 GFP to be an effective plasmid delivery reagent in a range of cells. As plasmid DNA is a larger cargo than siRNA, proportionately more +36 GFP protein is required to effectively complex plasmids. For effective plasmid delivery Applicants have developed a variant of +36 GFP bearing a C-terminal HA2 peptide tag, a known endosome-disrupting peptide derived from the influenza virus hemagglutinin protein. The following protocol has been effective in a variety of cells, but as above it is advised that plasmid DNA and supercharged protein doses be optimized for specific cell lines and delivery applications.

(1) One day before treatment, plate 1 x 10^5 per well in a 48-well plate.

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

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

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

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

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

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

[00265] See also, e.g., McNaughton et al., Proc. Natl. Acad. Sci. USA 106, 6111-6116 (2009); Cronican et al., ACS Chemical Biology 5, 747-752 (2010); Cronican et al., Chemistry & Biology 18, 833-838 (2011); Thompson et al., Methods in Enzymology 503, 293-319 (2012); Thompson, D.B., et al., Chemistry & Biology 19 (7), 831-843 (2012). The methods of the super charged proteins may be used and/or adapted for delivery of the CRISPR Cas system of the present invention.

Implantable devices

[00266] In another embodiment, implantable devices are also contemplated for delivery of the CRISPR Cas system. For example, US Patent Publication 20110195123 discloses an implantable medical device which elutes a drug locally and in prolonged period is provided, including several types of such a device, the treatment modes of implementation and methods of implantation. The device comprising of polymeric substrate, such as a matrix for example, that is used as the device body, and drugs, and in some cases additional scaffolding materials, such as metals or additional polymers, and materials to enhance visibility and imaging. The selection of drug is based on the advantageous of releasing drug locally and in prolonged period, where drug is released directly to the extracellular matrix (ECM) of the diseased area such as tumor, inflammation, degeneration or for symptomatic objectives, or to injured smooth muscle cells, or for prevention. One kind of drug is the gene silencing drugs based on RNA interference (RNAi), including but not limited to si RNA, sh RNA, or antisense RNA/DNA, ribozyme and nucleoside analogs. Therefore, this system may be used/and or adapted to the CRISPR Cas system of the present invention. The modes of implantation in some embodiments are existing implantation procedures that are developed and used today for other treatments, including brachytherapy and needle biopsy. In such cases the dimensions of the new implant described in this invention are similar to the original implant. Typically a few devices are implanted during the same treatment procedure.

[00267] As described in US Patent Publication 20110195123, there is provided a drug delivery implantable or insertable system, including systems applicable to a cavity such as the abdominal cavity and/or any other type of administration in which the drug delivery system is not anchored or attached, comprising a biostable and/or degradable and/or bioabsorbable polymeric substrate, which may for example optionally be a matrix. It should be noted that the term "insertion" also includes implantation. The drug delivery system is preferably implemented as a "Loder" as described in US Patent Publication 20110195123.

[00268] The polymer or plurality of polymers are biocompatible, incorporating an agent and/or plurality of agents, enabling the release of agent at a controlled rate, wherein the total volume of the polymeric substrate, such as a matrix for example, in some embodiments is optionally and preferably no greater than a maximum volume that permits a therapeutic level of the agent to be reached. As a non-limiting example, such a volume is preferably within the range of 0.1 m^3 to 1000 mm^3, as required by the volume for the agent load. The Loder may optionally

be larger, for example when incorporated with a device whose size is determined by functionality, for example and without limitation, a knee joint, an intra-uterine or cervical ring and the like.

[00269] The drug delivery system (for delivering the composition) is designed in some embodiments to preferably employ degradable polymers, wherein the main release mechanism is bulk erosion; or in some embodiments, non degradable, or slowly degraded polymers are used, wherein the main release mechanism is diffusion rather than bulk erosion, so that the outer part functions as membrane, and its internal part functions as a drug reservoir, which practically is not affected by the surroundings for an extended period (for example from about a week to about a few months). Combinations of different polymers with different release mechanisms may also optionally be used. The concentration gradient at the surface is preferably maintained effectively constant during a significant period of the total drug releasing period, and therefore the diffusion rate is effectively constant (termed "zero mode" diffusion). By the term "constant" it is meant a diffusion rate that is preferably maintained above the lower threshold of therapeutic effectiveness, but which may still optionally feature an initial burst and/or fluctuate, for example increasing and decreasing to a certain degree. The diffusion rate is preferably so maintained for a prolonged period, and it can be considered constant to a certain level to optimize the therapeutically effective period, for example the effective silencing period.

[00270] The drug delivery system optionally and preferably is designed to shield the nucleotide based therapeutic agent from degradation, whether chemical in nature or due to attack from enzymes and other factors in the body of the subject.

[00271] The drug delivery system as described in US Patent Publication 20110195123 is optionally associated with sensing and/or activation appliances that are operated at and/or after implantation of the device, by non and/or minimally invasive methods of activation and/or acceleration/deceleration, for example optionally including but not limited to thermal heating and cooling, laser beams, and ultrasonic, including focused ultrasound and/or RF (radiofrequency) methods or devices.

[00272] According to some embodiments of US Patent Publication 20110195123, the site for local delivery may optionally include target sites characterized by high abnormal proliferation of cells, and suppressed apoptosis, including tumors, active and or chronic inflammation and infection including autoimmune diseases states, degenerating tissue including muscle and

nervous tissue, chronic pain, degenerative sites, and location of bone fractures and other wound locations for enhancement of regeneration of tissue, and injured cardiac, smooth and striated muscle. The site for local delivery also may optionally include sites enabling performing preventive activities including pregnancy, prevention of infection and aging.

[00273] The site for implantation of the composition, or target site, preferably features a radius, area and/or volume that is sufficiently small for targeted local delivery. For example, the target site optionally has a diameter in a range of from about 0.1 mm to about 5 cm.

[00274] The location of the target site is preferably selected for maximum therapeutic efficacy. For example, the composition of the drug delivery system (optionally with a device for implantation as described above) is optionally and preferably implanted within or in the proximity of a tumor environment, or the blood supply associated thereof.

[00275] For example the composition (optionally with the device) is optionally implanted within or in the proximity to pancreas, prostate, breast, liver, via the nipple, within the vascular system and so forth.

[00276] The target location is optionally selected from the group consisting of (as non-limiting examples only, as optionally any site within the body may be suitable for implanting a Loder): 1. brain at degenerative sites like in Parkinson or Alzheimer disease at the basal ganglia, white and gray matter; 2. spine as in the case of amyotrophic lateral sclerosis (ALS); 3. uterine cervix to prevent HPV infection; 4. active and chronic inflammatory joints; 5. dermis as in the case of psoriasis; 6. sympathetic and sensoric nervous sites for analgesic effect; 7. Intra osseous implantation; 8. acute and chronic infection sites; 9. Intra vaginal; 10. Inner ear--auditory system, labyrinth of the inner ear, vestibular system; 11. Intra tracheal; 12. Intra-cardiac; coronary, epicardiac; 13. urinary bladder; 14. biliary system; 15. parenchymal tissue including and not limited to the kidney, liver, spleen; 16. lymph nodes; 17. salivary glands; 18. dental gums; 19. Intra-articular (into joints); 20. Intra-ocular; 21. Brain tissue; 22. Brain ventricles; 23. Cavities, including abdominal cavity (for example but without limitation, for ovary cancer); 24. Intra esophageal and 25. Intra rectal.

[00277] Optionally insertion of the system (for example a device containing the composition) is associated with injection of material to the ECM at the target site and the vicinity of that site to affect local pH and/or temperature and/or other biological factors affecting the diffusion of the drug and/or drug kinetics in the ECM, of the target site and the vicinity of such a site.

[00278] Optionally, according to some embodiments, the release of said agent could be associated with sensing and/or activation appliances that are operated prior and/or at and/or after insertion, by non and/or minimally invasive and/or else methods of activation and/or acceleration/deceleration, including laser beam, radiation, thermal heating and cooling, and ultrasonic, including focused ultrasound and/or RF (radiofrequency) methods or devices, and chemical activators.

[00279] According to other embodiments of US Patent Publication 20110195123, the drug preferably comprises a gene silencing biological RNAi drug, for example for localized cancer cases in breast, pancreas, brain, kidney, bladder, lung, and prostate as described below. Moreover, many drugs other than siRNA are applicable to be encapsulated in Loder, and can be used in association with this invention, as long as such drugs can be encapsulated with the Loder substrate, such as a matrix for example. Such drugs include approved drugs that are delivered today by methods other than of this invention, including Amphotericin B for fungal infection; antibiotics such as in osteomyelitis; pain killers such as narcotics; anti degenerative such as in Alzheimer or Parkinson diseases in a Loder implanted in the vicinity of the spine in the case of back pain. Such a system may be used and/or adapted to deliver the CRISPR Cas system of the present invention.

[00280] For example, for specific applications such as prevention of growth or regrowth of smooth muscle cells (that are injured during a stenting procedure and as a result tend to proliferate), the drug may optionally be siRNA that silence smooth muscle cells, including H19 silencing, or a drug selected from the group consisting of taxol, rapamycin and rapamycin-analogs. In such cases the Loder is preferably either a Drug Eluting Stent (DES), with prolonged release at constant rate, or a dedicated device that is implanted separately, in association to the stent. All of this may be used/and or adapted to the CRISPR Cas system of the present invention.

[00281] As another example of a specific application, neuro and muscular degenerative diseases develop due to abnormal gene expression. Local delivery of silencing RNAs may have therapeutic properties for interfering with such abnormal gene expression. Local delivery of anti apoptotic, anti inflammatory and anti degenerative drugs including small drugs and macromolecules may also optionally be therapeutic. In such cases the Loder is applied for

prolonged release at constant rate and/or through a dedicated device that is implanted separately. All of this may be used and/or adapted to the CRISPR Cas system of the present invention.

[00282] As yet another example of a specific application, psychiatric and cognitive disorders are treated with gene modifiers. Gene knockdown with silencing RNA is a treatment option. Loders locally delivering nucleotide based agents to central nervous system sites are therapeutic options for psychiatric and cognitive disorders including but not limited to psychosis, bi-polar diseases, neurotic disorders and behavioral maladies. The Loders could also deliver locally drugs including small drugs and macromolecules upon implantation at specific brain sites. All of this may be used and/or adapted to the CRISPR Cas system of the present invention.

[00283] As another example of a specific application, silencing of innate and/or adaptive immune mediators at local sites enables the prevention of organ transplant rejection. Local delivery of silencing RNAs and immunomodulating reagents with the Loder implanted into the transplanted organ and/or the implanted site renders local immune suppression by repelling immune cells such as CD8 activated against the transplanted organ. All of this may be used/and or adapted to the CRISPR Cas system of the present invention.

[00284] As another example of a specific application, vascular growth factors including VEGFs and angiogenin and others are essential for neovascularization. Local delivery of the factors, peptides, peptidomimetics, or suppressing their repressors is an important therapeutic modality; silencing the repressors and local delivery of the factors, peptides, macromolecules and small drugs stimulating angiogenesis with the Loder is therapeutic for peripheral, systemic and cardiac vascular disease.

[00285] The method of insertion, such as implantation, may optionally already be used for other types of tissue implantation and/or for insertions and/or for sampling tissues, optionally without modifications, or alternatively optionally only with non-major modifications in such methods. Such methods optionally include but are not limited to brachytherapy methods, biopsy, endoscopy with and/or without ultrasound, such as ERCP, stereotactic methods into the brain tissue, Laparoscopy, including implantation with a laparoscope into joints, abdominal organs, the bladder wall and body cavities.

CRISPR enzyme mRNA and guide RNA

[00286] CRISPR enzyme mRNA and guide RNA might also be delivered separately. CRISPR enzyme mRNA can be delivered prior to the guide RNA to give time for CRISPR enzyme to be

expressed. CRISPR enzyme mRNA might be administered 1-12 hours (preferably around 2-6 hours) prior to the administration of guide RNA.

[00287] Alternatively, CRISPR enzyme mRNA and guide RNA can be administered together. Advantageously, a second booster dose of guide RNA can be administered 1-12 hours (preferably around 2-6 hours) after the initial administration of CRISPR enzyme mRNA + guide RNA.

[00288] Additional administrations of CRISPR enzyme mRNA and/or guide RNA might be useful to achieve the most efficient levels of genome modification.

[00289] For minimization of toxicity and off-target effect, it will be important to control the concentration of CRISPR enzyme mRNA and guide RNA delivered. Optimal concentrations of CRISPR enzyme mRNA and guide RNA can be determined by testing different concentrations in a cellular or animal model and using deep sequencing the analyze the extent of modification at potential off-target genomic loci. For example, for the guide sequence targeting 5'-GAGTCCGAGCAGAAGAAGAA-3' in the EMX1 gene of the human genome, deep sequencing can be used to assess the level of modification at the following two off-target loci, 1: 5'-GAGTCCTAGCAGGAGAAGAA-3' and 2: 5'-GAGTCTAAGCAGAAGAAGAA-3'. The concentration that gives the highest level of on-target modification while minimizing the level of off-target modification should be chosen for in vivo delivery.

[00290] Alternatively, to minimize the level of toxicity and off-target effect, CRISPR enzyme nickase mRNA (for example S. pyogenes Cas9 with the D10A mutation) can be delivered with a pair of guide RNAs targeting a site of interest. The two guide RNAs need to be spaced as follows. Guide sequences in red (single underline) and blue (double underline) respectively (these examples are based on the PAM requirement for *Streptococcus pyogenes* Cas9).

Overhang length (bp)	Guide RNA design (guide sequence and PAM color coded)
14	5′- NNNNNNNNNNNNNNNNNNNNCCNNNNNNNNNNNNNNNNNNNNGGNNNNNNNNNNNN N-3′ 3′- NNNNNNNNNNNNNNNNNNNNGGNNNNNNNNNNNNNNNNNNNNCCNNNNNNNNNNNNNN N-5′
12	5′- NNNNNNNNNNNNNNNNNNNNCCNNNNNNNNNNNNNNNNNNNNGGNNNNNNNNNNNNNN N-3′
11	3′- NNNNNNNNNNNNNNNNNNNNGGNNNNNNNNNNNNNNNNNNNNCCNNNNNNNNNNNNNN N-5′
10	5′- NNNNNNNNNNNNNNNNNNNNCCNNNNNNNNNNNNNNNNNNNNGGNNNNNNNNNNNNNN N-3′
9	3′- NNNNNNNNNNNNNNNNNNNNGGNNNNNNNNNNNNNNNNNNNNCCNNNNNNNNNNNNNN N-5′
8	5′- NNNNNNNNNNNNNNNNNNNNCCNNNNNNNNNNNNNNNNNNNNGGNNNNNNNNNNNNNN N-3′ 3′- NNNNNNNNNNNNNNNNNNNNGGNNNNNNNNNNNNNNNNNNNNCCNNNNNNNNNNNNNN N-5′
7	5′- NNNNNNNNNNNNNNNNNNNNCCNNNNNNNNNNNNNNNNNNNNGGNNNNNNNNNNNNNN N-3′ 3′- NNNNNNNNNNNNNNNNNNNNGGNNNNNNNNNNNNNNNNNNNNCCNNNNNNNNNNNNNN N-5′
6	5′- NNNNNNNNNNNNNNNNNNNNCCNNNNNNNNNNNNNNNNNNNNGGNNNNNNNNNNNNNN N-3′ 3′- NNNNNNNNNNNNNNNNNNNNGGNNNNNNNNNNNNNNNNNNNNCCNNNNNNNNNNNNNN N-5′
5	5′- NNNNNNNNNNNNNNNNNNNNCCNNNNNNNNNNNNNNNNNNNNGGNNNNNNNNNNNNNN N-3′
4	3′- NNNNNNNNNNNNNNNNNNNNGGNNNNNNNNNNNNNNNNNNNNCCNNNNNNNNNNNNNN N-5′

3	5′- NNNNNNNNNNNNNNNNNNNNNNCCNNNNNNNNNNNNNNNNNNNNNGGNNNNNNNNNNNNNNNNNN
2	N-3′ 3′- NNNNNNNNNNNNNNNNNNNNGGNNNNNNNNNNNNNNNNNNCCNNNNNNNNNNNNNNNNNN
1	N-5′
blunt	5′- NNNNNNNNNNNNNNNNNNNNCCNNNNNNNNNNNNNNNNNNNNNGGNNNNNNNNNNNNNNNNNN N-3′ 3′- NNNNNNNNNNNNNNNNNNNNGGNNNNNNNNNNNNNNNNNNCCNNNNNNNNNNNNNNNNNN N-5′
2	5′- NNNNNNNNNNNNNNNNNNNNCCNNNNNNNNNNNNNNNNNNNNNGGNNNNNNNNNNNNNNNNNN N-3′
3	3′- NNNNNNNNNNNNNNNNNNNNGGNNNNNNNNNNNNNNNNNNCCNNNNNNNNNNNNNNNNNNNN N-5′
4	5′- NNNNNNNNNNNNNNNNNNNNCCNNNNNNNNNNNNNNNNNNNNNGGNNNNNNNNNNNNNNNNNN
5	N-3′ 3′- NNNNNNNNNNNNNNNNNNNNGGNNNNNNNNNNNNNNNNNNCCNNNNNNNNNNNNNNNNNN
6	N-5′
7	5′- NNNNNNNNNNNNNNNNNNNNCCNNNNNNNNNNNNNNNNNNNNNGGNNNNNNNNNNNNNNNNNNNN N-3′ 3′-
8	NNNNNNNNNNNNNNNNNNNNGGNNNNNNNNNNNNNNNNNNCCNNNNNNNNNNNNNNNNNNNN N-5′
12	5′-

	NNNNNNNNNNNNNNNNNNNNNCCNNNNNNNNNNNNNGGNNNNNNNNNNNNNNNNNNNNNNN
	N-3′
13	3′-
	NNNNNNNNNNNNNNNNNNNNNGGNNNNNNNNNNNNNCCNNNNNNNNNNNNNNNNNNNNNNN
	N-5′
14	
	5′-
	NNNNNNNNNNNNNNNNNNNNNCCNNNNNNNNNNNNNGGNNNNNNNNNNNNNNNNNNNNNNN
15	N-3′
	3′-
	NNNNNNNNNNNNNNNNNNNNNGGNNNNNNNNNNNNNCCNNNNNNNNNNNNNNNNNNNNNNN
16	N-5′
	5′-
17	NNNNNNNNNNNNNNNNNNNNNCCNNNNNNNNNNNNNGGNNNNNNNNNNNNNNNNNNNNNNN
	N-3′
	3′-
	NNNNNNNNNNNNNNNNNNNNNGGNNNNNNNNNNNNNCCNNNNNNNNNNNNNNNNNNNNNNN
	N-5′
	5′-
	NNNNNNNNNNNNNNNNNNNNNCCNNNNNNNNNNNNNGGNNNNNNNNNNNNNNNNNNNNNNN
	N-3′
	3′-
	NNNNNNNNNNNNNNNNNNNNNGGNNNNNNNNNNNNNCCNNNNNNNNNNNNNNNNNNNNNNN
	N-5′
	5′-
	NNNNNNNNNNNNNNNNNNNNNCCNNNNNNNNNNNNNGGNNNNNNNNNNNNNNNNNNNNNNN
	N-3′

	3′- NNNNNNNNNNNNNNNNNNNNGGNNNNNNNCCNNNNNNNNNNNNNNNNNNNNNNNNNNNNN N-5′ 5′- NNNNNNNNNNNNNNNNNNNNCCNNNNNNNGGNNNNNNNNNNNNNNNNNNNNNNNNNNNNN N-3′ 3′- NNNNNNNNNNNNNNNNNNNNGGNNNNNCCNNNNNNNNNNNNNNNNNNNNNNNNNNNNN N-5′ 5′- NNNNNNNNNNNNNNNNNNNNCCNNNNNNGGNNNNNNNNNNNNNNNNNNNNNNNNNNNNN N-3′ 3′- NNNNNNNNNNNNNNNNNNNNGGNNNNCCNNNNNNNNNNNNNNNNNNNNNNNNNNNNN N-5′ 5′- NNNNNNNNNNNNNNNNNNNNCCNNNNGGNNNNNNNNNNNNNNNNNNNNNNNNNNNNN N-3′ 3′- NNNNNNNNNNNNNNNNNNNNGGNNNCCNNNNNNNNNNNNNNNNNNNNNNNNNNNNN N-5′ 5′- NNNNNNNNNNNNNNNNNNNNCCNNNGGNNNNNNNNNNNNNNNNNNNNNNNNNNNNN N-3′ 3′- NNNNNNNNNNNNNNNNNNNNGGNNCCNNNNNNNNNNNNNNNNNNNNNNNNNNNNN N-5′ 5′- NNNNNNNNNNNNNNNNNNNNCCNNGGNNNNNNNNNNNNNNNNNNNNNNNNNNNNN N-3′ 3′- NNNNNNNNNNNNNNNNNNNNGGNNCCNNNNNNNNNNNNNNNNNNNNNNNNNNNNN

	N-5′ 5′- NNNNNNNNNNNNNNNNNNNNNNCCNGGNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNN N-3′ 3′- NNNNNNNNNNNNNNNNNNNNNGGNCCNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNN N-5′ 5′- NNNNNNNNNNNNNNNNNNNNNNCCGGNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNN N-3′ 3′- NNNNNNNNNNNNNNNNNNNNNGGCCNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNN N-5′ 5′- NNNNNNNNNNNNNNNNNNNNNNNNNGGNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNN N-3′ 3′- NNNNNNNNNNNNNNNNNNNNNNCCGGNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNN N-5′ 5′- NNNNNNNNNNNNNNNNNNNNNNNNCGGNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNN N-3′ 3′- NNNNNNNNNNNNNNNNNNNNNNCCNGGNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNN N-5′ 5′- NNNNNNNNNNNNNNNNNNNNNNNNCGGNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNN N-3′ 3′- NNNNNNNNNNNNNNNNNNNNNNCCNNGGNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNN N-5′

	5′ – NNNNNNNNNNNNNNNNNNNNNNNNCGGNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNN N–3′ 3′ – NNNNNNNNNNNNNNNNNNNNNNNNCCNNNGGNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNN N–5′ 5′ – NNNNNNNNNNNNNNNNNNNNNNNNCGGNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNN N–3′ 3′ – NNNNNNNNNNNNNNNNNNNNNNNNCCNNNNGGNNNNNNNNNNNNNNNNNNNNNNNNNNNNNN N–5′ 5′ – NNNNNNNNNNNNNNNNNNNNNNNNCGGNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNN N–3′ 3′ – NNNNNNNNNNNNNNNNNNNNNNNNCCNNNNGGNNNNNNNNNNNNNNNNNNNNNNNNNNNNNN N–5′

[00291] Further interrogation of the system have given Applicants evidence of the 5' overhang (see, e.g., Ran et al., Cell. 2013 Sep 12;154(6):1380-9 and US Provisional Patent Application Serial No. 61/871,301 filed August 28, 2013). Applicants have further identified parameters that relate to efficient cleavage by the Cas9 nickase mutant when combined with two guide RNAs and these parameters include but are not limited to the length of the 5' overhang. In embodiments of the invention the 5' overhang is at most 200 base pairs, preferably at most 100 base pairs, or more preferably at most 50 base pairs. In embodiments of the invention the 5' overhang is at least 26 base pairs, preferably at least 30 base pairs or more preferably 34-50 base pairs or 1-34 base pairs. In other preferred methods of the invention the first guide sequence directing cleavage of one strand of the DNA duplex near the first target sequence and the second guide sequence directing cleavage of other strand near the second target sequence results in a blunt cut or a 3' overhang. In embodiments of the invention the 3' overhang is at most 150, 100 or 25 base pairs or at least 15, 10 or 1 base pairs. In preferred embodiments the 3' overhang is 1-100 basepairs.

[00292] Aspects of the invention relate to the expression of the gene product being decreased or a template polynucleotide being further introduced into the DNA molecule encoding the gene product or an intervening sequence being excised precisely by allowing the two 5' overhangs to reanneal and ligate or the activity or function of the gene product being altered or the expression of the gene product being increased. In an embodiment of the invention, the gene product is a protein.

[00293] Only sgRNA pairs creating 5' overhangs with less than 8bp overlap between the guide sequences (offset greater than -8 bp) were able to mediate detectable indel formation. Importantly, each guide used in these assays is able to efficiently induce indels when paired with wildtype Cas9, indicating that the relative positions of the guide pairs are the most important parameters in predicting double nicking activity.

[00294] Since Cas9n and Cas9H840A nick opposite strands of DNA, substitution of Cas9n with Cas9H840A with a given sgRNA pair should result in the inversion of the overhang type. For example, a pair of sgRNAs that will generate a 5' overhang with Cas9n should in principle generate the corresponding 3' overhang instead. Therefore, sgRNA pairs that lead to the generation of a 3' overhang with Cas9n might be used with Cas9H840A to generate a 5' overhang. Unexpectedly, Applicants tested Cas9H840A with a set of sgRNA pairs designed to generate both 5' and 3' overhangs (offset range from –278 to +58 bp), but were unable to observe indel formation. Further work may be needed to identify the necessary design rules for sgRNA pairing to allow double nicking by Cas9H840A.

Liver, proprotein convertase subtilisin kexin 9 (PCSK9)

[00295] Proprotein convertase subtilisin kexin 9 (PCSK9) is a member of the subtilisin serine protease family. PCSK9 is primarily expressed by the liver and is critical for the down regulation of hepatocyte LDL receptor expression. LDL-C levels in plasma are highly elevated in humans with gain of function mutations in PCSK9, classifying them as having severe hypercholesterolemia. Therefore, PCSK9 is an attractive target for CRISPR. PCS9K-targeted CRISPR may be formulated in a lipid particle and for example administered at about 15, 45, 90, 150, 250 and 400 μg/kg intravenously (see, e.g., http://www.alnylam.com/capella/wp-content/uploads/2013/08/ALN-PCS02-001-Protocol-Lancet.pdf).

[00296] Bailey et al. (J Mol Med (Berl). 1999 Jan;77(1):244-9) discloses insulin delivery by ex-vivo somatic cell gene therapy involves the removal of non-B-cell somatic cells (e.g.

fibroblasts) from a diabetic patient, and genetically altering them in vitro to produce and secrete insulin. The cells can be grown in culture and returned to the donor as a source of insulin replacement. Cells modified in this way could be evaluated before implantation, and reserve stocks could be cryopreserved. By using the patient's own cells, the procedure should obviate the need for immunosuppression and overcome the problem of tissue supply, while avoiding a recurrence of cell destruction. Ex-vivo somatic cell gene therapy requires an accessible and robust cell type that is amenable to multiple transfections and subject to controlled proliferation. Special problems associated with the use of non-B-cell somatic cells include the processing of proinsulin to insulin, and the conferment of sensitivity to glucose-stimulated proinsulin biosynthesis and regulated insulin release. Preliminary studies using fibroblasts, pituitary cells, kidney (COS) cells and ovarian (CHO) cells suggest that these challenges could be met, and that ex-vivo somatic cell gene therapy offers a feasible approach to insulin replacement therapy. The system of Bailey et al. may be used/and or adapted to the CRISPR Cas system of the present invention for delivery to the liver.

[00297] The methods of Sato et al. (Nature Biotechnology Volume 26 Number 4 April 2008, Pages 431-442) may be applied to the CRISPR Cas system of the present invention for delivery to the liver. Sato et al. found that treatments with the siRNA-bearing vitamin A–coupled liposomes almost completely resolved liver fibrosis and prolonged survival in rats with otherwise lethal dimethylnitrosamine-induced liver cirrhosis in a dose- and duration-dependent manner. Cationic liposomes (Lipotrust) containing O,O'-ditetradecanoyl-N-(a-trimethylammonioacetyl) diethanolamine chloride (DC-6-14) as a cationic lipid, cholesterol and dioleoylphosphatidylethanolamine at a molar ratio of 4:3:3 (which has shown high transfection efficiency under serumcontaining conditions for in vitro and in vivo gene delivery) were purchased from Hokkaido System Science. The liposomes were manufactured using a freeze-dried empty liposomes method and prepared at a concentration of 1 mM (DC-16-4) by addition of double-distilled water (DDW) to the lyophilized lipid mixture under vortexing before use. To prepare VA-coupled liposomes, 200 nmol of vitamin A (retinol, Sigma) dissolved in DMSO was mixed with the liposome suspensions (100 nmol as DC-16-4) by vortexing in a 1.5 ml tube at 25 1C. To prepare VA-coupled liposomes carrying siRNAgp46 (VA-lip-siRNAgp46), a solution of siRNAgp46 (580 pmol/ml in DDW) was added to the retinol-coupled liposome solution with stirring at 25 C. The ratio of siRNA to DC-16-4 was 1:11.5 (mol/mol) and the siRNA to

liposome ratio (wt/wt) was 1:1. Any free vitamin A or siRNA that was not taken up by liposomes were separated from liposomal preparations using a micropartition system (VIVASPIN 2 concentrator 30,000 MWCO PES, VIVASCIENCE). The liposomal suspension was added to the filters and centrifuged at 1,500g for 5 min 3 times at 25 1C. Fractions were collected and the material trapped in the filter was reconstituted with PBS to achieve the desired dose for in vitro or in vivo use. Three injections of 0.75 mg/kg siRNA were given every other day to rats. The system of Sato et al. may be used/and or adapted to the CRISPR Cas system of the present invention for delivery to the liver by delivering about 0.5 to 1 mg/kg of CRISPR Cas RNA in the liposomes as described by Sato et al. to humans.

[00298] The methods of Rozema et al. (PNAS, August 7, 2007, vol. 104, no. 32) for a vehicle for the delivery of siRNA to hepatocytes both in vitro and in vivo, which Rozema et al. have named siRNA Dynamic PolyConjugates may also be applied to the present invention. Key features of the Dynamic Poly-Conjugate technology include a membrane-active polymer, the ability to reversibly mask the activity of this polymer until it reaches the acidic environment of endosomes, and the ability to target this modified polymer and its siRNA cargo specifically to hepatocytes in vivo after simple, low-pressure i.v. injection. SATA-modified siRNAs are synthesized by reaction of 5' aminemodified siRNA with 1 weight equivalents (wt eq) of Nsuccinimidyl-S-acetylthioacetate (SATA) reagent (Pierce) and 0.36 wt eq of $NaHCO_3$ in water at 4°C for 16 h. The modified siRNAs are then precipitated by the addition of 9 vol of ethanol and incubation at 80°C for 2 h. The precipitate is resuspended in 1X siRNA buffer (Dharmacon) and quantified by measuring absorbance at the 260-nm wavelength. PBAVE (30 mg/ml in 5mMTAPS, pH 9) is modified by addition of 1.5 wt % SMPT (Pierce). After a 1-h incubation, 0.8 mg of SMPT-PBAVE was added to 400 µl of isotonic glucose solution containing 5 mM TAPS (pH 9). To this solution was added 50 µg of SATA-modified siRNA. For the dose--response experiments where [PBAVE] was constant, different amounts of siRNA are added. The mixture is then incubated for 16 h. To the solution is then added 5.6 mg of Hepes free base followed by a mixture of 3.7 mg ofCDM-NAGand 1.9mg of CDM-PEG. The solution is then incubated for at least 1 h at room temperature before injection. CDM-PEG and CDM-NAG are synthesized from the acid chloride generated by using oxalyl chloride. To the acid chloride is added 1.1 molar equivalents polyethylene glycol monomethyl ether (molecular weight average of 450) to generate CDM-PEG or (aminoethoxy)ethoxy-2-(acetylamino)-2-deoxy-β-D-

glucopyranoside to generate CDM-NAG. The final product is purified by using reverse-phase HPLC with a 0.1% TFA water/acetonitrile gradient. About 25 to 50 µg of siRNA was delivered to mice. The system of Rozema et al. may be applied to the CRISPR Cas system of the present invention for delivery to the liver, for example by envisioning a dosage of about 50 to about 200 mg of CRISPR Cas for delivery to a human.

Bone

[00299] Oakes and Lieberman (Clin Orthop Relat Res. 2000 Oct;(379 Suppl):S101-12) discusses delivery of genes to the bone. By transferring genes into cells at a specific anatomic site, the osteoinductive properties of growth factors can be used at physiologic doses for a sustained period to facilitate a more significant healing response. The specific anatomic site, the quality of the bone, and the soft-tissue envelope, influences the selection of the target cells for regional gene therapy. Gene therapy vectors delivered to a treatment site in osteoconductive carriers have yielded promising results. Several investigators have shown exciting results using ex vivo and in vivo regional gene therapy in animal models. Such a system may be used/and or adapted to the CRISPR Cas system for delivery to the bone.

Brain

[00300] Delivery options for the brain include encapsulation of CRISPR enzyme and guide RNA in the form of either DNA or RNA into liposomes and conjugating to molecular Trojan horses for trans-blood brain barrier (BBB) delivery. Molecular Trojan horses have been shown to be effective for delivery of B-gal expression vectors into the brain of non-human primates. The same approach can be used to delivery vectors containing CRISPR enzyme and guide RNA. For instance, Xia CF and Boado RJ, Pardridge WM ("Antibody-mediated targeting of siRNA via the human insulin receptor using avidin-biotin technology." Mol Pharm. 2009 May-Jun;6(3):747-51. doi: 10.1021/mp800194) describes how delivery of short interfering RNA (siRNA) to cells in culture, and in vivo, is possible with combined use of a receptor-specific monoclonal antibody (mAb) and avidin-biotin technology. The authors also report that because the bond between the targeting mAb and the siRNA is stable with avidin-biotin technology, and RNAi effects at distant sites such as brain are observed in vivo following an intravenous administration of the targeted siRNA.

[00301] Zhang et al. (Mol Ther. 2003 Jan;7(1):11-8.)) describe how expression plasmids encoding reporters such as luciferase were encapsulated in the interior of an "artificial virus"

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

[00302] Other means of delivery or RNA are also preferred, such as via nanoparticles (Cho, S., Goldberg, M., Son, S., Xu, Q., Yang, F., Mei, Y., Bogatyrev, S., Langer, R. and Anderson, D., Lipid-like nanoparticles for small interfering RNA delivery to endothelial cells, Advanced Functional Materials, 19: 3112-3118, 2010) or exosomes (Schroeder, A., Levins, C., Cortez, C., Langer, R., and Anderson, D., Lipid-based nanotherapeutics for siRNA delivery, Journal of Internal Medicine, 267: 9-21, 2010, PMID: 20059641).

[00303] Indeed, exosomes have been shown to be particularly useful in delivery siRNA, a system with some parallels to the CRISPR system. For instance, El-Andaloussi S, et al. ("Exosome-mediated delivery of siRNA in vitro and in vivo." Nat Protoc. 2012 Dec;7(12):2112-26. doi: 10.1038/nprot.2012.131. Epub 2012 Nov 15.) describe how exosomes are promising tools for drug delivery across different biological barriers and can be harnessed for delivery of siRNA in vitro and in vivo. Their approach is to generate targeted exosomes through transfection of an expression vector, comprising an exosomal protein fused with a peptide ligand. The exosomes are then purify and characterized from transfected cell supernatant, then siRNA is loaded into the exosomes. Delivery or administration according to the invention can be performed with exosomes, in particular but not limited to the brain.

[00304] Vitamin E (α-tocopherol) may be conjugated with CRISPR Cas and delivered to the brain along with high density lipoprotein (HDL), for example in a similar manner as was done by Uno et al. (HUMAN GENE THERAPY 22:711–719 (June 2011)) for delivering short-interfering RNA (siRNA) to the brain. Mice were infused via Osmotic minipumps (model 1007D; Alzet, Cupertino, CA) filled with phosphate-buffered saline (PBS) or free TocsiBACE or Toc-

siBACE/HDL and connected with Brain Infusion Kit 3 (Alzet). A brain-infusion cannula was placed about 0.5mm posterior to the bregma at midline for infusion into the dorsal third ventricle. Uno et al. found that as little as 3 nmol of Toc-siRNA with HDL could induce a target reduction in comparable degree by the same ICV infusion method. A similar dosage of CRISPR Cas conjugated to α-tocopherol and co-administered with HDL targeted to the brain may be contemplated for humans in the present invention, for example, about 3 nmol to about 3 μmol of CRISPR Cas targeted to the brain may becontemplated.

[00305] Zou et al. ((HUMAN GENE THERAPY 22:465-475 (April 2011)) describes a method of lentiviral-mediated delivery of short-hairpin RNAs targeting PKCγ for in vivo gene silencing in the spinal cord of rats. Zou et al. administered about 10 μl of a recombinant lentivirus having a titer of 1 x 10^9 transducing units (TU)/ml by an intrathecal catheter. A similar dosage of CRISPR Cas expressed in a lentiviral vector targeted to the brain may be contemplated for humans in the present invention, for example, about 10-50 ml of CRISPR Cas targeted to the brain in a lentivirus having a titer of 1 x 10^9 transducing units (TU)/ml may becontemplated.

Targeted deletion, therapeutic applications

[00306] Targeted deletion of genes is preferred. Examples are exemplified in Example 18. Preferred are, therefore, genes involved in cholesterol biosynthesis, fatty acid biosynthesis, and other metabolic disorders, genes encoding mis-folded proteins involved in amyloid and other diseases, oncogenes leading to cellular transformation, latent viral genes, and genes leading to dominant-negative disorders, amongst other disorders. As exemplified here, Applicants prefer gene delivery of a CRISPR-Cas system to the liver, brain, ocular, epithelial, hematopoetic, or another tissue of a subject or a patient in need thereof, suffering from metabolic disorders, amyloidosis and protein-aggregation related diseases, cellular transformation arising from genetic mutations and translocations, dominant negative effects of gene mutations, latent viral infections, and other related symptoms, using either viral or nanoparticle delivery system.

[00307] Therapeutic applications of the CRISPR-Cas system include Glaucoma, Amyloidosis, and Huntington's disease. These are exemplified in Example 20 and the features described therein are preferred alone or in combination.

[00308] Another example of a polyglutamine expansion disease that may be treated by the present invention includes spinocerebellar ataxia type 1 (SCA1). Upon intracerebellar injection, recombinant adenoassociated virus (AAV) vectors expressing short hairpin RNAs profoundly

improve motor coordination, restored cerebellar morphology and resolved characteristic ataxin-1 inclusions in Purkinje cells of SCA1 mice (see, e.g., Xia et al., Nature Medicine, Vol. 10, No. 8, Aug. 2004). In particular, AAV1 and AAV5 vectors are preferred and AAV titers of about 1 x 10^{12} vector genomes/ml are desirable.

[00309] As an example, chronic infection by HIV-1 may be treated or prevented. In order to accomplish this, one may generate CRISPR-Cas guide RNAs that target the vast majority of the HIV-1 genome while taking into account HIV-1 strain variants for maximal coverage and effectiveness. One may accomplish delivery of the CRISPR-Cas system by conventional adenoviral or lentiviral-mediated infection of the host immune system. Depending on approach, host immune cells could be a) isolated, transduced with CRISPR-Cas, selected, and re-introduced in to the host or b) transduced in vivo by systemic delivery of the CRISPR-Cas system. The first approach allows for generation of a resistant immune population whereas the second is more likely to target latent viral reservoirs within the host. This is discussed in more detail in the Examples section.

[00310] In another example, US Patent Publication No. 20130171732 assigned to Sangamo BioSciences, Inc. relates to insertion of an anti-HIV transgene into the genome, methods of which may be applied to the CRISPR Cas system of the present invention. In another embodiment, the CXCR4 gene may be targeted and the TALE system of US Patent Publication No. 20100291048 assigned to Sangamo BioSciences, Inc. may be modified to the CRISPR Cas system of the present invention. The method of US Patent Publication Nos. 20130137104 and 20130122591 assigned to Sangamo BioSciences, Inc. and US Patent Publication No. 20100146651 assigned to Cellectis may be more generally applicable for transgene expression as it involves modifying a hypoxanthine-guanine phosphoribosyltransferase (HPRT) locus for increasing the frequency of gene modification.

[00311] It is also envisaged that the present invention generates a gene knockout cell library. Each cell may have a single gene knocked out. This is exemplified in Example 23.

[00312] One may make a library of ES cells where each cell has a single gene knocked out, and the entire library of ES cells will have every single gene knocked out. This library is useful for the screening of gene function in cellular processes as well as diseases. To make this cell library, one may integrate Cas9 driven by an inducible promoter (e.g. doxycycline inducible promoter) into the ES cell. In addition, one may integrate a single guide RNA targeting a specific

gene in the ES cell. To make the ES cell library, one may simply mix ES cells with a library of genes encoding guide RNAs targeting each gene in the human genome. One may first introduce a single BxB1 attB site into the AAVS1 locus of the human ES cell. Then one may use the BxB1 integrase to facilitate the integration of individual guide RNA genes into the BxB1 attB site in AAVS1 locus. To facilitate integration, each guide RNA gene may be contained on a plasmid that carries of a single attP site. This way BxB1 will recombine the attB site in the genome with the attP site on the guide RNA containing plasmid. To generate the cell library, one may take the library of cells that have single guide RNAs integrated and induce Cas9 expression. After induction, Cas9 mediates double strand break at sites specified by the guide RNA.

[00313] Chronic administration of protein therapeutics may elicit unacceptable immune responses to the specific protein. The immunogenicity of protein drugs can be ascribed to a few immunodominant helper T lymphocyte (HTL) epitopes. Reducing the MHC binding affinity of these HTL epitopes contained within these proteins can generate drugs with lower immunogenicity (Tangri S, et al. ("Rationally engineered therapeutic proteins with reduced immunogenicity" J Immunol. 2005 Mar 15;174(6):3187-96.) In the present invention, the immunogenicity of the CRISPR enzyme in particular may be reduced following the approach first set out in Tangri et al with respect to erythropoietin and subsequently developed. Accordingly, directed evolution or rational design may be used to reduce the immunogenicity of the CRISPR enzyme (for instance a Cas9) in the host species (human or other species).

[00314] In Example 28, Applicants used 3 guideRNAs of interest and able to visualize efficient DNA cleavage *in vivo* occurring only in a small subset of cells. Essentially, what Applicants have shown here is targeted *in vivo* cleavage. In particular, this provides proof of concept that specific targeting in higher organisms such as mammals can also be achieved. It also highlights multiplex aspect in that multiple guide sequences (i.e. separate targets) can be used simultaneously (in the sense of co-delivery). In other words, Applicants used a multiple approach, with several different sequences targeted at the same time, but independently.

[00315] A suitable example of a protocol for producing AAV, a preferred vector of the invention is provided in Example 34.

[00316] Trinucleotide repeat disorders are preferred conditions to be treated. These are also exemplified herein.

[00317] For example, US Patent Publication No. 20110016540, describes use of zinc finger nucleases to genetically modify cells, animals and proteins associated with trinucleotide repeat expansion disorders. Trinucleotide repeat expansion disorders are complex, progressive disorders that involve developmental neurobiology and often affect cognition as well as sensori-motor functions.

[00318] Trinucleotide repeat expansion proteins are a diverse set of proteins associated with susceptibility for developing a trinucleotide repeat expansion disorder, the presence of a trinucleotide repeat expansion disorder, the severity of a trinucleotide repeat expansion disorder or any combination thereof. Trinucleotide repeat expansion disorders are divided into two categories determined by the type of repeat. The most common repeat is the triplet CAG, which, when present in the coding region of a gene, codes for the amino acid glutamine (Q). Therefore, these disorders are referred to as the polyglutamine (polyQ) disorders and comprise the following diseases: Huntington Disease (HD); Spinobulbar Muscular Atrophy (SBMA); Spinocerebellar Ataxias (SCA types 1, 2, 3, 6, 7, and 17); and Dentatorubro-Pallidoluysian Atrophy (DRPLA). The remaining trinucleotide repeat expansion disorders either do not involve the CAG triplet or the CAG triplet is not in the coding region of the gene and are, therefore, referred to as the non-polyglutamine disorders. The non-polyglutamine disorders comprise Fragile X Syndrome (FRAXA); Fragile XE Mental Retardation (FRAXE); Friedreich Ataxia (FRDA); Myotonic Dystrophy (DM); and Spinocerebellar Ataxias (SCA types 8, and 12).

[00319] The proteins associated with trinucleotide repeat expansion disorders are typically selected based on an experimental association of the protein associated with a trinucleotide repeat expansion disorder to a trinucleotide repeat expansion disorder. For example, the production rate or circulating concentration of a protein associated with a trinucleotide repeat expansion disorder may be elevated or depressed in a population having a trinucleotide repeat expansion disorder relative to a population lacking the trinucleotide repeat expansion disorder. Differences in protein levels may be assessed using proteomic techniques including but not limited to Western blot, immunohistochemical staining, enzyme linked immunosorbent assay (ELISA), and mass spectrometry. Alternatively, the proteins associated with trinucleotide repeat expansion disorders may be identified by obtaining gene expression profiles of the genes encoding the proteins using genomic techniques including but not limited to DNA microarray

analysis, serial analysis of gene expression (SAGE), and quantitative real-time polymerase chain reaction (Q-PCR).

[00320] Non-limiting examples of proteins associated with trinucleotide repeat expansion disorders include AR (androgen receptor), FMR1 (fragile X mental retardation 1), HTT (huntingtin), DMPK (dystrophia myotonica-protein kinase), FXN (frataxin), ATXN2 (ataxin 2), ATN1 (atrophin 1), FEN1 (flap structure-specific endonuclease 1), TNRC6A (trinucleotide repeat containing 6A), PABPN1 (poly(A) binding protein, nuclear 1), JPH3 (junctophilin 3), MED15 (mediator complex subunit 15), ATXN1 (ataxin 1), ATXN3 (ataxin 3), TBP (TATA box binding protein), CACNA1A (calcium channel, voltage-dependent, P/Q type, alpha 1A subunit), ATXN80S (ATXN8 opposite strand (non-protein coding)), PPP2R2B (protein phosphatase 2, regulatory subunit B, beta), ATXN7 (ataxin 7), TNRC6B (trinucleotide repeat containing 6B), TNRC6C (trinucleotide repeat containing 6C), CELF3 (CUGBP, Elav-like family member 3), MAB21L1 (mab-21-like 1 (C. elegans)), MSH2 (mutS homolog 2, colon cancer, nonpolyposis type 1 (E. coli)), TMEM185A (transmembrane protein 185A), SIX5 (SIX homeobox 5), CNPY3 (canopy 3 homolog (zebrafish)), FRAXE (fragile site, folic acid type, rare, fra(X)(q28) E), GNB2 (guanine nucleotide binding protein (G protein), beta polypeptide 2), RPL14 (ribosomal protein L14), ATXN8 (ataxin 8), INSR (insulin receptor), TTR (transthyretin), EP400 (E1A binding protein p400), GIGYF2 (GRB10 interacting GYF protein 2), OGG1 (8-oxoguanine DNA glycosylase), STC1 (stanniocalcin 1), CNDP1 (carnosine dipeptidase 1 (metallopeptidase M20 family)), C10orf2 (chromosome 10 open reading frame 2), MAML3 mastermind-like 3 (Drosophila), DKC1 (dyskeratosis congenita 1, dyskerin), PAXIP1 (PAX interacting (with transcription-activation domain) protein 1), CASK (calcium/calmodulin-dependent serine protein kinase (MAGUK family)), MAPT (microtubule-associated protein tau), SP1 (Sp1 transcription factor), POLG (polymerase (DNA directed), gamma), AFF2 (AF4/FMR2 family, member 2), THBS1 (thrombospondin 1), TP53 (tumor protein p53), ESR1 (estrogen receptor 1), CGGBP1 (CGG triplet repeat binding protein 1), ABT1 (activator of basal transcription 1), KLK3 (kallikrein-related peptidase 3), PRNP (prion protein), JUN (jun oncogene), KCNN3 (potassium intermediate/small conductance calcium-activated channel, subfamily N, member 3), BAX (BCL2-associated X protein), FRAXA (fragile site, folic acid type, rare, fra(X)(q27.3) A (macroorchidism, mental retardation)), KBTBD10 (kelch repeat and BTB (POZ) domain containing 10), MBNL1 (muscleblind-like (Drosophila)), RAD51 (RAD51 homolog (RecA

homolog, E. coli) (S. cerevisiae)), NCOA3 (nuclear receptor coactivator 3), ERDA1 (expanded repeat domain, CAG/CTG 1), TSC1 (tuberous sclerosis 1), COMP (cartilage oligomeric matrix protein), GCLC (glutamate-cysteine ligase, catalytic subunit), RRAD (Ras-related associated with diabetes), MSH3 (mutS homolog 3 (E. coli)), DRD2 (dopamine receptor D2), CD44 (CD44 molecule (Indian blood group)), CTCF (CCCTC-binding factor (zinc finger protein)), CCND1 (cyclin D1), CLSPN (claspin homolog (Xenopus laevis)), MEF2A (myocyte enhancer factor 2A), PTPRU (protein tyrosine phosphatase, receptor type, U), GAPDH (glyceraldehyde-3-phosphate dehydrogenase), TRIM22 (tripartite motif-containing 22), WT1 (Wilms tumor 1), AHR (aryl hydrocarbon receptor), GPX1 (glutathione peroxidase 1), TPMT (thiopurine S-methyltransferase), NDP (Norrie disease (pseudoglioma)), ARX (aristaless related homeobox), MUS81 (MUS81 endonuclease homolog (S. cerevisiae)), TYR (tyrosinase (oculocutaneous albinism IA)), EGR1 (early growth response 1), UNG (uracil-DNA glycosylase), NUMBL (numb homolog (Drosophila)-like), FABP2 (fatty acid binding protein 2, intestinal), EN2 (engrailed homeobox 2), CRYGC (crystallin, gamma C), SRP14 (signal recognition particle 14 kDa (homologous Alu RNA binding protein)), CRYGB (crystallin, gamma B), PDCD1 (programmed cell death 1), HOXA1 (homeobox A1), ATXN2L (ataxin 2-like), PMS2 (PMS2 postmeiotic segregation increased 2 (S. cerevisiae)), GLA (galactosidase, alpha), CBL (Cas-Br-M (murine) ecotropic retroviral transforming sequence), FTH1 (ferritin, heavy polypeptide 1), IL12RB2 (interleukin 12 receptor, beta 2), OTX2 (orthodenticle homeobox 2), HOXA5 (homeobox A5), POLG2 (polymerase (DNA directed), gamma 2, accessory subunit), DLX2 (distal-less homeobox 2), SIRPA (signal-regulatory protein alpha), OTX1 (orthodenticle homeobox 1), AHRR (aryl-hydrocarbon receptor repressor), MANF (mesencephalic astrocyte-derived neurotrophic factor), TMEM158 (transmembrane protein 158 (gene/pseudogene)), and ENSG00000078687.

[00321] Preferred proteins associated with trinucleotide repeat expansion disorders include HTT (Huntingtin), AR (androgen receptor), FXN (frataxin), Atxn3 (ataxin), Atxn1 (ataxin), Atxn2 (ataxin), Atxn7 (ataxin), Atxn10 (ataxin), DMPK (dystrophia myotonica-protein kinase), Atn1 (atrophin 1), CBP (creb binding protein), VLDLR (very low density lipoprotein receptor), and any combination thereof.

[00322] According to another aspect, a method of gene therapy for the treatment of a subject having a mutation in the CFTR gene is provided and comprises administering a therapeutically

effective amount of a CRISPR-Cas gene therapy particle, optionally via a biocompatible pharmaceutical carrier, to the cells of a subject. Preferably, the target DNA comprises the mutation deltaF508. In general, it is of preferred that the mutation is repaired to the wildtype. In this case, the mutation is a deletion of the three nucleotides that comprise the codon for phenylalanine (F) at position 508. Accordingly, repair in this instance requires reintroduction of the missing codon into the mutant.

[00323] To implement this Gene Repair Strategy, it is preferred that an adenovirus/AAV vector system is introduced into the host cell, cells or patient. Preferably, the system comprises a Cas9 (or Cas9 nickase) and the guide RNA along with a adenovirus/AAV vector system comprising the homology repair template containing the F508 residue. This may be introduced into the subject via one of the methods of delivery discussed earlier. The CRISPR-Cas system may be guided by the CFTRdelta 508 chimeric guide RNA. It targets a specific site of the CFTR genomic locus to be nicked or cleaved. After cleavage, the repair template is inserted into the cleavage site via homologous recombination correcting the deletion that results in cystic fibrosis or causes cystic fibrosis related symptoms. This strategy to direct delivery and provide systemic introduction of CRISPR systems with appropriate guide RNAs can be employed to target genetic mutations to edit or otherwise manipulate genes that cause metabolic, liver, kidney and protein diseases and disorders such as those in Table B.

Genome editing

[00324] The CRISPR/Cas9 systems of the present invention can be used to correct genetic mutations that were previously attempted with limited success using TALEN and ZFN. For example, WO2013163628 A2, Genetic Correction of Mutated Genes, published application of Duke University describes efforts to correct, for example, a frameshift mutation which causes a premature stop codon and a truncated gene product that can be corrected via nuclease mediated non-homologous end joining such as those responsible for Duchenne Muscular Dystrophy, ("DMD") a recessive, fatal, X-linked disorder that results in muscle degeneration due to mutations in the dystrophin gene. The majority of dystrophin mutations that cause DMD are deletions of exons that disrupt the reading frame and cause premature translation termination in the dystrophin gene. Dystrophin is a cytoplasmic protein that provides structural stability to the dystroglycan complex of the cell membrane that is responsible for regulating muscle cell integrity and function. The dystrophin gene or "DMD gene" as used interchangeably herein is 2.2

megabases at locus Xp21. The primary transcription measures about 2,400 kb with the mature mRNA being about 14 kb. 79 exons code for the protein which is over 3500 amino acids. Exon 51 is frequently adjacent to frame-disrupting deletions in DMD patients and has been targeted in clinical trials for oligonucleotide-based exon skipping. A clinical trial for the exon 51 skipping compound eteplirsen recently reported a significant functional benefit across 48 weeks, with an average of 47% dystrophin positive fibers compared to baseline. Mutations in exon 51 are ideally suited for permanent correction by NHEJ-based genome editing.

[00325] The methods of US Patent Publication No. 20130145487 assigned to Cellectis, which relates to meganuclease variants to cleave a target sequence from the human dystrophin gene (DMD), may also be modified to for the CRISPR Cas system of the present invention.

Blood

[00326] The present invention also contemplates delivering the CRISPR-Cas system to the blood.

[00327] The plasma exosomes of Wahlgren et al. (Nucleic Acids Research, 2012, Vol. 40, No. 17 e130) were previously described and may be utilized to deliver the CRISPR Cas system to the blood.

[00328] The CRISPR Cas system of the present invention is also contemplated to treat hemoglobinopathies, such as thalassemias and sickle cell disease. See, e.g., International Patent Publication No. WO 2013/126794 for potential targets that may be targeted by the CRISPR Cas system of the present invention.

[00329] US Patent Publication Nos. 20110225664, 20110091441, 20100229252, 20090271881 and 20090222937 assigned to Cellectis, relates to CREI variants , wherein at least one of the two I-CreI monomers has at least two substitutions, one in each of the two functional subdomains of the LAGLIDADG core domain situated respectively from positions 26 to 40 and 44 to 77 of I-CreI, said variant being able to cleave a DNA target sequence from the human interleukin-2 receptor gamma chain (IL2RG) gene also named common cytokine receptor gamma chain gene or gamma C gene. The target sequences identified in US Patent Publication Nos. 20110225664, 20110091441, 20100229252, 20090271881 and 20090222937 may be utilized for the CRISPR Cas system of the present invention.

[00330] Severe Combined Immune Deficiency (SCID) results from a defect in lymphocytes T maturation, always associated with a functional defect in lymphocytes B (Cavazzana-Calvo et

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

[00331] Since the ADA gene has been shown to be mutated in SCID patients (Giblett et al., Lancet, 1972, 2, 1067-1069), several other genes involved in SCID have been identified (Cavazzana-Calvo et al., Annu. Rev. Med., 2005, 56, 585-602; Fischer et al., Immunol. Rev., 2005, 203, 98-109). There are four major causes for SCID: (i) the most frequent form of SCID, SCID-X1 (X-linked SCID or X-SCID), is caused by mutation in the IL2RG gene, resulting in the absence of mature T lymphocytes and NK cells. IL2RG encodes the gamma C protein (Noguchi, et al., Cell, 1993, 73, 147-157), a common component of at least five interleukin receptor complexes. These receptors activate several targets through the JAK3 kinase (Macchi et al., Nature, 1995, 377, 65-68), which inactivation results in the same syndrome as gamma C inactivation; (ii) mutation in the ADA gene results in a defect in purine metabolism that is lethal for lymphocyte precursors, which in turn results in the quasi absence of B, T and NK cells; (iii) V(D)J recombination is an essential step in the maturation of immunoglobulins and T lymphocytes receptors (TCRs). Mutations in Recombination Activating Gene 1 and 2 (RAG1 and RAG2) and Artemis, three genes involved in this process, result in the absence of mature T and B lymphocytes; and (iv) Mutations in other genes such as CD45, involved in T cell specific signaling have also been reported, although they represent a minority of cases (Cavazzana-Calvo et al., Annu. Rev. Med., 2005, 56, 585-602; Fischer et al., Immunol. Rev., 2005, 203, 98-109).

[00332] Since when their genetic bases have been identified, the different SCID forms have become a paradigm for gene therapy approaches (Fischer et al., Immunol. Rev., 2005, 203, 98-109) for two major reasons. First, as in all blood diseases, an ex vivo treatment can be envisioned. Hematopoietic Stem Cells (HSCs) can be recovered from bone marrow, and keep their pluripotent properties for a few cell divisions. Therefore, they can be treated in vitro, and then reinjected into the patient, where they repopulate the bone marrow. Second, since the maturation of lymphocytes is impaired in SCID patients, corrected cells have a selective

advantage. Therefore, a small number of corrected cells can restore a functional immune system. This hypothesis was validated several times by (i) the partial restoration of immune functions associated with the reversion of mutations in SCID patients (Hirschhorn et al., Nat. Genet., 1996, 13, 290-295; Stephan et al., N. Engl. J. Med., 1996, 335, 1563-1567; Bousso et al., Proc. Natl., Acad. Sci. USA, 2000, 97, 274-278; Wada et al., Proc. Natl. Acad. Sci. USA, 2001, 98, 8697-8702; Nishikomori et al., Blood, 2004, 103, 4565-4572), (ii) the correction of SCID-X1 deficiencies in vitro in hematopoietic cells (Candotti et al., Blood, 1996, 87, 3097-3102; Cavazzana-Calvo et al., Blood, 1996, Blood, 88, 3901-3909; Taylor et al., Blood, 1996, 87, 3103-3107; Hacein-Bey et al., Blood, 1998, 92, 4090-4097), (iii) the correction of SCID-X1 (Soudais et al., Blood, 2000, 95, 3071-3077; Tsai et al., Blood, 2002, 100, 72-79), JAK-3 (Bunting et al., Nat. Med., 1998, 4, 58-64; Bunting et al., Hum. Gene Ther., 2000, 11, 2353-2364) and RAG2 (Yates et al., Blood, 2002, 100, 3942-3949) deficiencies in vivo in animal models and (iv) by the result of gene therapy clinical trials (Cavazzana-Calvo et al., Science, 2000, 288, 669-672; Aiuti et al., Nat. Med., 2002; 8, 423-425; Gaspar et al., Lancet, 2004, 364, 2181-2187).

[00333] US Patent Publication No. 20110182867 assigned to the Children's Medical Center Corporation and the President and Fellows of Harvard College relates to methods and uses of modulating fetal hemoglobin expression (HbF) in a hematopoietic progenitor cells via inhibitors of BCL11A expression or activity, such as RNAi and antibodies. The targets disclosed in US Patent Publication No. 20110182867, such as BCL11A, may be targeted by the CRISPR Cas system of the present invention for modulating fetal hemoglobin expression. See also Bauer et al. (Science 11 October 2013: Vol. 342 no. 6155 pp. 253-257) and Xu et al. (Science 18 November 2011: Vol. 334 no. 6058 pp. 993-996) for additional BCL11A targets.

Ears

[00334] The present invention also contemplates delivering the CRISPR-Cas system to one or both ears.

[00335] Researchers are looking into whether gene therapy could be used to aid current deafness treatments—namely, cochlear implants. Deafness is often caused by lost or damaged hair cells that cannot relay signals to auditory neurons. In such cases, cochlear implants may be used to respond to sound and transmit electrical signals to the nerve cells. But these neurons

often degenerate and retract from the cochlea as fewer growth factors are released by impaired hair cells.

[00336] US patent application 20120328580 describes injection of a pharmaceutical composition into the ear (e.g., auricular administration), such as into the luminae of the cochlea (e.g., the Scala media, Sc vestibulae, and Sc tympani), e.g., using a syringe, e.g., a single-dose syringe. For example, one or more of the compounds described herein can be administered by intratympanic injection (e.g., into the middle ear), and/or injections into the outer, middle, and/or inner ear. Such methods are routinely used in the art, for example, for the administration of steroids and antibiotics into human ears. Injection can be, for example, through the round window of the ear or through the cochlear capsule. Other inner ear administration methods are known in the art (see, e.g., Salt and Plontke, Drug Discovery Today, 10:1299-1306, 2005).

[00337] In another mode of administration, the pharmaceutical composition can be administered in situ, via a catheter or pump. A catheter or pump can, for example, direct a pharmaceutical composition into the cochlear luminae or the round window of the ear and/or the lumen of the colon. Exemplary drug delivery apparatus and methods suitable for administering one or more of the compounds described herein into an ear, e.g., a human ear, are described by McKenna et al., (U.S. Publication No. 2006/0030837) and Jacobsen et al., (U.S. Pat. No. 7,206,639). In some embodiments, a catheter or pump can be positioned, e.g., in the ear (e.g., the outer, middle, and/or inner ear) of a patient during a surgical procedure. In some embodiments, a catheter or pump can be positioned, e.g., in the ear (e.g., the outer, middle, and/or inner ear) of a patient without the need for a surgical procedure.

[00338] Alternatively or in addition, one or more of the compounds described herein can be administered in combination with a mechanical device such as a cochlear implant or a hearing aid, which is worn in the outer ear. An exemplary cochlear implant that is suitable for use with the present invention is described by Edge et al., (U.S. Publication No. 2007/0093878).

[00339] In some embodiments, the modes of administration described above may be combined in any order and can be simultaneous or interspersed.

[00340] Alternatively or in addition, the present invention may be administered according to any of the Food and Drug Administration approved methods, for example, as described in CDER Data Standards Manual, version number 004 (which is available at fda.give/cder/dsm/DRG/drg00301.htm).

[00341] In general, the cell therapy methods described in US patent application 20120328580 can be used to promote complete or partial differentiation of a cell to or towards a mature cell type of the inner ear (e.g., a hair cell) in vitro. Cells resulting from such methods can then be transplanted or implanted into a patient in need of such treatment. The cell culture methods required to practice these methods, including methods for identifying and selecting suitable cell types, methods for promoting complete or partial differentiation of selected cells, methods for identifying complete or partially differentiated cell types, and methods for implanting complete or partially differentiated cells are described below.

[00342] Cells suitable for use in the present invention include, but are not limited to, cells that are capable of differentiating completely or partially into a mature cell of the inner ear, e.g., a hair cell (e.g., an inner and/or outer hair cell), when contacted, e.g., in vitro, with one or more of the compounds described herein. Exemplary cells that are capable of differentiating into a hair cell include, but are not limited to stem cells (e.g., inner ear stem cells, adult stem cells, bone marrow derived stem cells, embryonic stem cells, mesenchymal stem cells, skin stem cells, iPS cells, and fat derived stem cells), progenitor cells (e.g., inner ear progenitor cells), support cells (e.g., Deiters' cells, pillar cells, inner phalangeal cells, tectal cells and Hensen's cells), and/or germ cells. The use of stem cells for the replacement of inner ear sensory cells is described in Li et al., (U.S. Publication No. 2005/0287127) and Li et al., (U.S. patent Ser. No. 11/953,797). The use of bone marrow derived stem cells for the replacement of inner ear sensory cells is described in Edge et al., PCT/US2007/084654. iPS cells are described, e.g., at Takahashi et al., Cell, Volume 131, Issue 5, Pages 861-872 (2007); Takahashi and Yamanaka, Cell 126, 663-76 (2006); Okita et al., Nature 448, 260-262 (2007); Yu, J. et al., Science 318(5858):1917-1920 (2007); Nakagawa et al., Nat. Biotechnol. 26:101-106 (2008); and Zaehres and Scholer, Cell 131(5):834-835 (2007).

[00343] Such suitable cells can be identified by analyzing (e.g., qualitatively or quantitatively) the presence of one or more tissue specific genes. For example, gene expression can be detected by detecting the protein product of one or more tissue-specific genes. Protein detection techniques involve staining proteins (e.g., using cell extracts or whole cells) using antibodies against the appropriate antigen. In this case, the appropriate antigen is the protein product of the tissue-specific gene expression. Although, in principle, a first antibody (i.e., the antibody that binds the antigen) can be labeled, it is more common (and improves the visualization) to use a

second antibody directed against the first (e.g., an anti-IgG). This second antibody is conjugated either with fluorochromes, or appropriate enzymes for colorimetric reactions, or gold beads (for electron microscopy), or with the biotin-avidin system, so that the location of the primary antibody, and thus the antigen, can be recognized.

[00344] The CRISPR Cas molecules of the present invention may be delivered to the ear by direct application of pharmaceutical composition to the outer ear, with compositions modified from US Published application, 20110142917. In some embodiments the pharmaceutical composition is applied to the ear canal. Delivery to the ear may also be refered to as aural or otic delivery.

[00345] In some embodiments the RNA molecules of the invention are delivered in liposome or lipofectin formulations and the like and can be prepared by methods well known to those skilled in the art. Such methods are described, for example, in U.S. Pat. Nos. 5,593,972, 5,589,466, and 5,580,859, which are herein incorporated by reference.

[00346] Delivery systems aimed specifically at the enhanced and improved delivery of siRNA into mammalian cells have been developed, (see, for example, Shen et al FEBS Let. 2003, 539:111-114; Xia et al., Nat. Biotech. 2002, 20:1006-1010; Reich et al., Mol. Vision. 2003, 9: 210-216; Sorensen et al., J. Mol. Biol. 2003, 327: 761-766; Lewis et al., Nat. Gen. 2002, 32: 107-108 and Simeoni et al., NAR 2003, 31, 11: 2717-2724) and may be applied to the present invention. siRNA has recently been successfully used for inhibition of gene expression in primates (see for example. Tolentino et al., Retina 24(4):660 which may also be applied to the present invention.

[00347] Qi et al. discloses methods for efficient siRNA transfection to the inner ear through the intact round window by a novel proteidic delivery technology which may be applied to the CRISPR Cas system of the present invention (see, e.g., Qi et al., Gene Therapy (2013), 1-9). In particular, a TAT double stranded RNA-binding domains (TAT-DRBDs), which can transfect Cy3-labeled siRNA into cells of the inner ear, including the inner and outer hair cells, crista ampullaris, macula utriculi and macula sacculi, through intact round-window permeation was successful for delivering double stranded siRNAs in vivo for treating various inner ear ailments and preservation of hearing function. About 40 µl of 10mM RNA may be contemplated as the dosage for administration to the ear.

[00348] According to Rejali et al. (Hear Res. 2007 Jun;228(1-2):180-7), cochlear implant function can be improved by good preservation of the spiral ganglion neurons, which are the target of electrical stimulation by the implant and brain derived neurotrophic factor (BDNF) has previously been shown to enhance spiral ganglion survival in experimentally deafened ears. Rejali et al. tested a modified design of the cochlear implant electrode that includes a coating of fibroblast cells transduced by a viral vector with a BDNF gene insert. To accomplish this type of ex vivo gene transfer, Rejali et al. transduced guinea pig fibroblasts with an adenovirus with a BDNF gene cassette insert, and determined that these cells secreted BDNF and then attached BDNF-secreting cells to the cochlear implant electrode via an agarose gel, and implanted the electrode in the scala tympani. Rejali et al. determined that the BDNF expressing electrodes were able to preserve significantly more spiral ganglion neurons in the basal turns of the cochlea after 48 days of implantation when compared to control electrodes and demonstrated the feasibility of combining cochlear implant therapy with ex vivo gene transfer for enhancing spiral ganglion neuron survival. Such a system may be applied to the CRISPR Cas system of the present invention for delivery to the ear.

[00349] Mukherjea et al. (Antioxidants & Redox Signaling, Volume 13, Number 5, 2010) document that knockdown of NOX3 using short interfering (si) RNA abrogated cisplatin ototoxicity, as evidenced by protection of OHCs from damage and reduced threshold shifts in auditory brainstem responses (ABRs). Different doses of siNOX3 (0.3, 0.6, and 0.9 μg) were administered to rats and NOX3 expression was evaluated by real time RT-PCR. The lowest dose of NOX3 siRNA used (0.3 μg) did not show any inhibition of NOX3 mRNA when compared to transtympanic administration of scrambled siRNA or untreated cochleae. However, administration of the higher doses of NOX3 siRNA (0.6 and 0.9 μg) reduced NOX3 expression compared to control scrambled siRNA. Such a system may be applied to the CRISPR Cas system of the present invention for transtympanic administration with a dosage of about 2 mg to about 4 mg of CRISPR Cas for administration to a human.

[00350] Jung et al. (Molecular Therapy, vol. 21 no. 4, 834–841 apr. 2013) demonstrate that Hes5 levels in the utricle decreased after the application of siRNA and that the number of hair cells in these utricles was significantly larger than following control treatment. The data suggest that siRNA technology may be useful for inducing repair and regeneration in the inner ear and that the Notch signaling pathway is a potentially useful target for specific gene expression

inhibition. Jung et al. injected 8 μg of Hes5 siRNA in 2 μl volume, prepared by adding sterile normal saline to the lyophilized siRNA to a vestibular epithelium of the ear. Such a system may be applied to the CRISPR Cas system of the present invention for administration to the vestibular epithelium of the ear with a dosage of about 1 to about 30 mg of CRISPR Cas for administration to a human.

Eyes

[00351] The present invention also contemplates delivering the CRISPR-Cas system to one or both eyes.

[00352] In yet another aspect of the invention, the CRISPR-Cas system may be used to correct ocular defects that arise from several genetic mutations further described in Genetic Diseases of the Eye, Second Edition, edited by Elias I. Traboulsi, Oxford University Press, 2012.

[00353] For administration to the eye, lentiviral vectors, in particular equine infectious anemia viruses (EIAV) are particularly preferred.

[00354] In another embodiment, minimal non-primate lentiviral vectors based on the equine infectious anemia virus (EIAV) are also contemplated, especially for ocular gene therapy (see, e.g., Balagaan, J Gene Med 2006; 8: 275 – 285, Published online 21 November 2005 in Wiley InterScience (www.interscience.wiley.com). DOI: 10.1002/jgm.845). The vectors are contemplated to have cytomegalovirus (CMV) promoter driving expression of the target gene. Intracameral, subretinal, intraocular and intravitreal injections are all contemplated (see, e.g., Balagaan, J Gene Med 2006; 8: 275 – 285, Published online 21 November 2005 in Wiley InterScience (www.interscience.wiley.com). DOI: 10.1002/jgm.845). Intraocular injections may be performed with the aid of an operating microscope. For subretinal and intravitreal injections, eyes may be prolapsed by gentle digital pressure and fundi visualised using a contact lens system consisting of a drop of a coupling medium solution on the cornea covered with a glass microscope slide coverslip. For subretinal injections, the tip of a 10-mm 34-gauge needle, mounted on a 5-μl Hamilton syringe may be advanced under direct visualisation through the superior equatorial sclera tangentially towards the posterior pole until the aperture of the needle was visible in the subretinal space. Then, 2 μl of vector suspension may be injected to produce a superior bullous retinal detachment, thus confirming subretinal vector administration. This approach creates a self-sealing sclerotomy allowing the vector suspension to be retained in the subretinal space until it is absorbed by the RPE, usually within 48 h of the procedure. This

procedure may be repeated in the inferior hemisphere to produce an inferior retinal detachment. This technique results in the exposure of approximately 70% of neurosensory retina and RPE to the vector suspension. For intravitreal injections, the needle tip may be advanced through the sclera 1 mm posterior to the corneoscleral limbus and 2 µl of vector suspension injected into the vitreous cavity. For intracameral injections, the needle tip may be advanced through a corneoscleral limbal paracentesis, directed towards the central cornea, and 2 µl of vector suspension may be injected. For intracameral injections, the needle tip may be advanced through a corneoscleral limbal paracentesis, directed towards the central cornea, and 2 µl of vector suspension may be injected. These vectors may be injected at titres of either $1.0–1.4 \times 10^{10}$ or $1.0–1.4 \times 10^{9}$ transducing units (TU)/ml.

[00355] In another embodiment, RetinoStat®, an equine infectious anemia virus-based lentiviral gene therapy vector that expresses angiostatic proteins endostain and angiostatin that is delivered via a subretinal injection for the treatment of the web form of age-related macular degeneration is also contemplated (see, e.g., Binley et al., HUMAN GENE THERAPY 23:980–991 (September 2012)). Such a vector may be modified for the CRISPR-Cas system of the present invention. Each eye may be treated with either RetinoStat® at a dose of 1.1×10^{5} transducing units per eye (TU/eye) in a total volume of 100 µl.

[00356] In another embodiment, an E1-, partial E3-, E4-deleted adenoviral vector may be contemplated for delivery to the eye. Twenty-eight patients with advanced neovascular age-related macular degeneration (AMD) were given a single intravitreous injection of an E1-, partial E3-, E4-deleted adenoviral vector expressing human pigment ep- ithelium-derived factor (AdPEDF.11) (see, e.g., Campochiaro et al., Human Gene Therapy 17:167-176 (February 2006)). Doses ranging from 10^{6} to $10^{9.5}$ particle units (PU) were investigated and there were no serious adverse events related to AdPEDF.11 and no dose-limiting toxicities (see, e.g., Campochiaro et al., Human Gene Therapy 17:167-176 (February 2006)). Adenoviral vector-mediated ocular gene transfer appears to be a viable approach for the treatment of ocular disorders and could be applied to the CRISPR Cas system.

[00357] In another embodiment, the sd-rxRNA® system of RXi Pharmaceuticals may be used/and or adapted for delivering CRISPR Cas to the eye. In this system, a single intravitreal administration of 3 µg of sd-rxRNA results in sequence-specific reduction of PPIB mRNA levels for 14 days. The the sd-rxRNA® system may be applied to the CRISPR Cas system of the

present invention, contemplating a dose of about 3 to 20 mg of CRISPR administered to a human.

[00358] Millington-Ward et al. (Molecular Therapy, vol. 19 no. 4, 642–649 apr. 2011) describes adeno-associated virus (AAV) vectors to deliver an RNA interference (RNAi)-based rhodopsin suppressor and a codon-modified rhodopsin replacement gene resistant to suppression due to nucleotide alterations at degenerate positions over the RNAi target site. An injection of either 6.0 x 10^8 vp or 1.8 x 10^{10} vp AAV were subretinally injected into the eyes by Millington-Ward et al. The AAV vectors of Millington-Ward et al. may be applied to the CRISPR Cas system of the present invention, contemplating a dose of about 2 x 10^{11} to about 6 x 10^{13} vp administered to a human.

[00359] Dalkara et al. (Sci Transl Med 5, 189ra76 (2013)) also relates to in vivo directed evolution to fashion an AAV vector that delivers wild-type versions of defective genes throughout the retina after noninjurious injection into the eyes' vitreous humor. Dalkara describes a a 7mer peptide display library and an AAV library constructed by DNA shuffling of cap genes from AAV1, 2, 4, 5, 6, 8, and 9. The rcAAV libraries and rAAV vectors expressing GFP under a CAG or Rho promoter were packaged and and deoxyribonuclease-resistant genomic titers were obtained through quantitative PCR. The libraries were pooled, and two rounds of evolution were performed, each consisting of initial library diversification followed by three in vivo selection steps. In each such step, P30 rho-GFP mice were intravitreally injected with 2 ml of iodixanol-purified, phosphate-buffered saline (PBS)–dialyzed library with a genomic titer of about 1 × 10^{12} vg/ml. The AAV vectors of Dalkara et al. may be applied to the CRISPR Cas system of the present invention, contemplating a dose of about 1 x 10^{15} to about 1 x 10^{16} vg/ml administered to a human.

[00360] In another embodiment, the rhodopsin gene may be targeted for the treatment of retinitis pigmentosa (RP), wherein the system of US Patent Publication No. 20120204282 assigned to Sangamo BioSciences, Inc. may be modified in accordance of the CRISPR Cas system of the present invention.

[00361] In another embodiment, the methods of US Patent Publication No. 20130183282 assigned to Cellectis, which is directed to methods of cleaving a target sequence from the human rhodopsin gene, may also be modified to the CRISPR Cas system of the present invention.

[00362] US Patent Publication No. 20130202678 assigned to Academia Sinica relates to methods for treating retinopathies and sight-threatening ophthalmologic disorders relating to delivering of the Puf-A gene (which is expressed in retinal ganglion and pigmented cells of eye tissues and displays a unique anti-apoptotic activity) to the sub-retinal or intravitreal space in the eye. In particular, desirable targets are zgc:193933, prdm1a, spata2, tex10, rbb4, ddx3, zp2.2, Blimp-1 and HtrA2, all of which may be targeted by the CRISPR Cas system of the present invention.

[00363] Wu (Cell Stem Cell,13:659–62, 2013) designed a guide RNA that led Cas9 to a single base pair mutation that causes cataracts in mice, where it induced DNA cleavage. Then using either the other wild-type allele or oligos given to the zygotes repair mechanisms corrected the sequence of the broken allele and corrected the cataract-causing genetic defect in mutant mouse.

[00364] US Patent Publication No. 20120159653, describes use of zinc finger nucleases to genetically modify cells, animals and proteins associated with macular degeration (MD). Macular degeneration (MD) is the primary cause of visual impairment in the elderly, but is also a hallmark symptom of childhood diseases such as Stargardt disease, Sorsby fundus, and fatal childhood neurodegenerative diseases, with an age of onset as young as infancy. Macular degeneration results in a loss of vision in the center of the visual field (the macula) because of damage to the retina. Currently existing animal models do not recapitulate major hallmarks of the disease as it is observed in humans. The available animal models comprising mutant genes encoding proteins associated with MD also produce highly variable phenotypes, making translations to human disease and therapy development problematic.

[00365] One aspect of US Patent Publication No. 20120159653 relates to editing of any chromosomal sequences that encode proteins associated with MD which may be applied to the CRISPR Cas system of the present invention. The proteins associated with MD are typically selected based on an experimental association of the protein associated with MD to an MD disorder. For example, the production rate or circulating concentration of a protein associated with MD may be elevated or depressed in a population having an MD disorder relative to a population lacking the MD disorder. Differences in protein levels may be assessed using proteomic techniques including but not limited to Western blot, immunohistochemical staining, enzyme linked immunosorbent assay (ELISA), and mass spectrometry. Alternatively, the proteins associated with MD may be identified by obtaining gene expression profiles of the

genes encoding the proteins using genomic techniques including but not limited to DNA microarray analysis, serial analysis of gene expression (SAGE), and quantitative real-time polymerase chain reaction (Q-PCR).

[00366] By way of non-limiting example, proteins associated with MD include but are not limited to the following proteins: (ABCA4) ATP-binding cassette, sub-family A (ABC1), member 4 ACHM1 achromatopsia (rod monochromacy) 1 ApoE Apolipoprotein E (ApoE) C1QTNF5 (CTRP5) C1q and tumor necrosis factor related protein 5 (C1QTNF5) C2 Complement component 2 (C2) C3 Complement components (C3) CCL2 Chemokine (C-C motif) Ligand 2 (CCL2) CCR2 Chemokine (C-C motif) receptor 2 (CCR2) CD36 Cluster of Differentiation 36 CFB Complement factor B CFH Complement factor CFH H CFHR1 complement factor H-related 1 CFHR3 complement factor H-related 3 CNGB3 cyclic nucleotide gated channel beta 3 CP ceruloplasmin (CP) CRP C reactive protein (CRP) CST3 cystatin C or cystatin 3 (CST3) CTSD Cathepsin D (CTSD) CX3CR1 chemokine (C-X3-C motif) receptor 1 ELOVL4 Elongation of very long chain fatty acids 4 ERCC6 excision repair cross-complementing rodent repair deficiency, complementation group 6 FBLN5 Fibulin-5 FBLN5 Fibulin 5 FBLN6 Fibulin 6 FSCN2 fascin (FSCN2) HMCN1 Hemicentrin 1 HMCN1 hemicentin 1 HTRA1 HtrA serine peptidase 1 (HTRA1) HTRA1 HtrA serine peptidase 1 IL-6 Interleukin 6 IL-8 Interleukin 8 LOC387715 Hypothetical protein PLEKHA1 Pleckstrin homology domain-containing family A member 1 (PLEKHA1) PROM1 Prominin 1(PROM1 or CD133) PRPH2 Peripherin-2 RPGR retinitis pigmentosa GTPase regulator SERPING1 serpin peptidase inhibitor, clade G, member 1 (C1- inhibitor) TCOF1 Treacle TIMP3 Metalloproteinase inhibitor 3 (TIMP3) TLR3 Toll-like receptor 3

[00367] The identity of the protein associated with MD whose chromosomal sequence is edited can and will vary. In preferred embodiments, the proteins associated with MD whose chromosomal sequence is edited may be the ATP-binding cassette, sub-family A (ABC1) member 4 protein (ABCA4) encoded by the ABCR gene, the apolipoprotein E protein (APOE) encoded by the APOE gene, the chemokine (C-C motif) Ligand 2 protein (CCL2) encoded by the CCL2 gene, the chemokine (C-C motif) receptor 2 protein (CCR2) encoded by the CCR2 gene, the ceruloplasmin protein (CP) encoded by the CP gene, the cathepsin D protein (CTSD) encoded by the CTSD gene, or the metalloproteinase inhibitor 3 protein (TIMP3) encoded by the TIMP3 gene. In an exemplary embodiment, the genetically modified animal is a rat, and the

edited chromosomal sequence encoding the protein associated with MD may be: (ABCA4) ATP-binding cassette, NM_000350 sub-family A (ABC1), member 4 APOE Apolipoprotein E NM_138828 (APOE) CCL2 Chemokine (C-C NM_031530 motif) Ligand 2 (CCL2) CCR2 Chemokine (C-C NM_021866 motif) receptor 2 (CCR2) CP ceruloplasmin (CP) NM_012532 CTSD Cathepsin D (CTSD) NM_134334 TIMP3 Metalloproteinase NM_012886 inhibitor 3 (TIMP3) The animal or cell may comprise 1, 2, 3, 4, 5, 6, 7 or more disrupted chromosomal sequences encoding a protein associated with MD and zero, 1, 2, 3, 4, 5, 6, 7 or more chromosomally integrated sequences encoding the disrupted protein associated with MD.

[00368] The edited or integrated chromosomal sequence may be modified to encode an altered protein associated with MD. Several mutations in MD-related chromosomal sequences have been associated with MD. Non-limiting examples of mutations in chromosomal sequences associated with MD include those that may cause MD including in the ABCR protein, E471K (i.e. glutamate at position 471 is changed to lysine), R1129L (i.e. arginine at position 1129 is changed to leucine), T1428M (i.e. threonine at position 1428 is changed to methionine), R1517S (i.e. arginine at position 1517 is changed to serine), I1562T (i.e. isoleucine at position 1562 is changed to threonine), and G1578R (i.e. glycine at position 1578 is changed to arginine); in the CCR2 protein, V64I (i.e. valine at position 192 is changed to isoleucine); in CP protein, G969B (i.e. glycine at position 969 is changed to asparagine or aspartate); in TIMP3 protein, S156C (i.e. serine at position 156 is changed to cysteine), G166C (i.e. glycine at position 166 is changed to cysteine), G167C (i.e. glycine at position 167 is changed to cysteine), Y168C (i.e. tyrosine at position 168 is changed to cysteine), S170C (i.e. serine at position 170 is changed to cysteine), Y172C (i.e. tyrosine at position 172 is changed to cysteine) and S181C (i.e. serine at position 181 is changed to cysteine). Other associations of genetic variants in MD-associated genes and disease are known in the art.

Heart

[00369] The present invention also contemplates delivering the CRISPR-Cas system to the heart. For the heart, a myocardium tropic adena-associated virus (AAVM) is preferred, in particular AAVM41 which showed preferential gene transfer in the heart (see, e.g., Lin-Yanga et al., PNAS, March 10, 2009, vol. 106, no. 10). Administration may be systemic or local. A dosage of about 1-10 x 10^{14} vector genomes are contemplated for systemic administration. See also, e.g., Eulalio et al. (2012) Nature 492: 376 and Somasuntharam et al. (2013) Biomaterials 34: 7790.

[00370] For example, US Patent Publication No. 20110023139, describes use of zinc finger nucleases to genetically modify cells, animals and proteins associated with cardiovascular disease. Cardiovascular diseases generally include high blood pressure, heart attacks, heart failure, and stroke and TIA. Any chromosomal sequence involved in cardiovascular disease or the protein encoded by any chromosomal sequence involved in cardiovascular disease may be utilized in the methods described in this disclosure. The cardiovascular-related proteins are typically selected based on an experimental association of the cardiovascular-related protein to the development of cardiovascular disease. For example, the production rate or circulating concentration of a cardiovascular-related protein may be elevated or depressed in a population having a cardiovascular disorder relative to a population lacking the cardiovascular disorder. Differences in protein levels may be assessed using proteomic techniques including but not limited to Western blot, immunohistochemical staining, enzyme linked immunosorbent assay (ELISA), and mass spectrometry. Alternatively, the cardiovascular-related proteins may be identified by obtaining gene expression profiles of the genes encoding the proteins using genomic techniques including but not limited to DNA microarray analysis, serial analysis of gene expression (SAGE), and quantitative real-time polymerase chain reaction (Q-PCR).

[00371] By way of example, the chromosomal sequence may comprise, but is not limited to, IL1B (interleukin 1, beta), XDH (xanthine dehydrogenase), TP53 (tumor protein p53), PTGIS (prostaglandin 12 (prostacyclin) synthase), MB (myoglobin), IL4 (interleukin 4), ANGPT1 (angiopoietin 1), ABCG8 (ATP-binding cassette, sub-family G (WHITE), member 8), CTSK (cathepsin K), PTGIR (prostaglandin 12 (prostacyclin) receptor (IP)), KCNJ11 (potassium inwardly-rectifying channel, subfamily J, member 11), INS (insulin), CRP (C-reactive protein, pentraxin-related), PDGFRB (platelet-derived growth factor receptor, beta polypeptide), CCNA2 (cyclin A2), PDGFB (platelet-derived growth factor beta polypeptide (simian sarcoma viral (v-sis) oncogene homolog)), KCNJ5 (potassium inwardly-rectifying channel, subfamily J, member 5), KCNN3 (potassium intermediate/small conductance calcium-activated channel, subfamily N, member 3), CAPN10 (calpain 10), PTGES (prostaglandin E synthase), ADRA2B (adrenergic, alpha-2B-, receptor), ABCG5 (ATP-binding cassette, sub-family G (WHITE), member 5), PRDX2 (peroxiredoxin 2), CAPN5 (calpain 5), PARP14 (poly (ADP-ribose) polymerase family, member 14), MEX3C (mex-3 homolog C (C. elegans)), ACE angiotensin I converting enzyme (peptidyl-dipeptidase A) 1), TNF (tumor necrosis factor (TNF superfamily, member 2)), IL6

(interleukin 6 (interferon, beta 2)), STN (statin), SERPINE1 (serpin peptidase inhibitor, clade E (nexin, plasminogen activator inhibitor type 1), member 1), ALB (albumin), ADIPOQ (adiponectin, C1Q and collagen domain containing), APOB (apolipoprotein B (including Ag(x) antigen)), APOE (apolipoprotein E), LEP (leptin), MTHFR (5,10-methylenetetrahydrofolate reductase (NADPH)), APOA1 (apolipoprotein A-I), EDN1 (endothelin 1), NPPB (natriuretic peptide precursor B), NOS3 (nitric oxide synthase 3 (endothelial cell)), PPARG (peroxisome proliferator-activated receptor gamma), PLAT (plasminogen activator, tissue), PTGS2 (prostaglandin-endoperoxide synthase 2 (prostaglandin G/H synthase and cyclooxygenase)), CETP (cholesteryl ester transfer protein, plasma), AGTR1 (angiotensin II receptor, type 1), HMGCR (3-hydroxy-3-methylglutaryl-Coenzyme A reductase), IGF1 (insulin-like growth factor 1 (somatomedin C)), SELE (selectin E), REN (renin), PPARA (peroxisome proliferator-activated receptor alpha), PON1 (paraoxonase 1), KNG1 (kininogen 1), CCL2 (chemokine (C-C motif) ligand 2), LPL (lipoprotein lipase), VWF (von Willebrand factor), F2 (coagulation factor II (thrombin)), ICAM1 (intercellular adhesion molecule 1), TGFB1 (transforming growth factor, beta 1), NPPA (natriuretic peptide precursor A), IL10 (interleukin 10), EPO (erythropoietin), SOD1 (superoxide dismutase 1, soluble), VCAM1 (vascular cell adhesion molecule 1), IFNG (interferon, gamma), LPA (lipoprotein, Lp(a)), MPO (myeloperoxidase), ESR1 (estrogen receptor 1), MAPK1 (mitogen-activated protein kinase 1), HP (haptoglobin), F3 (coagulation factor III (thromboplastin, tissue factor)), CST3 (cystatin C), COG2 (component of oligomeric golgi complex 2), MMP9 (matrix metallopeptidase 9 (gelatinase B, 92 kDa gelatinase, 92 kDa type IV collagenase)), SERPINC1 (serpin peptidase inhibitor, clade C (antithrombin), member 1), F8 (coagulation factor VIII, procoagulant component), HMOX1 (heme oxygenase (decycling) 1), APOC3 (apolipoprotein C-III), IL8 (interleukin 8), PROK1 (prokineticin 1), CBS (cystathionine-beta-synthase), NOS2 (nitric oxide synthase 2, inducible), TLR4 (toll-like receptor 4), SELP (selectin P (granule membrane protein 140 kDa, antigen CD62)), ABCA1 (ATP-binding cassette, sub-family A (ABC1), member 1), AGT (angiotensinogen (serpin peptidase inhibitor, clade A, member 8)), LDLR (low density lipoprotein receptor), GPT (glutamic-pyruvate transaminase (alanine aminotransferase)), VEGFA (vascular endothelial growth factor A), NR3C2 (nuclear receptor subfamily 3, group C, member 2), IL18 (interleukin 18 (interferon-gamma-inducing factor)), NOS1 (nitric oxide synthase 1 (neuronal)), NR3C1 (nuclear receptor subfamily 3, group C, member 1 (glucocorticoid receptor)), FGB (fibrinogen

beta chain), HGF (hepatocyte growth factor (hepapoietin A; scatter factor)), IL1A (interleukin 1, alpha), RETN (resistin), AKT1 (v-akt murine thymoma viral oncogene homolog 1), LIPC (lipase, hepatic), HSPD1 (heat shock 60 kDa protein 1 (chaperonin)), MAPK14 (mitogen-activated protein kinase 14), SPP1 (secreted phosphoprotein 1), ITGB3 (integrin, beta 3 (platelet glycoprotein 111a, antigen CD61)), CAT (catalase), UTS2 (urotensin 2), THBD (thrombomodulin), F10 (coagulation factor X), CP (ceruloplasmin (ferroxidase)), TNFRSF11B (tumor necrosis factor receptor superfamily, member 11b), EDNRA (endothelin receptor type A), EGFR (epidermal growth factor receptor (erythroblastic leukemia viral (v-erb-b) oncogene homolog, avian)), MMP2 (matrix metallopeptidase 2 (gelatinase A, 72 kDa gelatinase, 72 kDa type IV collagenase)), PLG (plasminogen), NPY (neuropeptide Y), RHOD (ras homolog gene family, member D), MAPK8 (mitogen-activated protein kinase 8), MYC (v-myc myelocytomatosis viral oncogene homolog (avian)), FN1 (fibronectin 1), CMA1 (chymase 1, mast cell), PLAU (plasminogen activator, urokinase), GNB3 (guanine nucleotide binding protein (G protein), beta polypeptide 3), ADRB2 (adrenergic, beta-2-, receptor, surface), APOA5 (apolipoprotein A-V), SOD2 (superoxide dismutase 2, mitochondrial), F5 (coagulation factor V (proaccelerin, labile factor)), VDR (vitamin D (1,25-dihydroxyvitamin D3) receptor), ALOX5 (arachidonate 5-lipoxygenase), HLA-DRB1 (major histocompatibility complex, class II, DR beta 1), PARP1 (poly (ADP-ribose) polymerase 1), CD40LG (CD40 ligand), PON2 (paraoxonase 2), AGER (advanced glycosylation end product-specific receptor), IRS1 (insulin receptor substrate 1), PTGS1 (prostaglandin-endoperoxide synthase 1 (prostaglandin G/H synthase and cyclooxygenase)), ECE1 (endothelin converting enzyme 1), F7 (coagulation factor VII (serum prothrombin conversion accelerator)), URN (interleukin 1 receptor antagonist), EPHX2 (epoxide hydrolase 2, cytoplasmic), IGFBP1 (insulin-like growth factor binding protein 1), MAPK10 (mitogen-activated protein kinase 10), FAS (Fas (TNF receptor superfamily, member 6)), ABCB1 (ATP-binding cassette, sub-family B (MDR/TAP), member 1), JUN (jun oncogene), IGFBP3 (insulin-like growth factor binding protein 3), CD14 (CD14 molecule), PDE5A (phosphodiesterase 5A, cGMP-specific), AGTR2 (angiotensin II receptor, type 2), CD40 (CD40 molecule, TNF receptor superfamily member 5), LCAT (lecithin-cholesterol acyltransferase), CCR5 (chemokine (C-C motif) receptor 5), MMP1 (matrix metallopeptidase 1 (interstitial collagenase)), TIMP1 (TIMP metallopeptidase inhibitor 1), ADM (adrenomedullin), DYT10 (dystonia 10), STAT3 (signal transducer and activator of transcription 3 (acute-phase response

factor)), MMP3 (matrix metallopeptidase 3 (stromelysin 1, progelatinase)), ELN (elastin), USF1 (upstream transcription factor 1), CFH (complement factor H), HSPA4 (heat shock 70 kDa protein 4), MMP12 (matrix metallopeptidase 12 (macrophage elastase)), MME (membrane metallo-endopeptidase), F2R (coagulation factor II (thrombin) receptor), SELL (selectin L), CTSB (cathepsin B), ANXA5 (annexin A5), ADRB1 (adrenergic, beta-1-, receptor), CYBA (cytochrome b-245, alpha polypeptide), FGA (fibrinogen alpha chain), GGT1 (gamma-glutamyltransferase 1), LIPG (lipase, endothelial), HIF1A (hypoxia inducible factor 1, alpha subunit (basic helix-loop-helix transcription factor)), CXCR4 (chemokine (C-X-C motif) receptor 4), PROC (protein C (inactivator of coagulation factors Va and VIIIa)), SCARB1 (scavenger receptor class B, member 1), CD79A (CD79a molecule, immunoglobulin-associated alpha), PLTP (phospholipid transfer protein), ADD1 (adducin 1 (alpha)), FGG (fibrinogen gamma chain), SAA1 (serum amyloid A1), KCNH2 (potassium voltage-gated channel, subfamily H (eag-related), member 2), DPP4 (dipeptidyl-peptidase 4), G6PD (glucose-6-phosphate dehydrogenase), NPR1 (natriuretic peptide receptor A/guanylate cyclase A (atrionatriuretic peptide receptor A)), VTN (vitronectin), KIAA0101 (KIAA0101), FOS (FBJ murine osteosarcoma viral oncogene homolog), TLR2 (toll-like receptor 2), PPIG (peptidylprolyl isomerase G (cyclophilin G)), IL1R1 (interleukin 1 receptor, type I), AR (androgen receptor), CYP1A1 (cytochrome P450, family 1, subfamily A, polypeptide 1), SERPINA1 (serpin peptidase inhibitor, clade A (alpha-1 antiproteinase, antitrypsin), member 1), MTR (5-methyltetrahydrofolate-homocysteine methyltransferase), RBP4 (retinol binding protein 4, plasma), APOA4 (apolipoprotein A-IV), CDKN2A (cyclin-dependent kinase inhibitor 2A (melanoma, p16, inhibits CDK4)), FGF2 (fibroblast growth factor 2 (basic)), EDNRB (endothelin receptor type B), ITGA2 (integrin, alpha 2 (CD49B, alpha 2 subunit of VLA-2 receptor)), CABIN1 (calcineurin binding protein 1), SHBG (sex hormone-binding globulin), HMGB1 (high-mobility group box 1), HSP90B2P (heat shock protein 90 kDa beta (Grp94), member 2 (pseudogene)), CYP3A4 (cytochrome P450, family 3, subfamily A, polypeptide 4), GJA1 (gap junction protein, alpha 1, 43 kDa), CAV1 (caveolin 1, caveolae protein, 22 kDa), ESR2 (estrogen receptor 2 (ER beta)), LTA (lymphotoxin alpha (TNF superfamily, member 1)), GDF15 (growth differentiation factor 15), BDNF (brain-derived neurotrophic factor), CYP2D6 (cytochrome P450, family 2, subfamily D, polypeptide 6), NGF (nerve growth factor (beta polypeptide)), SP1 (Sp1 transcription factor), TGIF1 (TGFB-induced factor homeobox 1), SRC

(v-src sarcoma (Schmidt-Ruppin A-2) viral oncogene homolog (avian)), EGF (epidermal growth factor (beta-urogastrone)), PIK3CG (phosphoinositide-3-kinase, catalytic, gamma polypeptide), HLA-A (major histocompatibility complex, class I, A), KCNQ1 (potassium voltage-gated channel, KQT-like subfamily, member 1), CNR1 (cannabinoid receptor 1 (brain)), FBN1 (fibrillin 1), CHKA (choline kinase alpha), BEST1 (bestrophin 1), APP (amyloid beta (A4) precursor protein), CTNNB1 (catenin (cadherin-associated protein), beta 1, 88 kDa), IL2 (interleukin 2), CD36 (CD36 molecule (thrombospondin receptor)), PRKAB1 (protein kinase, AMP-activated, beta 1 non-catalytic subunit), TPO (thyroid peroxidase), ALDH7A1 (aldehyde dehydrogenase 7 family, member A1), CX3CR1 (chemokine (C-X3-C motif) receptor 1), TH (tyrosine hydroxylase), F9 (coagulation factor IX), GH1 (growth hormone 1), TF (transferrin), HFE (hemochromatosis), IL17A (interleukin 17A), PTEN (phosphatase and tensin homolog), GSTM1 (glutathione S-transferase mu 1), DMD (dystrophin), GATA4 (GATA binding protein 4), F13A1 (coagulation factor XIII, A1 polypeptide), TTR (transthyretin), FABP4 (fatty acid binding protein 4, adipocyte), PON3 (paraoxonase 3), APOC1 (apolipoprotein C-I), INSR (insulin receptor), TNFRSF1B (tumor necrosis factor receptor superfamily, member 1B), HTR2A (5-hydroxytryptamine (serotonin) receptor 2A), CSF3 (colony stimulating factor 3 (granulocyte)), CYP2C9 (cytochrome P450, family 2, subfamily C, polypeptide 9), TXN (thioredoxin), CYP11B2 (cytochrome P450, family 11, subfamily B, polypeptide 2), PTH (parathyroid hormone), CSF2 (colony stimulating factor 2 (granulocyte-macrophage)), KDR (kinase insert domain receptor (a type III receptor tyrosine kinase)), PLA2G2A (phospholipase A2, group IIA (platelets, synovial fluid)), B2M (beta-2-microglobulin), THBS1 (thrombospondin 1), GCG (glucagon), RHOA (ras homolog gene family, member A), ALDH2 (aldehyde dehydrogenase 2 family (mitochondrial)), TCF7L2 (transcription factor 7-like 2 (T-cell specific, HMG-box)), BDKRB2 (bradykinin receptor B2), NFE2L2 (nuclear factor (erythroid-derived 2)-like 2), NOTCH1 (Notch homolog 1, translocation-associated (Drosophila)), UGT1A1 (UDP glucuronosyltransferase 1 family, polypeptide A1), IFNA1 (interferon, alpha 1), PPARD (peroxisome proliferator-activated receptor delta), SIRT1 (sirtuin (silent mating type information regulation 2 homolog) 1 (S. cerevisiae)), GNRH1 (gonadotropin-releasing hormone 1 (luteinizing-releasing hormone)), PAPPA (pregnancy-associated plasma protein A, pappalysin 1), ARR3 (arrestin 3, retinal (X-arrestin)), NPPC (natriuretic peptide precursor C), AHSP (alpha hemoglobin stabilizing protein), PTK2 (PTK2 protein tyrosine kinase 2), IL13 (interleukin 13),

MTOR (mechanistic target of rapamycin (serine/threonine kinase)), ITGB2 (integrin, beta 2 (complement component 3 receptor 3 and 4 subunit)), GSTT1 (glutathione S-transferase theta 1), IL6ST (interleukin 6 signal transducer (gp130, oncostatin M receptor)), CPB2 (carboxypeptidase B2 (plasma)), CYP1A2 (cytochrome P450, family 1, subfamily A, polypeptide 2), HNF4A (hepatocyte nuclear factor 4, alpha), SLC6A4 (solute carrier family 6 (neurotransmitter transporter, serotonin), member 4), PLA2G6 (phospholipase A2, group VI (cytosolic, calcium-independent)), TNFSF11 (tumor necrosis factor (ligand) superfamily, member 11), SLC8A1 (solute carrier family 8 (sodium/calcium exchanger), member 1), F2RL1 (coagulation factor II (thrombin) receptor-like 1), AKR1A1 (aldo-keto reductase family 1, member A1 (aldehyde reductase)), ALDH9A1 (aldehyde dehydrogenase 9 family, member A1), BGLAP (bone gamma-carboxyglutamate (gla) protein), MTTP (microsomal triglyceride transfer protein), MTRR (5-methyltetrahydrofolate-homocysteine methyltransferase reductase), SULT1A3 (sulfotransferase family, cytosolic, 1A, phenol-preferring, member 3), RAGE (renal tumor antigen), C4B (complement component 4B (Chido blood group), P2RY12 (purinergic receptor P2Y, G-protein coupled, 12), RNLS (renalase, FAD-dependent amine oxidase), CREB1 (cAMP responsive element binding protein 1), POMC (proopiomelanocortin), RAC1 (ras-related C3 botulinum toxin substrate 1 (rho family, small GTP binding protein Rac1)), LMNA (lamin NC), CD59 (CD59 molecule, complement regulatory protein), SCN5A (sodium channel, voltage-gated, type V, alpha subunit), CYP1B1 (cytochrome P450, family 1, subfamily B, polypeptide 1), MIF (macrophage migration inhibitory factor (glycosylation-inhibiting factor)), MMP13 (matrix metallopeptidase 13 (collagenase 3)), TIMP2 (TIMP metallopeptidase inhibitor 2), CYP19A1 (cytochrome P450, family 19, subfamily A, polypeptide 1), CYP21A2 (cytochrome P450, family 21, subfamily A, polypeptide 2), PTPN22 (protein tyrosine phosphatase, non-receptor type 22 (lymphoid)), MYH14 (myosin, heavy chain 14, non-muscle), MBL2 (mannose-binding lectin (protein C) 2, soluble (opsonic defect)), SELPLG (selectin P ligand), AOC3 (amine oxidase, copper containing 3 (vascular adhesion protein 1)), CTSL1 (cathepsin L1), PCNA (proliferating cell nuclear antigen), IGF2 (insulin-like growth factor 2 (somatomedin A)), ITGB1 (integrin, beta 1 (fibronectin receptor, beta polypeptide, antigen CD29 includes MDF2, MSK12)), CAST (calpastatin), CXCL12 (chemokine (C-X-C motif) ligand 12 (stromal cell-derived factor 1)), IGHE (immunoglobulin heavy constant epsilon), KCNE1 (potassium voltage-gated channel, Isk-related family, member 1), TFRC (transferrin receptor (p90, CD71)), COL1A1 (collagen, type I,

alpha 1), COL1A2 (collagen, type I, alpha 2), IL2RB (interleukin 2 receptor, beta), PLA2G10 (phospholipase A2, group X), ANGPT2 (angiopoietin 2), PROCR (protein C receptor, endothelial (EPCR)), NOX4 (NADPH oxidase 4), HAMP (hepcidin antimicrobial peptide), PTPN11 (protein tyrosine phosphatase, non-receptor type 11), SLC2A1 (solute carrier family 2 (facilitated glucose transporter), member 1), IL2RA (interleukin 2 receptor, alpha), CCL5 (chemokine (C-C motif) ligand 5), IRF1 (interferon regulatory factor 1), CFLAR (CASP8 and FADD-like apoptosis regulator), CALCA (calcitonin-related polypeptide alpha), EIF4E (eukaryotic translation initiation factor 4E), GSTP1 (glutathione S-transferase pi 1), JAK2 (Janus kinase 2), CYP3A5 (cytochrome P450, family 3, subfamily A, polypeptide 5), HSPG2 (heparan sulfate proteoglycan 2), CCL3 (chemokine (C-C motif) ligand 3), MYD88 (myeloid differentiation primary response gene (88)), VIP (vasoactive intestinal peptide), SOAT1 (sterol O-acyltransferase 1), ADRBK1 (adrenergic, beta, receptor kinase 1), NR4A2 (nuclear receptor subfamily 4, group A, member 2), MMP8 (matrix metallopeptidase 8 (neutrophil collagenase)), NPR2 (natriuretic peptide receptor B/guanylate cyclase B (atrionatriuretic peptide receptor B)), GCH1 (GTP cyclohydrolase 1), EPRS (glutamyl-prolyl-tRNA synthetase), PPARGC1A (peroxisome proliferator-activated receptor gamma, coactivator 1 alpha), F12 (coagulation factor XII (Hageman factor)), PECAM1 (platelet/endothelial cell adhesion molecule), CCL4 (chemokine (C-C motif) ligand 4), SERPINA3 (serpin peptidase inhibitor, clade A (alpha-1 antiproteinase, antitrypsin), member 3), CASR (calcium-sensing receptor), GJA5 (gap junction protein, alpha 5, 40 kDa), FABP2 (fatty acid binding protein 2, intestinal), TTF2 (transcription termination factor, RNA polymerase II), PROS1 (protein S (alpha)), CTF1 (cardiotrophin 1), SGCB (sarcoglycan, beta (43 kDa dystrophin-associated glycoprotein)), YME1L1 (YME1-like 1 (S. cerevisiae)), CAMP (cathelicidin antimicrobial peptide), ZC3H12A (zinc finger CCCH-type containing 12A), AKR1B1 (aldo-keto reductase family 1, member B1 (aldose reductase)), DES (desmin), MMP7 (matrix metallopeptidase 7 (matrilysin, uterine)), AHR (aryl hydrocarbon receptor), CSF1 (colony stimulating factor 1 (macrophage)), HDAC9 (histone deacetylase 9), CTGF (connective tissue growth factor), KCNMA1 (potassium large conductance calcium-activated channel, subfamily M, alpha member 1), UGT1A (UDP glucuronosyltransferase 1 family, polypeptide A complex locus), PRKCA (protein kinase C, alpha), COMT (catechol-.beta.-methyltransferase), S100B (S100 calcium binding protein B), EGR1 (early growth response 1), PRL (prolactin), IL15 (interleukin 15), DRD4 (dopamine receptor D4), CAMK2G

(calcium/calmodulin-dependent protein kinase II gamma), SLC22A2 (solute carrier family 22 (organic cation transporter), member 2), CCL11 (chemokine (C-C motif) ligand 11), PGF (B321 placental growth factor), THPO (thrombopoietin), GP6 (glycoprotein VI (platelet)), TACR1 (tachykinin receptor 1), NTS (neurotensin), HNF1A (HNF1 homeobox A), SST (somatostatin), KCND1 (potassium voltage-gated channel, Shal-related subfamily, member 1), LOC646627 (phospholipase inhibitor), TBXAS1 (thromboxane A synthase 1 (platelet)), CYP2J2 (cytochrome P450, family 2, subfamily J, polypeptide 2), TBXA2R (thromboxane A2 receptor), ADH1C (alcohol dehydrogenase 1C (class I), gamma polypeptide), ALOX12 (arachidonate 12-lipoxygenase), AHSG (alpha-2-HS-glycoprotein), BHMT (betaine-homocysteine methyltransferase), GJA4 (gap junction protein, alpha 4, 37 kDa), SLC25A4 (solute carrier family 25 (mitochondrial carrier; adenine nucleotide translocator), member 4), ACLY (ATP citrate lyase), ALOX5AP (arachidonate 5-lipoxygenase-activating protein), NUMA1 (nuclear mitotic apparatus protein 1), CYP27B1 (cytochrome P450, family 27, subfamily B, polypeptide 1), CYSLTR2 (cysteinyl leukotriene receptor 2), SOD3 (superoxide dismutase 3, extracellular), LTC4S (leukotriene C4 synthase), UCN (urocortin), GHRL (ghrelin/obestatin prepropeptide), APOC2 (apolipoprotein C-II), CLEC4A (C-type lectin domain family 4, member A), KBTBD10 (kelch repeat and BTB (POZ) domain containing 10), TNC (tenascin C), TYMS (thymidylate synthetase), SHC1 (SHC (Src homology 2 domain containing) transforming protein 1), LRP1 (low density lipoprotein receptor-related protein 1), SOCS3 (suppressor of cytokine signaling 3), ADH1B (alcohol dehydrogenase 1B (class I), beta polypeptide), KLK3 (kallikrein-related peptidase 3), HSD11B1 (hydroxysteroid (11-beta) dehydrogenase 1), VKORC1 (vitamin K epoxide reductase complex, subunit 1), SERPINB2 (serpin peptidase inhibitor, clade B (ovalbumin), member 2), TNS1 (tensin 1), RNF19A (ring finger protein 19A), EPOR (erythropoietin receptor), ITGAM (integrin, alpha M (complement component 3 receptor 3 subunit)), PITX2 (paired-like homeodomain 2), MAPK7 (mitogen-activated protein kinase 7), FCGR3A (Fc fragment of IgG, low affinity IIIa, receptor (CD16a)), LEPR (leptin receptor), ENG (endoglin), GPX1 (glutathione peroxidase 1), GOT2 (glutamic-oxaloacetic transaminase 2, mitochondrial (aspartate aminotransferase 2)), HRH1 (histamine receptor H1), NR112 (nuclear receptor subfamily 1, group I, member 2), CRH (corticotropin releasing hormone), HTR1A (5-hydroxytryptamine (serotonin) receptor 1A), VDAC1 (voltage-dependent anion channel 1), HPSE (heparanase), SFTPD (surfactant protein D), TAP2 (transporter 2, ATP-binding cassette,

sub-family B (MDR/TAP)), RNF123 (ring finger protein 123), PTK2B (PTK2B protein tyrosine kinase 2 beta), NTRK2 (neurotrophic tyrosine kinase, receptor, type 2), IL6R (interleukin 6 receptor), ACHE (acetylcholinesterase (Yt blood group)), GLP1R (glucagon-like peptide 1 receptor), GHR (growth hormone receptor), GSR (glutathione reductase), NQO1 (NAD(P)H dehydrogenase, quinone 1), NR5A1 (nuclear receptor subfamily 5, group A, member 1), GJB2 (gap junction protein, beta 2, 26 kDa), SLC9A1 (solute carrier family 9 (sodium/hydrogen exchanger), member 1), MAOA (monoamine oxidase A), PCSK9 (proprotein convertase subtilisin/kexin type 9), FCGR2A (Fc fragment of IgG, low affinity IIa, receptor (CD32)), SERPINF1 (serpin peptidase inhibitor, clade F (alpha-2 antiplasmin, pigment epithelium derived factor), member 1), EDN3 (endothelin 3), DHFR (dihydrofolate reductase), GAS6 (growth arrest-specific 6), SMPD1 (sphingomyelin phosphodiesterase 1, acid lysosomal), UCP2 (uncoupling protein 2 (mitochondrial, proton carrier)), TFAP2A (transcription factor AP-2 alpha (activating enhancer binding protein 2 alpha)), C4BPA (complement component 4 binding protein, alpha), SERPINF2 (serpin peptidase inhibitor, clade F (alpha-2 antiplasmin, pigment epithelium derived factor), member 2), TYMP (thymidine phosphorylase), ALPP (alkaline phosphatase, placental (Regan isozyme)), CXCR2 (chemokine (C-X-C motif) receptor 2), SLC39A3 (solute carrier family 39 (zinc transporter), member 3), ABCG2 (ATP-binding cassette, sub-family G (WHITE), member 2), ADA (adenosine deaminase), JAK3 (Janus kinase 3), HSPA1A (heat shock 70 kDa protein 1A), FASN (fatty acid synthase), FGF1 (fibroblast growth factor 1 (acidic)), F11 (coagulation factor XI), ATP7A (ATPase, Cu++ transporting, alpha polypeptide), CR1 (complement component (3b/4b) receptor 1 (Knops blood group)), GFAP (glial fibrillary acidic protein), ROCK1 (Rho-associated, coiled-coil containing protein kinase 1), MECP2 (methyl CpG binding protein 2 (Rett syndrome)), MYLK (myosin light chain kinase), BCHE (butyrylcholinesterase), LIPE (lipase, hormone-sensitive), PRDX5 (peroxiredoxin 5), ADORA1 (adenosine A1 receptor), WRN (Werner syndrome, RecQ helicase-like), CXCR3 (chemokine (C-X-C motif) receptor 3), CD81 (CD81 molecule), SMAD7 (SMAD family member 7), LAMC2 (laminin, gamma 2), MAP3K5 (mitogen-activated protein kinase kinase kinase 5), CHGA (chromogranin A (parathyroid secretory protein 1)), IAPP (islet amyloid polypeptide), RHO (rhodopsin), ENPP1 (ectonucleotide pyrophosphatase/phosphodiesterase 1), PTHLH (parathyroid hormone-like hormone), NRG1 (neuregulin 1), VEGFC (vascular endothelial growth factor C), ENPEP (glutamyl aminopeptidase (aminopeptidase A)), CEBPB

(CCAAT/enhancer binding protein (C/EBP), beta), NAGLU (N-acetylglucosaminidase, alpha-), F2RL3 (coagulation factor II (thrombin) receptor-like 3), CX3CL1 (chemokine (C-X3-C motif) ligand 1), BDKRB1 (bradykinin receptor B1), ADAMTS13 (ADAM metallopeptidase with thrombospondin type 1 motif, 13), ELANE (elastase, neutrophil expressed), ENPP2 (ectonucleotide pyrophosphatase/phosphodiesterase 2), CISH (cytokine inducible SH2-containing protein), GAST (gastrin), MYOC (myocilin, trabecular meshwork inducible glucocorticoid response), ATP1A2 (ATPase, Na+/K+ transporting, alpha 2 polypeptide), NF1 (neurofibromin 1), GJB1 (gap junction protein, beta 1, 32 kDa), MEF2A (myocyte enhancer factor 2A), VCL (vinculin), BMPR2 (bone morphogenetic protein receptor, type II (serine/threonine kinase)), TUBB (tubulin, beta), CDC42 (cell division cycle 42 (GTP binding protein, 25 kDa)), KRT18 (keratin 18), HSF1 (heat shock transcription factor 1), MYB (v-myb myeloblastosis viral oncogene homolog (avian)), PRKAA2 (protein kinase, AMP-activated, alpha 2 catalytic subunit), ROCK2 (Rho-associated, coiled-coil containing protein kinase 2), TFPI (tissue factor pathway inhibitor (lipoprotein-associated coagulation inhibitor)), PRKG1 (protein kinase, cGMP-dependent, type I), BMP2 (bone morphogenetic protein 2), CTNND1 (catenin (cadherin-associated protein), delta 1), CTH (cystathionase (cystathionine gamma-lyase)), CTSS (cathepsin S), VAV2 (vav 2 guanine nucleotide exchange factor), NPY2R (neuropeptide Y receptor Y2), IGFBP2 (insulin-like growth factor binding protein 2, 36 kDa), CD28 (CD28 molecule), GSTA1 (glutathione S-transferase alpha 1), PPIA (peptidylprolyl isomerase A (cyclophilin A)), APOH (apolipoprotein H (beta-2-glycoprotein I)), S100A8 (S100 calcium binding protein A8), IL11 (interleukin 11), ALOX15 (arachidonate 15-lipoxygenase), FBLN1 (fibulin 1), NR1H3 (nuclear receptor subfamily 1, group H, member 3), SCD (stearoyl-CoA desaturase (delta-9-desaturase)), GIP (gastric inhibitory polypeptide), CHGB (chromogranin B (secretogranin 1)), PRKCB (protein kinase C, beta), SRD5A1 (steroid-5-alpha-reductase, alpha polypeptide 1 (3-oxo-5 alpha-steroid delta 4-dehydrogenase alpha 1)), HSD11B2 (hydroxysteroid (11-beta) dehydrogenase 2), CALCRL (calcitonin receptor-like), GALNT2 (UDP-N-acetyl-alpha-D-galactosamine:polypeptide N-acetylgalactosaminyltransferase 2 (GalNAc-T2)), ANGPTL4 (angiopoietin-like 4), KCNN4 (potassium intermediate/small conductance calcium-activated channel, subfamily N, member 4), PIK3C2A (phosphoinositide-3-kinase, class 2, alpha polypeptide), HBEGF (heparin-binding EGF-like growth factor), CYP7A1 (cytochrome P450, family 7, subfamily A, polypeptide 1), HLA-DRB5 (major

histocompatibility complex, class II, DR beta 5), BNIP3 (BCL2/adenovirus E1B 19 kDa interacting protein 3), GCKR (glucokinase (hexokinase 4) regulator), S100A12 (S100 calcium binding protein A12), PADI4 (peptidyl arginine deiminase, type IV), HSPA14 (heat shock 70 kDa protein 14), CXCR1 (chemokine (C-X-C motif) receptor 1), H19 (H19, imprinted maternally expressed transcript (non-protein coding)), KRTAP19-3 (keratin associated protein 19-3), IDDM2 (insulin-dependent diabetes mellitus 2), RAC2 (ras-related C3 botulinum toxin substrate 2 (rho family, small GTP binding protein Rac2)), RYR1 (ryanodine receptor 1 (skeletal)), CLOCK (clock homolog (mouse)), NGFR (nerve growth factor receptor (TNFR superfamily, member 16)), DBH (dopamine beta-hydroxylase (dopamine beta-monooxygenase)), CHRNA4 (cholinergic receptor, nicotinic, alpha 4), CACNA1C (calcium channel, voltage-dependent, L type, alpha 1C subunit), PRKAG2 (protein kinase, AMP-activated, gamma 2 non-catalytic subunit), CHAT (choline acetyltransferase), PTGDS (prostaglandin D2 synthase 21 kDa (brain)), NR1H2 (nuclear receptor subfamily 1, group H, member 2), TEK (TEK tyrosine kinase, endothelial), VEGFB (vascular endothelial growth factor B), MEF2C (myocyte enhancer factor 2C), MAPKAPK2 (mitogen-activated protein kinase-activated protein kinase 2), TNFRSF11A (tumor necrosis factor receptor superfamily, member 11a, NFKB activator), HSPA9 (heat shock 70 kDa protein 9 (mortalin)), CYSLTR1 (cysteinyl leukotriene receptor 1), MAT1A (methionine adenosyltransferase I, alpha), OPRL1 (opiate receptor-like 1), IMPA1 (inositol(myo)-1(or 4)-monophosphatase 1), CLCN2 (chloride channel 2), DLD (dihydrolipoamide dehydrogenase), PSMA6 (proteasome (prosome, macropain) subunit, alpha type, 6), PSMB8 (proteasome (prosome, macropain) subunit, beta type, 8 (large multifunctional peptidase 7)), CHI3L1 (chitinase 3-like 1 (cartilage glycoprotein-39)), ALDH1B1 (aldehyde dehydrogenase 1 family, member B1), PARP2 (poly (ADP-ribose) polymerase 2), STAR (steroidogenic acute regulatory protein), LBP (lipopolysaccharide binding protein), ABCC6 (ATP-binding cassette, sub-family C(CFTR/MRP), member 6), RGS2 (regulator of G-protein signaling 2, 24 kDa), EFNB2 (ephrin-B2), GJB6 (gap junction protein, beta 6, 30 kDa), APOA2 (apolipoprotein A-II), AMPD1 (adenosine monophosphate deaminase 1), DYSF (dysferlin, limb girdle muscular dystrophy 2B (autosomal recessive)), FDFT1 (farnesyl-diphosphate farnesyltransferase 1), EDN2 (endothelin 2), CCR6 (chemokine (C-C motif) receptor 6), GJB3 (gap junction protein, beta 3, 31 kDa), IL1RL1 (interleukin 1 receptor-like 1), ENTPD1 (ectonucleoside triphosphate diphosphohydrolase 1), BBS4 (Bardet-Biedl syndrome 4), CELSR2 (cadherin, EGF LAG seven-

pass G-type receptor 2 (flamingo homolog, Drosophila)), F11R (F11 receptor), RAPGEF3 (Rap guanine nucleotide exchange factor (GEF) 3), HYAL1 (hyaluronoglucosaminidase 1), ZNF259 (zinc finger protein 259), ATOX1 (ATX1 antioxidant protein 1 homolog (yeast)), ATF6 (activating transcription factor 6), KHK (ketohexokinase (fructokinase)), SAT1 (spermidine/spermine N1-acetyltransferase 1), GGH (gamma-glutamyl hydrolase (conjugase, folylpolygammaglutamyl hydrolase)), TIMP4 (TIMP metallopeptidase inhibitor 4), SLC4A4 (solute carrier family 4, sodium bicarbonate cotransporter, member 4), PDE2A (phosphodiesterase 2A, cGMP-stimulated), PDE3B (phosphodiesterase 3B, cGMP-inhibited), FADS1 (fatty acid desaturase 1), FADS2 (fatty acid desaturase 2), TMSB4X (thymosin beta 4, X-linked), TXNIP (thioredoxin interacting protein), LIMS1 (LIM and senescent cell antigen-like domains 1), RHOB (ras homolog gene family, member B), LY96 (lymphocyte antigen 96), FOXO1 (forkhead box O1), PNPLA2 (patatin-like phospholipase domain containing 2), TRH (thyrotropin-releasing hormone), GJC1 (gap junction protein, gamma 1, 45 kDa), SLC17A5 (solute carrier family 17 (anion/sugar transporter), member 5), FTO (fat mass and obesity associated), GJD2 (gap junction protein, delta 2, 36 kDa), PSRC1 (proline/serine-rich coiled-coil 1), CASP12 (caspase 12 (gene/pseudogene)), GPBAR1 (G protein-coupled bile acid receptor 1), PXK (PX domain containing serine/threonine kinase), IL33 (interleukin 33), TRIB1 (tribbles homolog 1 (Drosophila)), PBX4 (pre-B-cell leukemia homeobox 4), NUPR1 (nuclear protein, transcriptional regulator, 1), 15-Sep(15 kDa selenoprotein), CILP2 (cartilage intermediate layer protein 2), TERC (telomerase RNA component), GGT2 (gamma-glutamyltransferase 2), MT-CO1 (mitochondrially encoded cytochrome c oxidase I), and UOX (urate oxidase, pseudogene).

[00372] In an additional embodiment, the chromosomal sequence may further be selected from Pon1 (paraoxonase 1), LDLR (LDL receptor), ApoE (Apolipoprotein E), Apo B-100 (Apolipoprotein B-100), ApoA (Apolipoprotein(a)), ApoA1 (Apolipoprotein A1), CBS (Cystathione B-synthase), Glycoprotein IIb/IIb, MTHRF (5,10-methylenetetrahydrofolate reductase (NADPH), and combinations thereof. In one iteration, the chromosomal sequences and proteins encoded by chromosomal sequences involved in cardiovascular disease may be chosen from Cacna1C, Sod1, Pten, Ppar(alpha), Apo E, Leptin, and combinations thereof.

Kidneys

[00373] The present invention also contemplates delivering the CRISPR-Cas system to the kidney. Delivery strategies to induce cellular uptake of the therapeutic nucleic acid include

physical force or vector systems such as viral-, lipid- or complex- based delivery, or nanocarriers. From the initial applications with less possible clinical relevance, when nucleic acids were addressed to renal cells with hydrodynamic high pressure injection systemically, a wide range of gene therapeutic viral and non-viral carriers have been applied already to target posttranscriptional events in different animal kidney disease models in vivo (Csaba Révész and Péter Hamar (2011). Delivery Methods to Target RNAs in the Kidney, Gene Therapy Applications, Prof. Chunsheng Kang (Ed.), ISBN: 978-953-307-541-9, InTech, Available from: http://www.intechopen.com/books/gene-therapy-applications/delivery-methods-to-target-rnas-in-the-kidney). Delivery methods to the kidney are summarized as follows:

Delivery method	Carrier	Target RNA	Disease	Model	Functional assays	Author
Hydrodynamic / Lipid	TransIT In Vivo Gene Delivery System, DOTAP	p85α	Acute renal injury	Ischemia-reperfusion	Uptake, biodistribution	Larson et al., Surgery, (Aug 2007), Vol. 142, No. 2, pp. (262-269)
Hydrodynamic / Lipid	Lipofectamine 2000	Fas	Acute renal injury	Ischemia-reperfusion	Blood urea nitrogen, Fas Immunohistochemistry, apoptosis, histological scoring	Hamar et al., Proc Natl Acad Sci, (Oct 2004), Vol. 101, No. 41, pp. (14883-14888)
Hydrodynamic	n.a.	Apoptosis cascade elements	Acute renal injury	Ischemia-reperfusion	n.a.	Zheng et al., Am J Pathol, (Oct 2008), Vol. 173, No. 4, pp. (973–980)
Hydrodynamic	n.a.	Nuclear factor kappa-b (NFkB)	Acute renal injury	Ischemia-reperfusion	n.a.	Feng et al., Transplantation, (May 2009), Vol. 87, No. 9, pp. (1283–1289)
Hydrodynamic /Viral	Lipofectamine 2000	Apoptosis antagonizing transcription factor (AATF)	Acute renal injury	Ischemia-reperfusion	Apoptosis, oxidative stress, caspase activation, membrane lipid peroxidation	Xie & Guo, Am Soc Nephrol, (Dec 2006), Vol. 17, No. 12, pp. (3336–3346)
Hydrodynamic	pBAsi mU6 Neo/ TransIT-EE Hydrodynamic Delivery System	Gremlin	Diabetic nephropathy	Streptozotozin -induced diabetes	Proteinuria, serum creatinine, glomerular and tubular diameter, collagen type IV/BMP7 expression	Q. Zhang et al., PloS ONE, (Jul 2010), Vol. 5, No. 7, e11709, pp. (1-13)
Viral/Lipid	pSUPER vector/Lipofectamine	TGF-β type II receptor	Interstitial renal fibrosis	Unilateral urethral obstruction	α-SMA expression, collagen content,	Kushibikia et al., J Controlled Release, (Jul 2005), Vol. 105, No. 3, pp. (318-331)

Delivery method	Carrier	Target RNA	Disease	Model	Functional assays	Author
Viral	Adeno-associated virus-2	Mineral corticoid receptor	Hyper-tension caused renal damage	Cold-induced hypertension	blood pressure, serum albumin, serum urea nitrogen, serum creatinine, kidney weight, urinary sodium	Wang et al., Gene Therapy, (Jul 2006), Vol. 13, No. 14, pp. (1097-1103)
Hydrodynamic /Viral	pU6 vector	Luciferase	n.a.	n.a.	uptake	Kobayashi et al., Journal of Pharmacology and Experimental Therapeutics, (Feb 2004), Vol. 308, No. 2, pp. (688-693)
Lipid	Lipoproteins, albumin	apoB1, apoM	n.a.	n.a.	Uptake, binding affinity to lipoproteins and albumin	Wolfrum et al., Nature Biotechnology, (Sep 2007), Vol. 25, No. 10, pp. (1149-1157)
Lipid	Lipofectamine2000	p53	Acute renal injury	Ischemic and cisplatin-induced acute injury	Histological scoring, apoptosis	Molitoris et al., J Am Soc Nephrol, (Aug 2009), Vol. 20, No. 8, pp. (1754-1764)
Lipid	DOTAP/DOPE, DOTAP/DOPE/DOPE-PEG2000	COX-2	Breast adeno-carcinoma	MDA-MB-231 breast cancer xenograft-bearing mouse	Cell viability, uptake	Mikhaylova et al., Cancer Gene Therapy (Mar 2011), Vol. 16, No. 3, pp. (217-226)
Lipid	Cholesterol	12/15-lipoxygenase	Diabetic nephro- pathy	Streptozotocin -induced diabetes	Albuminuria, urinary creatinine, histology, type I and IV collagen, TGF-β, fibronectin, plasminogen activator inhibitor 1	Yuan et al., Am J Physiol Renal Physiol, (Jun 2008), Vol. 295, pp. (F605-F617)
Lipid	Lipofectamine 2000	Mitochondrial membrane 44 (TIM44)	Diabetic nephro- pathy	Streptozotocin -induced diabetes	Cell proliferation and apoptosis, histology, ROS, mitochondrial import of Mn-SOD and glutathione peroxidase, cellular membrane polarization	Y. Zhang et al., J Am Soc Nephrol, (Apr 2006), Vol. 17, No. 4, pp. (1090–1101)
Hydrodynamic / Lipid	Proteolipo-some	RLIP76	Renal carcinoma	Caki-2 kidney cancer xenograft-bearing mouse	uptake	Singhal et al., Cancer Res, (May 2009), Vol. 69, No. 10, pp. (4244-4251)

Delivery method	Carrier	Target RNA	Disease	Model	Functional assays	Author
Polymer	PEGylated PEI	Luciferase pGL3	n.a.	n.a.	Uptake, biodistribution, erythrocyte aggregation	Malek et al., Toxicology and Applied Pharmacology, (Apr 2009), Vol. 236, No. 1, pp. (97-108)
Polymer	PEGylated poly-L-lysine	MAPK1	Lupus glomerulo-nephritis	Glomerulo-nephritis	Proteinuria, glomerulosclerosis, TGF- β, fibronectin, plasminogen activator inhibitor 1	Shimizu et al., J Am Soc Nephrology, (Apr 2010), Vol. 21, No. 4, pp. (622-633)
Polymer/Nano particle	Hyaluronic acid/ Quantum dot/ PEI	VEGF	Kidney cancer/ melanoma	B16F1 melanoma tumor-bearing mouse	Biodistribution, citotoxicity, tumor volume, endocytosis	Jiang et al., Molecular Pharmaceutics, (May-Jun 2009), Vol. 6, No. 3, pp. (727-737)
Polymer/Nano particle	PEGylated polycapro- lactone nanofiber	GAPDH	n.a.	n.a.	cell viability, uptake	Cao et al, J Controlled Release, (Jun 2010), Vol. 144, No. 2, pp. (203-212)
Aptamer	Spiegelmer mNOX-E36	CC chemokine ligand 2	Glomerulo sclerosis	Uninephrecto - mized mouse	urinary albumin, urinary creatinine, histopathology, glomerular filtration rate, macrophage count, serum Ccl2, Mac-2+, Ki-67+	Ninichuk et al., Am J Pathol, (Mar 2008), Vol. 172, No. 3, pp. (628-637)
Aptamer	Aptamer NOX-F37	vasopressin (AVP)	Congestive heart failure	n.a.	Binding affinity to D-AVP, Inhibition of AVP Signaling, Urine osmolality and sodium concentration,	Purschke et al., Proc Natl Acad Sci, (Mar 2006), Vol. 103, No. 13, pp. (5173-5178)

[00374] Yuan et al. (Am J Physiol Renal Physiol 295: F605–F617, 2008) investigated whether in vivo delivery of small interfering RNAs (siRNAs) targeting the 12/15-lipoxygenase (12/15-LO) pathway of arachidonate acid metabolism can ameliorate renal injury and diabetic nephropathy (DN) in a streptozotocininjected mouse model of type 1 diabetes. To achieve greater in vivo access and siRNA expression in the kidney, Yuan et al. used double-stranded 12/15-LO siRNA oligonucleotides conjugated with cholesterol. About 400 μg of siRNA was injected subcutaneously into mice. The method of Yuang et al. may be applied to the CRISPR Cas system of the present invention contemplating a 1-2 g subcutaneous injection of CRISPR Cas conjugated with cholesterol to a human for delivery to the kidneys.

[00375] Molitoris et al. (J Am Soc Nephrol 20: 1754–1764, 2009) exploited proximal tubule cells (PTCs), as the site of oligonucleotide reabsorption within the kidney to test the efficacy of siRNA targeted to p53, a pivotal protein in the apoptotic pathway, to prevent kidney injury. Naked synthetic siRNA to p53 injected intravenously 4 h after ischemic injury maximally protected both PTCs and kidney function. Molitoris et al.'s data indicates that rapid delivery of siRNA to proximal tubule cells follows intravenous administration. For dose-response analysis, rats were injected with doses of siP53, 0.33; 1, 3, or 5mg/kg, given at the same four time points, resulting in cumulative doses of 1.32; 4, 12, and 20 mg/kg, respectively. All siRNA doses tested produced a SCr reducing effect on day one with higher doses being effective over approximately five days compared with PBS-treated ischemic control rats. The 12 and 20 mg/kg cumulative doses provided the best protective effect. The method of Molitoris et al. may be applied to the CRISPR Cas system of the present invention contemplating 12 and 20 mg/kg cumulative doses to a human for delivery to the kidneys.

[00376] Thompson et al. (Nucleic Acid Therapeutics, Volume 22, Number 4, 2012) reports the toxicological and pharmacokinetic properties of the synthetic, small interfering RNA I5NP following intravenous administration in rodents and nonhuman primates. I5NP is designed to act via the RNA interference (RNAi) pathway to temporarily inhibit expression of the pro-apoptotic protein p53 and is being developed to protect cells from acute ischemia/reperfusion injuries such as acute kidney injury that can occur during major cardiac surgery and delayed graft function that can occur following renal transplantation. Doses of 800mg/kg I5NP in rodents, and 1,000 mg/kg I5NP in nonhuman primates, were required to elicit adverse effects, which in the monkey were isolated to direct effects on the blood that included a sub-clinical activation of complement and slightly increased clotting times. In the rat, no additional adverse effects were observed with a rat analogue of I5NP, indicating that the effects likely represent class effects of synthetic RNA duplexes rather than toxicity related to the intended pharmacologic activity of I5NP. Taken together, these data support clinical testing of intravenous administration of I5NP for the preservation of renal function following acute ischemia/reperfusion injury. The no observed adverse effect level (NOAEL) in the monkey was 500 mg/kg. No effects on cardiovascular, respiratory, and neurologic parameters were observed in monkeys following i.v. administration at dose levels up to 25 mg/kg. Therefore, a similar dosage may be contemplated for intravenous administration of CRISPR Cas to the kidneys of a human.

[00377] Shimizu et al. (J Am Soc Nephrol 21: 622–633, 2010) developed a system to target delivery of siRNAs to glomeruli via poly(ethylene glycol)-poly(L-lysine)-based vehicles. The siRNA/nanocarrier complex was approximately 10 to 20 nm in diameter, a size that would allow it to move across the fenestrated endothelium to access to the mesangium. After intraperitoneal injection of fluorescence-labeled siRNA/nanocarrier complexes, Shimizu et al. detected siRNAs in the blood circulation for a prolonged time. Repeated intraperitoneal administration of a mitogen-activated protein kinase 1 (MAPK1) siRNA/nanocarrier complex suppressed glomerular MAPK1 mRNA and protein expression in a mouse model of glomerulonephritis. For the investigation of siRNA accumulation, Cy5-labeled siRNAs complexed with PIC nanocarriers (0.5 ml, 5 nmol of siRNA content), naked Cy5-labeled siRNAs (0.5 ml, 5 nmol), or Cy5-labeled siRNAs encapsulated in HVJ-E (0.5 ml, 5 nmol of siRNA content) were administrated to BALB-c mice. The method of Shimizu et al. may be applied to the CRISPR Cas system of the present invention contemplating a dose of about of 10-20 μmol CRISPR Cas complexed with nanocarriers in about 1-2 liters to a human for intraperitoneal administration and delivery to the kidneys.

Lungs

[00378] The present invention also contemplates delivering the CRISPR-Cas system to oneor both lungs.

[00379] Although AAV-2-based vectors were originally proposed for CFTR delivery to CF airways, other serotypes such as AAV-1, AAV-5, AAV-6, and AAV-9 exhibit improved gene transfer efficiency in a variety of models of the lung epithelium (see, e.g., Li et al., Molecular Therapy, vol. 17 no. 12, 2067-2077 Dec 2009). AAV-1 was demonstrated to be ~100-fold more efficient than AAV-2 and AAV-5 at transducing human airway epithelial cells in vitro,5 although AAV-1 transduced murine tracheal airway epithelia in vivo with an efficiency equal to that of AAV-5. Other studies have shown that AAV-5 is 50-fold more efficient than AAV-2 at gene delivery to human airway epithelium (HAE) in vitro and significantly more efficient in the mouse lung airway epithelium in vivo. AAV-6 has also been shown to be more efficient than AAV-2 in human airway epithelial cells in vitro and murine airways in vivo.8 The more recent isolate, AAV-9, was shown to display greater gene transfer efficiency than AAV-5 in murine nasal and alveolar epithelia in vivo with gene expression detected for over 9 months suggesting AAV may enable long-term gene expression in vivo, a desirable property for a CFTR gene

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

[00380] Zamora et al. (Am J Respir Crit Care Med Vol 183. pp 531–538, 2011) reported an example of the application of an RNA interference therapeutic to the treatment of human infectious disease and also a randomized trial of an antiviral drug in respiratory syncytial virus (RSV)-infected lung transplant recipients. Zamora et al. performed a randomized, double-blind, placebocontrolled trial in LTX recipients with RSV respiratory tract infection. Patients were permitted to receive standard of care for RSV. Aerosolized ALN-RSV01 (0.6 mg/kg) or placebo was administered daily for 3 days. This study demonstrates that an RNAi therapeutic targeting RSV can be safely administered to LTX recipients with RSV infection. Three daily doses of ALN-RSV01 did not result in any exacerbation of respiratory tract symptoms or impairment of lung function and did not exhibit any systemic proinflammatory effects, such as induction of cytokines or CRP. Pharmacokinetics showed only low, transient systemic exposure after inhalation, consistent with preclinical animal data showing that ALN-RSV01, administered intravenously or by inhalation, is rapidly cleared from the circulation through exonucleasemediated digestion and renal excretion. The method of Zamora et al. may be applied to the CRISPR Cas system of the present invention and an aerosolized CRISPR Cas, for example with a dosage of 0.6 mg/kg, may be contemplated for the present invention.

[00381] For an example of CFTRdelta508 chimeric guide RNA, see Example 22 which demonstrates gene transfer or gene delivery of a CRISPR-Cas system in airways of subject or a patient in need thereof, suffering from cystic fibrosis or from cystic fibrosis (CF) related symptoms, using adeno-associated virus (AAV) particles. In particular, they exemplify a repair strategy for Cystic Fibrosis delta F508 mutation. This type of strategy should apply across all organisms. With particular reference to CF, suitable patients may include: Human, non-primate human, canine, feline, bovine, equine and other domestic animals. In this instance, Applicants

utilized a CRISPR-Cas system comprising a Cas9 enzyme to target deltaF508 or other CFTR-inducing mutations.

[00382] The treated subjects in this instance receive pharmaceutically effective amount of aerosolized AAV vector system per lung endobronchially delivered while spontaneously breathing. As such, aerosolized delivery is preferred for AAV delivery in general. An adenovirus or an AAV particle may be used for delivery. Suitable gene constructs, each operably linked to one or more regulatory sequences, may be cloned into the delivery vector. In this instance, the following constructs are provided as examples: Cbh or EF1a promoter for Cas9, U6 or H1 promoter for chimeric guide RNA),: A preferred arrangement is to use a CFTRdelta508 targeting chimeric guide, a repair template for deltaF508 mutation and a codon optimized Cas9 enzyme (preferred Cas9s are those with nuclease or nickase activity) with optionally one or more nuclear localization signal or sequence(s) (NLS(s)), e.g., two (2) NLSs. Constructs without NLS are also envisaged.

[00383] In order to identify the Cas9 target site, Applicants analyzed the human CFTR genomic locus and identified the Cas9 target site. Preferably, in general and in this CF case, the PAM may contain a NGG or a NNAGAAW motif.

[00384] Accordingly, in the case of CF, the present method comprises manipulation of a target sequence in a genomic locus of interest comprising

delivering a non-naturally occurring or engineered composition comprising a viral vector system comprising one or more viral vectors operably encoding a composition for expression thereof, wherein the composition comprises:

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

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

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

(b) a tracr mate sequence, and

(c) a tracr sequence, and

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

wherein (a), (b) and (c) are arranged in a 5' to 3' orientation,
wherein components I and II are located on the same or different vectors of the system,
wherein when transcribed, the tracr mate sequence hybridizes to the tracr sequence and the guide sequence directs sequence-specific binding of a CRISPR complex to the target sequence, and wherein the CRISPR complex comprises the CRISPR enzyme complexed with (1) the guide sequence that is hybridized to the target sequence, and (2) the tracr mate sequence that is hybridized to the tracr sequence. In respect of CF, preferred target DNA sequences comprise the CFTRdelta508 mutation. A preferred PAM is described above. A preferred CRISPR enzyme is any Cas (described herein, but particularly that described in Example 22).

[00385] Alternatives to CF include any genetic disorder and examples of these are well known. Another preferred method or use of the invention is for correcting defects in the EMP2A and EMP2B genes that have been identified to be associated with Lafora disease.

[00386] In some embodiments, a "guide sequence" may be distinct from "guide RNA". A guide sequence may refer to an approx. 20bp sequence, within the guide RNA, that specifies the target site.

[00387] In some embodiments, the Cas9 is (or is derived from) SpCas9. In such embodiments, preferred mutations are at any or all or positions 10, 762, 840, 854, 863 and/or 986 of SpCas9 or corresponding positions in other Cas9s (which may be ascertained for instance by standard sequence comparison tools. In particular, any or all of the following mutations are preferred in SpCas9: D10A, E762A, H840A, N854A, N863A and/or D986A; as well as conservative substitution for any of the replacement amino acids is also envisaged. The same (or conservative substitutions of these mutations) at corresponding positions in other Cas9s are also preferred. Particularly preferred are D10 and H840 in SpCas9. However, in other Cas9s, residues corresponding to SpCas9 D10 and H840 are also preferred. These are advantageous as they provide nickase activity. Such mutations may be applied to all aspects of the present invention, not only treatment of CF.

[00388] Schwank et al. (Cell Stem Cell, 13:653–58, 2013) used CRISPR/Cas9 to correct a defect associated with cystic fibrosis in human stem cells. The team's target was the gene for an ion channel, cystic fibrosis transmembrane conductor receptor (CFTR). A deletion in CFTR causes the protein to misfold in cystic fibrosis patients. Using cultured intestinal stem cells developed from cell samples from two children with cystic fibrosis, Schwank et al. were able to

correct the defect using CRISPR along with a donor plasmid containing the reparative sequence to be inserted. The researchers then grew the cells into intestinal "organoids," or miniature guts, and showed that they functioned normally. In this case, about half of clonal organoids underwent the proper genetic correction.

Muscles

[00389] The present invention also contemplates delivering the CRISPR-Cas system to muscle(s).

[00390] Bortolanza et al. (Molecular Therapy vol. 19 no. 11, 2055–2064 Nov. 2011) shows that systemic delivery of RNA interference expression cassettes in the FRG1 mouse, after the onset of facioscapulohumeral muscular dystrophy (FSHD), led to a dose-dependent long-term FRG1 knockdown without signs of toxicity. Bortolanza et al. found that a single intravenous injection of 5×10^{12} vg of rAAV6-sh1FRG1 rescues muscle histopathology and muscle function of FRG1 mice. In detail, 200 µl containing 2×10^{12} or 5×10^{12} vg of vector in physiological solution were injected into the tail vein using a 25-gauge Terumo syringe. The method of Bortolanza et al. may be applied to an AAV expressing CRISPR Cas and injected into humans at a dosage of about 2×10^{15} or 2×10^{16} vg of vector.

[00391] Dumonceaux et al. (Molecular Therapy vol. 18 no. 5, 881–887 May 2010) inhibit the myostatin pathway using the technique of RNA interference directed against the myostatin receptor AcvRIIb mRNA (sh-AcvRIIb). The restoration of a quasi-dystrophin was mediated by the vectorized U7 exon-skipping technique (U7-DYS). Adeno-associated vectors carrying either the sh-AcvrIIb construct alone, the U7-DYS construct alone, or a combination of both constructs were injected in the tibialis anterior (TA) muscle of dystrophic mdx mice. The injections were performed with 10^{11} AAV viral genomes. The method of Dumonceaux et al. may be applied to an AAV expressing CRISPR Cas and injected into humans, for example, at a dosage of about 10^{14} to about 10^{15} vg of vector.

[00392] Kinouchi et al. (Gene Therapy (2008) 15, 1126–1130) report the effectiveness of in vivo siRNA delivery into skeletal muscles of normal or diseased mice through nanoparticle formation of chemically unmodified siRNAs with atelocollagen (ATCOL). ATCOL-mediated local application of siRNA targeting myostatin, a negative regulator of skeletal muscle growth, in mouse skeletal muscles or intravenously, caused a marked increase in the muscle mass within a few weeks after application. These results imply that ATCOL-mediated application of siRNAs

is a powerful tool for future therapeutic use for diseases including muscular atrophy. Mst-siRNAs (final concentration, 10 mM) were mixed with ATCOL (final concentration for local administration, 0.5%) (AteloGene, Kohken, Tokyo, Japan) according to the manufacturer's instructions. After anesthesia of mice (20-week-old male C57BL/6) by Nembutal (25 mg/kg, i.p.), the Mst-siRNA/ATCOL complex was injected into the masseter and biceps femoris muscles. The method of Kinouchi et al. may be applied to CRISPR Cas and injected into a human, for example, at a dosage of about 500 to 1000 ml of a 40 μM solution into the muscle.

[00393] Hagstrom et al. (Molecular Therapy Vol. 10, No. 2, August 2004) describe an intravascular, nonviral methodology that enables efficient and repeatable delivery of nucleic acids to muscle cells (myofibers) throughout the limb muscles of mammals. The procedure involves the injection of naked plasmid DNA or siRNA into a distal vein of a limb that is transiently isolated by a tourniquet or blood pressure cuff. Nucleic acid delivery to myofibers is facilitated by its rapid injection in sufficient volume to enable extravasation of the nucleic acid solution into muscle tissue. High levels of transgene expression in skeletal muscle were achieved in both small and large animals with minimal toxicity. Evidence of siRNA delivery to limb muscle was also obtained. For plasmid DNA intravenous injection into a rhesus monkey, a threeway stopcock was connected to two syringe pumps (Model PHD 2000; Harvard Instruments), each loaded with a single syringe. Five minutes after a papaverine injection, pDNA (15.5 to 25.7 mg in 40 –100 ml saline) was injected at a rate of 1.7 or 2.0 ml/s. This could be scaled up for plasmid DNA expressing CRISPR Cas of the present invention with an injection of about 300 to 500 mg in 800 to 2000 ml saline for a human. For adenoviral vector injections into a rat, 2 x 10^9 infectious particles were injected in 3 ml of normal saline solution (NSS). This could be scaled up for an adenoviral vector expressing CRISPR Cas of the present invention with an injection of about 1 x 10^{13} infectious particles were injected in 10 liters of NSS for a human. For siRNA, a rat was injected into the great saphenous vein with 12.5 μg of a siRNA and a primate was injected injected into the great saphenous vein with 750 μg of a siRNA. This could be scaled up for a CRISPR Cas of the present invention, for example, with an injection of about 15 to about 50 mg into the great saphenous vein of a human.

Skin

[00394] The present invention also contemplates delivering the CRISPR-Cas system to the skin.

[00395] Hickerson et al. (Molecular Therapy—Nucleic Acids (2013) 2, e129) relates to a motorized microneedle array skin delivery device for delivering self-delivery (sd)-siRNA to human and murine skin. The primary challenge to translating siRNA-based skin therapeutics to the clinic is the development of effective delivery systems. Substantial effort has been invested in a variety of skin delivery technologies with limited success. In a clinical study in which skin was treated with siRNA, the exquisite pain associated with the hypodermic needle injection precluded enrollment of additional patients in the trial, highlighting the need for improved, more "patient-friendly" (i.e., little or no pain) delivery approaches. Microneedles represent an efficient way to deliver large charged cargos including siRNAs across the primary barrier, the stratum corneum, and are generally regarded as less painful than conventional hypodermic needles. Motorized "stamp type" microneedle devices, including the motorized microneedle array (MMNA) device used by Hickerson et al., have been shown to be safe in hairless mice studies and cause little or no pain as evidenced by (i) widespread use in the cosmetic industry and (ii) limited testing in which nearly all volunteers found use of the device to be much less painful than a flushot, suggesting siRNA delivery using this device will result in much less pain than was experienced in the previous clinical trial using hypodermic needle injections. The MMNA device (marketed as Triple-M or Tri-M by Bomtech Electronic Co, Seoul, South Korea) was adapted for delivery of siRNA to mouse and human skin. sd-siRNA solution (up to 300 µl of 0.1 mg/ml RNA) was introduced into the chamber of the disposable Tri-M needle cartridge (Bomtech), which was set to a depth of 0.1 mm. For treating human skin, deidentified skin (obtained immediately following surgical procedures) was manually stretched and pinned to a cork platform before treatment. All intradermal injections were performed using an insulin syringe with a 28-gauge 0.5-inch needle. The MMNA device and method of Hickerson et al. could be used and/or adapted to deliver the CRISPR Cas of the present invention, for example, at a dosage of up to 300 µl of 0.1 mg/ml CRISPR Cas to the skin.

[00396] Leachman et al. (Molecular Therapy, vol. 18 no. 2, 442–446 Feb. 2010) relates to a phase Ib clinical trial for treatment of a rare skin disorder pachyonychia congenita (PC), an autosomal dominant syndrome that includes a disabling plantar keratoderma, utilizing the first short-interfering RNA (siRNA)-based therapeutic for skin. This siRNA, called TD101, specifically and potently targets the keratin 6a (K6a) N171K mutant mRNA without affecting wild-type K6a mRNA. The dose-escalation schedule is presented below:

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

[00397] Initially, 0.1 ml of a 1.0 mg/ml solution of TD101 or vehicle alone (Dulbecco's phosphate-buffered saline without calcium or magnesium) was administered to symmetric calluses. Six rising dose-volumes were completed without an adverse reaction to the increases: 0.1, 0.25, 0.5, 1.0, 1.5, and 2.0 ml of a 1.0 mg/ml solution of TD101 solution per injection. As the highest planned volume (2.0 ml) was well tolerated, the concentration of TD101 was then increased each week from 1 mg/ml up to a final concentration of 8.5 mg/ml. Similar dosages are contemplated for the administration of a CRISPR Cas that specifically and potently targets the keratin 6a (K6a) N171K mutant mRNA.

[00398] Zheng et al. (PNAS, July 24, 2012, vol. 109, no. 30, 11975–11980) show that spherical nucleic acid nanoparticle conjugates (SNA-NCs), gold cores surrounded by a dense shell of highly oriented, covalently immobilized siRNA, freely penetrate almost 100% of keratinocytes in vitro, mouse skin, and human epidermis within hours after application. Zheng et al. demonstrated that a single application of 25 nM epidermal growth factor receptor (EGFR) SNA-NCs for 60 h demonstrate effective gene knockdown in human skin. A similar dosage may be contemplated for CRISPR Cas immobilized in SNA-NCs for administration to the skin.

Hepatitis viruses

[00399] The present invention may also be applied to treat hepatitis B virus (HBV). However, the CRISPR Cas system must be adapted to avoid the shortcomings of RNAi, such as the risk of oversatring endogenous small RNA pathways, by for example, optimizing dose and sequence

(see, e.g., Grimm et al., Nature vol. 441, 26 May 2006). For example, low doses, such as about 1-10 x 10^{14} particles per humane are contemplated.

[00400] In another embodiment, the CRISPR Cas system directed against HBV may be administered in liposomes, such as a stable nucleic-acid-lipid particle (SNALP) (see, e.g., Morrissey et al., Nature Biotechnology, Vol. 23, No. 8, August 2005). Daily intravenous injections of about 1, 3 or 5 mg/kg/day of CRISPR Cas targeted to HBV RNA in a SNALP are contemplated. The daily treatment may be over about three days and then weekly for about five weeks.

[00401] In another embodiment, the system of Chen et al. (Gene Therapy (2007) 14, 11–19) may be used/and or adapted for the CRISPR Cas system of the present invention. Chen et al. use a double-stranded adenoassociated virus 8-pseudotyped vector (dsAAV2/8) to deliver shRNA. A single administration of dsAAV2/8 vector (1 x 10^{12} vector genomes per mouse), carrying HBV-specific shRNA, effectively suppressed the steady level of HBV protein, mRNA and replicative DNA in liver of HBV transgenic mice, leading to up to 2–3 $\log_{10}$ decrease in HBV load in the circulation. Significant HBV suppression sustained for at least 120 days after vector administration. The therapeutic effect of shRNA was target sequence dependent and did not involve activation of interferon. For the present invention, a CRISPR Cas system directed to HBV may be cloned into an AAV vector, such as a dsAAV2/8 vector and administered to a human, for example, at a dosage of about 1 x 10^{15} vector genomes to about 1 x 10^{16} vector genomes per human.

[00402] In another embodiment, the method of Wooddell et al. (Molecular Therapy vol. 21 no. 5, 973–985 May 2013) may be used/and or adapted to the CRISPR Cas system of the present invention. Woodell et al. show that simple coinjection of a hepatocyte-targeted, N-acetylgalactosamine-conjugated melittin-like peptide (NAG-MLP) with a liver-tropic cholesterol-conjugated siRNA (chol-siRNA) targeting coagulation factor VII (F7) results in efficient F7 knockdown in mice and nonhuman primates without changes in clinical chemistry or induction of cytokines. Using transient and transgenic mouse models of HBV infection, Wooddell et al. show that a single coinjection of NAG-MLP with potent chol-siRNAs targeting conserved HBV sequences resulted in multilog repression of viral RNA, proteins, and viral DNA with long duration of effect. Intraveinous coinjections, for example, of about 6 mg/kg of NAG-MLP and 6 mg/kg of HBV specific CRISPR Cas may be envisioned for the present invention. In

the alternative, about 3 mg/kg of NAG-MLP and 3 mg/kg of HBV specific CRISPR Cas may be delivered on day one, followed by administration of about about 2-3 mg/kg of NAG-MLP and 2-3 mg/kg of HBV specific CRISPR Cas two weeks later.

[00403] The present invention may also be applied to treat hepatitis C virus (HCV). The methods of Roelvinki et al. (Molecular Therapy vol. 20 no. 9, 1737-1749 Sep 2012) may be applied to the CRISPR Cas system. For example, an AAV vector such as AAV8 may be a contemplated vector and for example a dosage of about 1.25×10^{11} to 1.25×10^{13} vector genomes per kilogram body weight (vg/kg) may be contemplated.

[00404] It will be readily apparent that a host of other diseases can be treated in a similar fashion. Some examples of genetic diseases caused by mutations are provided herein, but many more are known. The above strategy can be applied to these diseases.

Huntington's Disease (HD)

[00405] RNA interference (RNAi) offers therapeutic potential for this disorder by reducing the expression of *HTT*, the disease-causing gene of Huntington's disease (see, e.g., McBride et al., Molecular Therapy vol. 19 no. 12 Dec. 2011, pp. 2152-2162), therefore Applicant postulates that it may be used/and or adapted to the CRISPR-Cas system. The CRISPR-Cas system may be generated using an algorithm to reduce the off-targeting potential of antisense sequences. The CRISPR-Cas sequences may target either a sequence in exon 52 of mouse, rhesus or human huntingtin and expressed in a viral vector, such as AAV. Animals, including humans, may be injected with about three microinjections per hemisphere (six injections total): the first 1 mm rostral to the anterior commissure (12 μl) and the two remaining injections (12 μl and 10 μl, respectively) spaced 3 and 6 mm caudal to the first injection with 1e12 vg/ml of AAV at a rate of about 1 μl/minute, and the needle was left in place for an additional 5 minutes to allow the injectate to diffuse from the needle tip.

[00406] DiFiglia et al. (PNAS, October 23, 2007, vol. 104, no. 43, 17204–17209) observed that single administration into the adult striatum of an siRNA targeting Htt can silence mutant Htt, attenuate neuronal pathology, and delay the abnormal behavioral phenotype observed in a rapid-onset, viral transgenic mouse model of HD. DiFiglia injected mice intrastriatally with 2 μl of Cy3-labeled cc-siRNA-Htt or unconjugated siRNA-Htt at 10 μM. A similar dosage of CRISPR Cas targeted to Htt may be contemplated for humans in the present invention, for example, about 5-10 ml of 10 μM CRISPR Cas targeted to Htt may be injected intrastriatally.

[00407] In another example, Boudreau et al. (Molecular Therapy vol. 17 no. 6 june 2009) injects 5 µl of recombinant AAV serotype 2/1 vectors expressing htt-specific RNAi virus (at 4 x 10^{12} viral genomes/ml) into the straiatum. A similar dosage of CRISPR Cas targeted to Htt may be contemplated for humans in the present invention, for example, about 10-20 ml of 4 x 10^{12} viral genomes/ml) CRISPR Cas targeted to Htt may be injected intrastriatally.

[00408] In another example, a CRISPR Cas targetd to HTT may be administered continuously (see, e.g., Yu et al., Cell 150, 895–908, August 31, 2012). Yu et al. utilizes osmotic pumps delivering 0.25 ml/hr (Model 2004) to deliver 300 mg/day of ss-siRNA or phosphate-buffered saline (PBS) (Sigma Aldrich) for 28 days, and pumps designed to deliver 0.5 µl/hr (Model 2002) were used to deliver 75 mg/day of the positive control MOE ASO for 14 days. Pumps (Durect Corporation) were filled with ss-siRNA or MOE diluted in sterile PBS and then incubated at 37 C for 24 or 48 (Model 2004) hours prior to implantation. Mice were anesthetized with 2.5% isofluorane, and a midline incision was made at the base of the skull. Using stereotaxic guides, a cannula was implanted into the right lateral ventricle and secured with Loctite adhesive. A catheter attached to an Alzet osmotic mini pump was attached to the cannula, and the pump was placed subcutaneously in the midscapular area. The incision was closed with 5.0 nylon sutures. A similar dosage of CRISPR Cas targeted to Htt may be contemplated for humans in the present invention, for example, about 500 to 1000 g/day CRISPR Cas targeted to Htt may be administered.

[00409] In another example of continuous infusion, Stiles et al. (Experimental Neurology 233 (2012) 463–471) implanted an intraparenchymal catheter with a titanium needle tip into the right putamen. The catheter was connected to a SynchroMed® II Pump (Medtronic Neurological, Minneapolis, MN) subcutaneously implanted in the abdomen. After a 7 day infusion of phosphate buffered saline at 6 µL/day, pumps were re-filled with test article and programmed for continuous delivery for 7 days. About 2.3 to 11.52 mg/d of siRNA were infused at varying infusion rates of about 0.1 to 0.5 µL/min. A similar dosage of CRISPR Cas targeted to Htt may be contemplated for humans in the present invention, for example, about 20 to 200 mg/day CRISPR Cas targeted to Htt may be administered.

[00410] In another example, the methods of US Patent Publication No. 20130253040 assigned to Sangamo may also be also be adapted from TALES to the CRISPR Cas system of the present invention for treating Huntington's Disease.

Nucleic acids, amino acids and proteins

[00411] The invention uses nucleic acids to bind target DNA sequences. This is advantageous as nucleic acids are much easier and cheaper to produce than proteins, and the specificity can be varied according to the length of the stretch where homology is sought. Complex 3-D positioning of multiple fingers, for example is not required.

[00412] The terms "polynucleotide", "nucleotide", "nucleotide sequence", "nucleic acid" and "oligonucleotide" are used interchangeably. They refer to a polymeric form of nucleotides of any length, either deoxyribonucleotides or ribonucleotides, or analogs thereof. Polynucleotides may have any three dimensional structure, and may perform any function, known or unknown. The following are non-limiting examples of polynucleotides: coding or non-coding regions of a gene or gene fragment, loci (locus) defined from linkage analysis, exons, introns, messenger RNA (mRNA), transfer RNA, ribosomal RNA, short interfering RNA (siRNA), short-hairpin RNA (shRNA), micro-RNA (miRNA), ribozymes, cDNA, recombinant polynucleotides, branched polynucleotides, plasmids, vectors, isolated DNA of any sequence, isolated RNA of any sequence, nucleic acid probes, and primers. The term also encompasses nucleic-acid-like structures with synthetic backbones, see, e.g., Eckstein, 1991; Baserga et al., 1992; Milligan, 1993; WO 97/03211; WO 96/39154; Mata, 1997; Strauss-Soukup, 1997; and Samstag, 1996. A polynucleotide may comprise one or more modified nucleotides, such as methylated nucleotides and nucleotide analogs. If present, modifications to the nucleotide structure may be imparted before or after assembly of the polymer. The sequence of nucleotides may be interrupted by non-nucleotide components. A polynucleotide may be further modified after polymerization, such as by conjugation with a labeling component.

[00413] As used herein the term "wild type" is a term of the art understood by skilled persons and means the typical form of an organism, strain, gene or characteristic as it occurs in nature as distinguished from mutant or variant forms.

[00414] As used herein the term "variant" should be taken to mean the exhibition of qualities that have a pattern that deviates from what occurs in nature.

[00415] The terms "non-naturally occurring" or "engineered" are used interchangeably and indicate the involvement of the hand of man. The terms, when referring to nucleic acid molecules or polypeptides mean that the nucleic acid molecule or the polypeptide is at least

substantially free from at least one other component with which they are naturally associated in nature and as found in nature.

[00416] "Complementarity" refers to the ability of a nucleic acid to form hydrogen bond(s) with another nucleic acid sequence by either traditional Watson-Crick base pairing or other non-traditional types. A percent complementarity indicates the percentage of residues in a nucleic acid molecule which can form hydrogen bonds (e.g., Watson-Crick base pairing) with a second nucleic acid sequence (e.g., 5, 6, 7, 8, 9, 10 out of 10 being 50%, 60%, 70%, 80%, 90%, and 100% complementary). "Perfectly complementary" means that all the contiguous residues of a nucleic acid sequence will hydrogen bond with the same number of contiguous residues in a second nucleic acid sequence. "Substantially complementary" as used herein refers to a degree of complementarity that is at least 60%, 65%, 70%, 75%, 80%, 85%, 90%, 95%, 97%, 98%, 99%, or 100% over a region of 8, 9, 10, 11, 12, 13, 14, 15, 16, 17, 18, 19, 20, 21, 22, 23, 24, 25, 30, 35, 40, 45, 50, or more nucleotides, or refers to two nucleic acids that hybridize under stringent conditions.

[00417] As used herein, "stringent conditions" for hybridization refer to conditions under which a nucleic acid having complementarity to a target sequence predominantly hybridizes with the target sequence, and substantially does not hybridize to non-target sequences. Stringent conditions are generally sequence-dependent, and vary depending on a number of factors. In general, the longer the sequence, the higher the temperature at which the sequence specifically hybridizes to its target sequence. Non-limiting examples of stringent conditions are described in detail in Tijssen (1993), Laboratory Techniques In Biochemistry And Molecular Biology-Hybridization With Nucleic Acid Probes Part I, Second Chapter "Overview of principles of hybridization and the strategy of nucleic acid probe assay", Elsevier, N.Y. Where reference is made to a polynucleotide sequence, then complementary or partially complementary sequences are also envisaged. These are preferably capable of hybridising to the reference sequence under highly stringent conditions. Generally, in order to maximize the hybridization rate, relatively low-stringency hybridization conditions are selected: about 20 to 25° C lower than the thermal melting point (T_m). The T_m is the temperature at which 50% of specific target sequence hybridizes to a perfectly complementary probe in solution at a defined ionic strength and pH. Generally, in order to require at least about 85% nucleotide complementarity of hybridized sequences, highly stringent washing conditions are selected to be about 5 to 15° C lower than the

T_m. In order to require at least about 70% nucleotide complementarity of hybridized sequences, moderately-stringent washing conditions are selected to be about 15 to 30° C lower than the T_m. Highly permissive (very low stringency) washing conditions may be as low as 50° C below the T_m, allowing a high level of mis-matching between hybridized sequences. Those skilled in the art will recognize that other physical and chemical parameters in the hybridization and wash stages can also be altered to affect the outcome of a detectable hybridization signal from a specific level of homology between target and probe sequences. Preferred highly stringent conditions comprise incubation in 50% formamide, 5×SSC, and 1% SDS at 42° C, or incubation in 5×SSC and 1% SDS at 65° C, with wash in 0.2×SSC and 0.1% SDS at 65° C.

[00418] "Hybridization" refers to a reaction in which one or more polynucleotides react to form a complex that is stabilized via hydrogen bonding between the bases of the nucleotide residues. The hydrogen bonding may occur by Watson Crick base pairing, Hoogstein binding, or in any other sequence specific manner. The complex may comprise two strands forming a duplex structure, three or more strands forming a multi stranded complex, a single self-hybridizing strand, or any combination of these. A hybridization reaction may constitute a step in a more extensive process, such as the initiation of PCR, or the cleavage of a polynucleotide by an enzyme. A sequence capable of hybridizing with a given sequence is referred to as the "complement" of the given sequence.

[00419] As used herein, the term "genomic locus" or "locus" (plural loci) is the specific location of a gene or DNA sequence on a chromosome. A "gene" refers to stretches of DNA or RNA that encode a polypeptide or an RNA chain that has functional role to play in an organism and hence is the molecular unit of heredity in living organisms. For the purpose of this invention it may be considered that genes include regions which regulate the production of the gene product, whether or not such regulatory sequences are adjacent to coding and/or transcribed sequences. Accordingly, a gene includes, but is not necessarily limited to, promoter sequences, terminators, translational regulatory sequences such as ribosome binding sites and internal ribosome entry sites, enhancers, silencers, insulators, boundary elements, replication origins, matrix attachment sites and locus control regions.

[00420] As used herein, "expression of a genomic locus" or "gene expression" is the process by which information from a gene is used in the synthesis of a functional gene product. The products of gene expression are often proteins, but in non-protein coding genes such as rRNA

genes or tRNA genes, the product is functional RNA. The process of gene expression is used by all known life - eukaryotes (including multicellular organisms), prokaryotes (bacteria and archaea) and viruses to generate functional products to survive. As used herein "expression" of a gene or nucleic acid encompasses not only cellular gene expression, but also the transcription and translation of nucleic acid(s) in cloning systems and in any other context. As used herein, "expression" also refers to the process by which a polynucleotide is transcribed from a DNA template (such as into and mRNA or other RNA transcript) and/or the process by which a transcribed mRNA is subsequently translated into peptides, polypeptides, or proteins. Transcripts and encoded polypeptides may be collectively referred to as "gene product." If the polynucleotide is derived from genomic DNA, expression may include splicing of the mRNA in a eukaryotic cell.

[00421] The terms "polypeptide", "peptide" and "protein" are used interchangeably herein to refer to polymers of amino acids of any length. The polymer may be linear or branched, it may comprise modified amino acids, and it may be interrupted by non amino acids. The terms also encompass an amino acid polymer that has been modified; for example, disulfide bond formation, glycosylation, lipidation, acetylation, phosphorylation, or any other manipulation, such as conjugation with a labeling component. As used herein the term "amino acid" includes natural and/or unnatural or synthetic amino acids, including glycine and both the D or L optical isomers, and amino acid analogs and peptidomimetics.

[00422] As used herein, the term "domain" or "protein domain" refers to a part of a protein sequence that may exist and function independently of the rest of the protein chain.

[00423] As described in aspects of the invention, sequence identity is related to sequence homology. Homology comparisons may be conducted by eye, or more usually, with the aid of readily available sequence comparison programs. These commercially available computer programs may calculate percent (%) homology between two or more sequences and may also calculate the sequence identity shared by two or more amino acid or nucleic acid sequences. In some preferred embodiments, the capping region of the dTALEs described herein have sequences that are at least 95% identical or share identity to the capping region amino acid sequences provided herein.

[00424] Sequence homologies may be generated by any of a number of computer programs known in the art, for example BLAST or FASTA, etc. A suitable computer program for carrying

out such an alignment is the GCG Wisconsin Bestfit package (University of Wisconsin, U.S.A; Devereux et al., 1984, Nucleic Acids Research 12:387). Examples of other software than may perform sequence comparisons include, but are not limited to, the BLAST package (see Ausubel et al., 1999 ibid – Chapter 18), FASTA (Atschul et al., 1990, J. Mol. Biol., 403-410) and the GENEWORKS suite of comparison tools. Both BLAST and FASTA are available for offline and online searching (see Ausubel et al., 1999 ibid, pages 7-58 to 7-60). However it is preferred to use the GCG Bestfit program.

[00425] Percentage (%) sequence homology may be calculated over contiguous sequences, i.e., one sequence is aligned with the other sequence and each amino acid or nucleotide in one sequence is directly compared with the corresponding amino acid or nucleotide in the other sequence, one residue at a time. This is called an "ungapped" alignment. Typically, such ungapped alignments are performed only over a relatively short number of residues.

[00426] Although this is a very simple and consistent method, it fails to take into consideration that, for example, in an otherwise identical pair of sequences, one insertion or deletion may cause the following amino acid residues to be put out of alignment, thus potentially resulting in a large reduction in % homology when a global alignment is performed. Consequently, most sequence comparison methods are designed to produce optimal alignments that take into consideration possible insertions and deletions without unduly penalizing the overall homology or identity score. This is achieved by inserting "gaps" in the sequence alignment to try to maximize local homology or identity.

[00427] However, these more complex methods assign "gap penalties" to each gap that occurs in the alignment so that, for the same number of identical amino acids, a sequence alignment with as few gaps as possible - reflecting higher relatedness between the two compared sequences - may achieve a higher score than one with many gaps. "Affinity gap costs" are typically used that charge a relatively high cost for the existence of a gap and a smaller penalty for each subsequent residue in the gap. This is the most commonly used gap scoring system. High gap penalties may, of course, produce optimized alignments with fewer gaps. Most alignment programs allow the gap penalties to be modified. However, it is preferred to use the default values when using such software for sequence comparisons. For example, when using the GCG Wisconsin Bestfit package the default gap penalty for amino acid sequences is -12 for a gap and -4 for each extension.

[00428] Calculation of maximum % homology therefore first requires the production of an optimal alignment, taking into consideration gap penalties. A suitable computer program for carrying out such an alignment is the GCG Wisconsin Bestfit package (Devereux et al., 1984 *Nuc. Acids Research* 12 p387). Examples of other software than may perform sequence comparisons include, but are not limited to, the BLAST package (see Ausubel et al., 1999 *Short Protocols in Molecular Biology*, 4th Ed. – Chapter 18), FASTA (Altschul et al., 1990 *J. Mol. Biol.* 403-410) and the GENEWORKS suite of comparison tools. Both BLAST and FASTA are available for offline and online searching (see Ausubel et al., 1999, *Short Protocols in Molecular Biology*, pages 7-58 to 7-60). However, for some applications, it is preferred to use the GCG Bestfit program. A new tool, called BLAST 2 Sequences is also available for comparing protein and nucleotide sequences (see *FEMS Microbiol Lett.* 1999 174(2): 247-50; *FEMS Microbiol Lett.* 1999 177(1): 187-8 and the website of the National Center for Biotechnology information at the website of the National Institutes for Health).

[00429] Although the final % homology may be measured in terms of identity, the alignment process itself is typically not based on an all-or-nothing pair comparison. Instead, a scaled similarity score matrix is generally used that assigns scores to each pair-wise comparison based on chemical similarity or evolutionary distance. An example of such a matrix commonly used is the BLOSUM62 matrix - the default matrix for the BLAST suite of programs. GCG Wisconsin programs generally use either the public default values or a custom symbol comparison table, if supplied (see user manual for further details). For some applications, it is preferred to use the public default values for the GCG package, or in the case of other software, the default matrix, such as BLOSUM62.

[00430] Alternatively, percentage homologies may be calculated using the multiple alignment feature in DNASIS™ (Hitachi Software), based on an algorithm, analogous to CLUSTAL (Higgins DG & Sharp PM (1988), *Gene* 73(1), 237-244). Once the software has produced an optimal alignment, it is possible to calculate % homology, preferably % sequence identity. The software typically does this as part of the sequence comparison and generates a numerical result.

[00431] The sequences may also have deletions, insertions or substitutions of amino acid residues which produce a silent change and result in a functionally equivalent substance. Deliberate amino acid substitutions may be made on the basis of similarity in amino acid properties (such as polarity, charge, solubility, hydrophobicity, hydrophilicity, and/or the

amphipathic nature of the residues) and it is therefore useful to group amino acids together in functional groups. Amino acids may be grouped together based on the properties of their side chains alone. However, it is more useful to include mutation data as well. The sets of amino acids thus derived are likely to be conserved for structural reasons. These sets may be described in the form of a Venn diagram (Livingstone C.D. and Barton G.J. (1993) "Protein sequence alignments: a strategy for the hierarchical analysis of residue conservation" *Comput. Appl. Biosci.* 9: 745-756) (Taylor W.R. (1986) "The classification of amino acid conservation" *J. Theor. Biol.* 119; 205-218). Conservative substitutions may be made, for example according to the table below which describes a generally accepted Venn diagram grouping of amino acids.

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

[00432] Embodiments of the invention include sequences (both polynucleotide or polypeptide) which may comprise homologous substitution (substitution and replacement are both used herein to mean the interchange of an existing amino acid residue or nucleotide, with an alternative residue or nucleotide) that may occur i.e., like-for-like substitution in the case of amino acids such as basic for basic, acidic for acidic, polar for polar, etc. Non-homologous substitution may also occur i.e., from one class of residue to another or alternatively involving the inclusion of unnatural amino acids such as ornithine (hereinafter referred to as Z), diaminobutyric acid ornithine (hereinafter referred to as B), norleucine ornithine (hereinafter referred to as O), pyriylalanine, thienylalanine, naphthylalanine and phenylglycine.

[00433] Variant amino acid sequences may include suitable spacer groups that may be inserted between any two amino acid residues of the sequence including alkyl groups such as methyl, ethyl or propyl groups in addition to amino acid spacers such as glycine or β-alanine residues. A further form of variation, which involves the presence of one or more amino acid residues in peptoid form, may be well understood by those skilled in the art. For the avoidance of doubt, "the peptoid form" is used to refer to variant amino acid residues wherein the α-carbon substituent group is on the residue's nitrogen atom rather than the α-carbon. Processes for preparing peptides in the peptoid form are known in the art, for example Simon RJ et al., *PNAS* (1992) 89(20), 9367-9371 and Horwell DC, *Trends Biotechnol.* (1995) 13(4), 132-134.

[00434] The practice of the present invention employs, unless otherwise indicated, conventional techniques of immunology, biochemistry, chemistry, molecular biology, microbiology, cell biology, genomics and recombinant DNA, which are within the skill of the art. See Sambrook, Fritsch and Maniatis, MOLECULAR CLONING: A LABORATORY MANUAL, 2nd edition (1989); CURRENT PROTOCOLS IN MOLECULAR BIOLOGY (F. M. Ausubel, et al. eds., (1987)); the series METHODS IN ENZYMOLOGY (Academic Press, Inc.): PCR 2: A PRACTICAL APPROACH (M.J. MacPherson, B.D. Hames and G.R. Taylor eds. (1995)), Harlow and Lane, eds. (1988) ANTIBODIES, A LABORATORY MANUAL, and ANIMAL CELL CULTURE (R.I. Freshney, ed. (1987)).

Vectors

[00435] In one aspect, the invention provides for vectors that are used in the engineering and optimization of CRISPR-Cas systems.

[00436] A used herein, a "vector" is a tool that allows or facilitates the transfer of an entity from one environment to another. It is a replicon, such as a plasmid, phage, or cosmid, into which another DNA segment may be inserted so as to bring about the replication of the inserted segment. Generally, a vector is capable of replication when associated with the proper control elements. In general, the term "vector" refers to a nucleic acid molecule capable of transporting another nucleic acid to which it has been linked. Vectors include, but are not limited to, nucleic acid molecules that are single-stranded, double-stranded, or partially double-stranded; nucleic acid molecules that comprise one or more free ends, no free ends (e.g. circular); nucleic acid molecules that comprise DNA, RNA, or both; and other varieties of polynucleotides known in the art. One type of vector is a "plasmid," which refers to a circular double stranded DNA loop

into which additional DNA segments can be inserted, such as by standard molecular cloning techniques. Another type of vector is a viral vector, wherein virally-derived DNA or RNA sequences are present in the vector for packaging into a virus (e.g. retroviruses, replication defective retroviruses, adenoviruses, replication defective adenoviruses, and adeno-associated viruses (AAVs)). Viral vectors also include polynucleotides carried by a virus for transfection into a host cell. Certain vectors are capable of autonomous replication in a host cell into which they are introduced (e.g. bacterial vectors having a bacterial origin of replication and episomal mammalian vectors). Other vectors (e.g., non-episomal mammalian vectors) are integrated into the genome of a host cell upon introduction into the host cell, and thereby are replicated along with the host genome. Moreover, certain vectors are capable of directing the expression of genes to which they are operatively-linked. Such vectors are referred to herein as "expression vectors." Common expression vectors of utility in recombinant DNA techniques are often in the form of plasmids.

[00437] 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.

[00438] Aspects of the invention relate to bicistronic vectors for chimeric RNA and Cas9. Bicistronic expression vectors for chimeric RNA and Cas9 are preferred. In general and particularly in this embodiment Cas9 is preferably driven by the CBh promoter. The chimeric RNA may preferably be driven by a U6 promoter. Ideally the two are combined. The chimeric guide RNA typically consists of a 20bp guide sequence (Ns) and this may be joined to the tracr sequence (running from the first "U" of the lower strand to the end of the transcript). The tracr sequence may be truncated at various positions as indicated. The guide and tracr sequences are

separated by the tracr-mate sequence, which may be GUUUUAGAGCUA. This may be followed by the loop sequence GAAA as shown. Both of these are preferred examples. Applicants have demonstrated Cas9-mediated indels at the human *EMX1* and *PVALB* loci by SURVEYOR assays. ChiRNAs are indicated by their "+n" designation, and crRNA refers to a hybrid RNA where guide and tracr sequences are expressed as separate transcripts. Throughout this application, chimeric RNA may also be called single guide, or synthetic guide RNA (sgRNA). The loop is preferably GAAA, but it is not limited to this sequence or indeed to being only 4bp in length. Indeed, preferred loop forming sequences for use in hairpin structures are four nucleotides in length, and most preferably have the sequence GAAA. However, longer or shorter loop sequences may be used, as may alternative sequences. The sequences preferably include a nucleotide triplet (for example, AAA), and an additional nucleotide (for example C or G). Examples of loop forming sequences include CAAA and AAAG.

[00439] The term "regulatory element" is intended to include promoters, enhancers, internal ribosomal entry sites (IRES), and other expression control elements (e.g. transcription termination signals, such as polyadenylation signals and poly-U sequences). Such regulatory elements are described, for example, in Goeddel, GENE EXPRESSION TECHNOLOGY: METHODS IN ENZYMOLOGY 185, Academic Press, San Diego, Calif. (1990). Regulatory elements include those that direct constitutive expression of a nucleotide sequence in many types of host cell and those that direct expression of the nucleotide sequence only in certain host cells (e.g., tissue-specific regulatory sequences). A tissue-specific promoter may direct expression primarily in a desired tissue of interest, such as muscle, neuron, bone, skin, blood, specific organs (e.g. liver, pancreas), or particular cell types (e.g. lymphocytes). Regulatory elements may also direct expression in a temporal-dependent manner, such as in a cell-cycle dependent or developmental stage-dependent manner, which may or may not also be tissue or cell-type specific. In some embodiments, a vector comprises one or more pol III promoter (e.g. 1, 2, 3, 4, 5, or more pol III promoters), one or more pol II promoters (e.g. 1, 2, 3, 4, 5, or more pol II promoters), one or more pol I promoters (e.g. 1, 2, 3, 4, 5, or more pol I promoters), or combinations thereof. Examples of pol III promoters include, but are not limited to, U6 and H1 promoters. Examples of pol II promoters include, but are not limited to, the retroviral Rous sarcoma virus (RSV) LTR promoter (optionally with the RSV enhancer), the cytomegalovirus (CMV) promoter (optionally with the CMV enhancer) [see, e.g., Boshart et al, Cell, 41:521-530

(1985)], the SV40 promoter, the dihydrofolate reductase promoter, the β-actin promoter, the phosphoglycerol kinase (PGK) promoter, and the EF1α promoter. Also encompassed by the term "regulatory element" are enhancer elements, such as WPRE; CMV enhancers; the R-U5' segment in LTR of HTLV-I (Mol. Cell. Biol., Vol. 8(1), p. 466-472, 1988); SV40 enhancer; and the intron sequence between exons 2 and 3 of rabbit β-globin (Proc. Natl. Acad. Sci. USA., Vol. 78(3), p. 1527-31, 1981). It will be appreciated by those skilled in the art that the design of the expression vector can depend on such factors as the choice of the host cell to be transformed, the level of expression desired, etc. A vector can be introduced into host cells to thereby produce transcripts, proteins, or peptides, including fusion proteins or peptides, encoded by nucleic acids as described herein (e.g., clustered regularly interspersed short palindromic repeats (CRISPR) transcripts, proteins, enzymes, mutant forms thereof, fusion proteins thereof, etc.). With regards to regulatory sequences, mention is made of U.S. patent application 10/491,026, the contents of which are incorporated by reference herein in their entirety. With regards to promoters, mention is made of PCT publication WO 2011/028929 and U.S. application 12/511,940, the contents of which are incorporated by reference herein in their entirety.

[00440] Vectors can be designed for expression of CRISPR transcripts (e.g. nucleic acid transcripts, proteins, or enzymes) in prokaryotic or eukaryotic cells. For example, CRISPR transcripts can be expressed in bacterial cells such as Escherichia coli, insect cells (using baculovirus expression vectors), yeast cells, or mammalian cells. Suitable host cells are discussed further in Goeddel, GENE EXPRESSION TECHNOLOGY: METHODS IN ENZYMOLOGY 185, Academic Press, San Diego, Calif. (1990). Alternatively, the recombinant expression vector can be transcribed and translated in vitro, for example using T7 promoter regulatory sequences and T7 polymerase.

[00441] Vectors may be introduced and propagated in a prokaryote or prokaryotic cell. In some embodiments, a prokaryote is used to amplify copies of a vector to be introduced into a eukaryotic cell or as an intermediate vector in the production of a vector to be introduced into a eukaryotic cell (e.g. amplifying a plasmid as part of a viral vector packaging system). In some embodiments, a prokaryote is used to amplify copies of a vector and express one or more nucleic acids, such as to provide a source of one or more proteins for delivery to a host cell or host organism. Expression of proteins in prokaryotes is most often carried out in Escherichia coli with vectors containing constitutive or inducible promoters directing the expression of either

fusion or non-fusion proteins. Fusion vectors add a number of amino acids to a protein encoded therein, such as to the amino terminus of the recombinant protein. Such fusion vectors may serve one or more purposes, such as: (i) to increase expression of recombinant protein; (ii) to increase the solubility of the recombinant protein; and (iii) to aid in the purification of the recombinant protein by acting as a ligand in affinity purification. Often, in fusion expression vectors, a proteolytic cleavage site is introduced at the junction of the fusion moiety and the recombinant protein to enable separation of the recombinant protein from the fusion moiety subsequent to purification of the fusion protein. Such enzymes, and their cognate recognition sequences, include Factor Xa, thrombin and enterokinase. Example fusion expression vectors include pGEX (Pharmacia Biotech Inc; Smith and Johnson, 1988. *Gene* 67: 31-40), pMAL (New England Biolabs, Beverly, Mass.) and pRIT5 (Pharmacia, Piscataway, N.J.) that fuse glutathione S-transferase (GST), maltose E binding protein, or protein A, respectively, to the target recombinant protein.

[00442] Examples of suitable inducible non-fusion E. coli expression vectors include pTrc (Amrann et al., (1988) Gene 69:301-315) and pET 11d (Studier et al., GENE EXPRESSION TECHNOLOGY: METHODS IN ENZYMOLOGY 185, Academic Press, San Diego, Calif. (1990) 60-89).

[00443] In some embodiments, a vector is a yeast expression vector. Examples of vectors for expression in yeast Saccharomyces cerivisae include pYepSec1 (Baldari, et al., 1987. EMBO J. 6: 229-234), pMFa (Kuijan and Herskowitz, 1982. Cell 30: 933-943), pJRY88 (Schultz et al., 1987. Gene 54: 113-123), pYES2 (Invitrogen Corporation, San Diego, Calif.), and picZ (InVitrogen Corp, San Diego, Calif.).

[00444] In some embodiments, a vector drives protein expression in insect cells using baculovirus expression vectors. Baculovirus vectors available for expression of proteins in cultured insect cells (e.g., SF9 cells) include the pAc series (Smith, et al., 1983. Mol. Cell. Biol. 3: 2156-2165) and the pVL series (Lucklow and Summers, 1989. Virology 170: 31-39).

[00445] In some embodiments, a vector is capable of driving expression of one or more sequences in mammalian cells using a mammalian expression vector. Examples of mammalian expression vectors include pCDM8 (Seed, 1987. Nature 329: 840) and pMT2PC (Kaufman, et al., 1987. EMBO J. 6: 187-195). When used in mammalian cells, the expression vector's control functions are typically provided by one or more regulatory elements. For example, commonly

used promoters are derived from polyoma, adenovirus 2, cytomegalovirus, simian virus 40, and others disclosed herein and known in the art. For other suitable expression systems for both prokaryotic and eukaryotic cells see, e.g., Chapters 16 and 17 of Sambrook, et al., MOLECULAR CLONING: A LABORATORY MANUAL. 2nd ed., Cold Spring Harbor Laboratory, Cold Spring Harbor Laboratory Press, Cold Spring Harbor, N.Y., 1989.

[00446] In some embodiments, the recombinant mammalian expression vector is capable of directing expression of the nucleic acid preferentially in a particular cell type (e.g., tissue-specific regulatory elements are used to express the nucleic acid). Tissue-specific regulatory elements are known in the art. Non-limiting examples of suitable tissue-specific promoters include the albumin promoter (liver-specific; Pinkert, et al., 1987. *Genes Dev.* 1: 268-277), lymphoid-specific promoters (Calame and Eaton, 1988. *Adv. Immunol.* 43: 235-275), in particular promoters of T cell receptors (Winoto and Baltimore, 1989. *EMBO J.* 8: 729-733) and immunoglobulins (Baneiji, et al., 1983. *Cell* 33: 729-740; Queen and Baltimore, 1983. *Cell* 33: 741-748), neuron-specific promoters (e.g., the neurofilament promoter; Byrne and Ruddle, 1989. *Proc. Natl. Acad. Sci. USA* 86: 5473-5477), pancreas-specific promoters (Edlund, et al., 1985. *Science* 230: 912-916), and mammary gland-specific promoters (e.g., milk whey promoter; U.S. Pat. No. 4,873,316 and European Application Publication No. 264,166). Developmentally-regulated promoters are also encompassed, e.g., the murine hox promoters (Kessel and Gruss, 1990. *Science* 249: 374-379) and the α-fetoprotein promoter (Campes and Tilghman, 1989. *Genes Dev.* 3: 537-546). With regards to these prokaryotic and eukaryotic vectors, mention is made of U.S. Patent 6,750,059, the contents of which are incorporated by reference herein in their entirety. Other embodiments of the invention may relate to the use of viral vectors, with regards to which mention is made of U.S. Patent application 13/092,085, the contents of which are incorporated by reference herein in their entirety. Tissue-specific regulatory elements are known in the art and in this regard, mention is made of U.S. Patent 7,776,321, the contents of which are incorporated by reference herein in their entirety.

Regulatory elements

[00447] In some embodiments, a regulatory element is operably linked to one or more elements of a CRISPR system so as to drive expression of the one or more elements of the CRISPR system. In general, CRISPRs (Clustered Regularly Interspaced Short Palindromic Repeats), also known as SPIDRs (SPacer Interspersed Direct Repeats), constitute a family of

DNA loci that are usually specific to a particular bacterial species. The CRISPR locus comprises a distinct class of interspersed short sequence repeats (SSRs) that were recognized in E. coli (Ishino et al., J. Bacteriol., 169:5429-5433 [1987]; and Nakata et al., J. Bacteriol., 171:3553-3556 [1989]), and associated genes. Similar interspersed SSRs have been identified in Haloferax mediterranei, Streptococcus pyogenes, Anabaena, and Mycobacterium tuberculosis (See, Groenen et al., Mol. Microbiol., 10:1057-1065 [1993]; Hoe et al., Emerg. Infect. Dis., 5:254-263 [1999]; Masepohl et al., Biochim. Biophys. Acta 1307:26-30 [1996]; and Mojica et al., Mol. Microbiol., 17:85-93 [1995]). The CRISPR loci typically differ from other SSRs by the structure of the repeats, which have been termed short regularly spaced repeats (SRSRs) (Janssen et al., OMICS J. Integ. Biol., 6:23-33 [2002]; and Mojica et al., Mol. Microbiol., 36:244-246 [2000]). In general, the repeats are short elements that occur in clusters that are regularly spaced by unique intervening sequences with a substantially constant length (Mojica et al., [2000], supra). Although the repeat sequences are highly conserved between strains, the number of interspersed repeats and the sequences of the spacer regions typically differ from strain to strain (van Embden et al., J. Bacteriol., 182:2393-2401 [2000]). CRISPR loci have been identified in more than 40 prokaryotes (See e.g., Jansen et al., Mol. Microbiol., 43:1565-1575 [2002]; and Mojica et al., [2005]) including, but not limited to Aeropyrum, Pyrobaculum, Sulfolobus, Archaeoglobus, Halocarcula, Methanobacterium, Methanococcus, Methanosarcina, Methanopyrus, Pyrococcus, Picrophilus, Thermoplasma, Corynebacterium, Mycobacterium, Streptomyces, Aquifex, Porphyromonas, Chlorobium, Thermus, Bacillus, Listeria, Staphylococcus, Clostridium, Thermoanaerobacter, Mycoplasma, Fusobacterium, Azarcus, Chromobacterium, Neisseria, Nitrosomonas, Desulfovibrio, Geobacter, Myxococcus, Campylobacter, Wolinella, Acinetobacter, Erwinia, Escherichia, Legionella, Methylococcus, Pasteurella, Photobacterium, Salmonella, Xanthomonas, Yersinia, Treponema, and Thermotoga.

[00448] In general, "CRISPR system" refers collectively to transcripts and other elements involved in the expression of or directing the activity of CRISPR-associated ("Cas") genes, including sequences encoding a Cas gene, a tracr (trans-activating CRISPR) sequence (e.g. tracrRNA or an active partial tracrRNA), a tracr-mate sequence (encompassing a "direct repeat" and a tracrRNA-processed partial direct repeat in the context of an endogenous CRISPR system), a guide sequence (also referred to as a "spacer" in the context of an endogenous CRISPR system), or other sequences and transcripts from a CRISPR locus. In embodiments of the

invention the terms guide sequence and guide RNA are used interchangeably. In some embodiments, one or more elements of a CRISPR system is derived from a type I, type II, or type III CRISPR system. In some embodiments, one or more elements of a CRISPR system is derived from a particular organism comprising an endogenous CRISPR system, such as Streptococcus pyogenes. In general, a CRISPR system is characterized by elements that promote the formation of a CRISPR complex at the site of a target sequence (also referred to as a protospacer in the context of an endogenous CRISPR system). In the context of formation of a CRISPR complex, "target sequence" refers to a sequence to which a guide sequence is designed to have complementarity, where hybridization between a target sequence and a guide sequence promotes the formation of a CRISPR complex. A target sequence may comprise any polynucleotide, such as DNA or RNA polynucleotides. In some embodiments, a target sequence is located in the nucleus or cytoplasm of a cell.

[00449] In some embodiments, direct repeats may be identified *in silico* by searching for repetitive motifs that fulfill any or all of the following criteria:

1. found in a 2Kb window of genomic sequence flanking the type II CRISPR locus;
2. span from 20 to 50 bp; and
3. interspaced by 20 to 50 bp.

[00450] In some embodiments, 2 of these criteria may be used, for instance 1 and 2, 2 and 3, or 1 and 3. In some embodiments, all 3 criteria may be used.

[00451] In some embodiments, candidate tracrRNA may be subsequently predicted by sequences that fulfill any or all of the following criteria:

1. sequence homology to direct repeats (motif search in Geneious with up to 18-bp mismatches);
2. presence of a predicted Rho-independent transcriptional terminator in direction of transcription; and
3. stable hairpin secondary structure between tracrRNA and direct repeat.

[00452] In some embodiments, 2 of these criteria may be used, for instance 1 and 2, 2 and 3, or 1 and 3. In some embodiments, all 3 criteria may be used.

[00453] In some embodiments, chimeric synthetic guide RNAs (sgRNAs) designs may incorporate at least 12 bp of duplex structure between the direct repeat and tracrRNA.

[00454] In preferred embodiments of the invention, the CRISPR system is a type II CRISPR system and the Cas enzyme is Cas9, which catalyzes DNA cleavage. Enzymatic action by Cas9 derived from Streptococcus pyogenes or any closely related Cas9 generates double stranded breaks at target site sequences which hybridize to 20 nucleotides of the guide sequence and that have a protospacer-adjacent motif (PAM) sequence (examples include NGG/NRG or a PAM that can be determined as described herein) following the 20 nucleotides of the target sequence. CRISPR activity through Cas9 for site-specific DNA recognition and cleavage is defined by the guide sequence, the tracr sequence that hybridizes in part to the guide sequence and the PAM sequence. More aspects of the CRISPR system are described in Karginov and Hannon, The CRISPR system: small RNA-guided defence in bacteria and archaea, Mole Cell 2010, January 15; 37(1): 7.

[00455] The type II CRISPR locus from Streptococcus pyogenes SF370, which contains a cluster of four genes Cas9, Cas1, Cas2, and Csn1, as well as two non-coding RNA elements, tracrRNA and a characteristic array of repetitive sequences (direct repeats) interspaced by short stretches of non-repetitive sequences (spacers, about 30bp each). In this system, targeted DNA double-strand break (DSB) is generated in four sequential steps (Fig. 2A). First, two non-coding RNAs, the pre-crRNA array and tracrRNA, are transcribed from the CRISPR locus. Second, tracrRNA hybridizes to the direct repeats of pre-crRNA, which is then processed into mature crRNAs containing individual spacer sequences. Third, the mature crRNA:tracrRNA complex directs Cas9 to the DNA target consisting of the protospacer and the corresponding PAM via heteroduplex formation between the spacer region of the crRNA and the protospacer DNA. Finally, Cas9 mediates cleavage of target DNA upstream of PAM to create a DSB within the protospacer (Fig. 2A). Fig. 2B demonstrates the nuclear localization of the codon optimized Cas9. To promote precise transcriptional initiation, the RNA polymerase III-based U6 promoter was selected to drive the expression of tracrRNA (Fig. 2C). Similarly, a U6 promoter-based construct was developed to express a pre-crRNA array consisting of a single spacer flanked by two direct repeats (DRs, also encompassed by the term "tracr-mate sequences"; Fig. 2C). The initial spacer was designed to target a 33-base-pair (bp) target site (30-bp protospacer plus a 3-bp CRISPR motif (PAM) sequence satisfying the NGG recognition motif of Cas9) in the human EMX1 locus (Fig. 2C), a key gene in the development of the cerebral cortex.

[00456] Typically, in the context of an endogenous CRISPR system, formation of a CRISPR complex (comprising a guide sequence hybridized to a target sequence and complexed with one or more Cas proteins) results in cleavage of one or both strands in or near (e.g. within 1, 2, 3, 4, 5, 6, 7, 8, 9, 10, 20, 50, or more base pairs from) the target sequence. Without wishing to be bound by theory, the tracr sequence, which may comprise or consist of all or a portion of a wild-type tracr sequence (e.g. about or more than about 20, 26, 32, 45, 48, 54, 63, 67, 85, or more nucleotides of a wild-type tracr sequence), may also form part of a CRISPR complex, such as by hybridization along at least a portion of the tracr sequence to all or a portion of a tracr mate sequence that is operably linked to the guide sequence. In some embodiments, one or more vectors driving expression of one or more elements of a CRISPR system are introduced into a host cell such that expression of the elements of the CRISPR system direct formation of a CRISPR complex at one or more target sites. For example, a Cas enzyme, a guide sequence linked to a tracr-mate sequence, and a tracr sequence could each be operably linked to separate regulatory elements on separate vectors. Alternatively, two or more of the elements expressed from the same or different regulatory elements, may be combined in a single vector, with one or more additional vectors providing any components of the CRISPR system not included in the first vector. CRISPR system elements that are combined in a single vector may be arranged in any suitable orientation, such as one element located 5' with respect to ("upstream" of) or 3' with respect to ("downstream" of) a second element. The coding sequence of one element may be located on the same or opposite strand of the coding sequence of a second element, and oriented in the same or opposite direction. In some embodiments, a single promoter drives expression of a transcript encoding a CRISPR enzyme and one or more of the guide sequence, tracr mate sequence (optionally operably linked to the guide sequence), and a tracr sequence embedded within one or more intron sequences (e.g. each in a different intron, two or more in at least one intron, or all in a single intron). In some embodiments, the CRISPR enzyme, guide sequence, tracr mate sequence, and tracr sequence are operably linked to and expressed from the same promoter.

[00457] In some embodiments, a vector comprises one or more insertion sites, such as a restriction endonuclease recognition sequence (also referred to as a "cloning site"). In some embodiments, one or more insertion sites (e.g. about or more than about 1, 2, 3, 4, 5, 6, 7, 8, 9, 10, or more insertion sites) are located upstream and/or downstream of one or more sequence

elements of one or more vectors. In some embodiments, a vector comprises an insertion site upstream of a tracr mate sequence, and optionally downstream of a regulatory element operably linked to the tracr mate sequence, such that following insertion of a guide sequence into the insertion site and upon expression the guide sequence directs sequence-specific binding of a CRISPR complex to a target sequence in a eukaryotic cell. In some embodiments, a vector comprises two or more insertion sites, each insertion site being located between two tracr mate sequences so as to allow insertion of a guide sequence at each site. In such an arrangement, the two or more guide sequences may comprise two or more copies of a single guide sequence, two or more different guide sequences, or combinations of these. When multiple different guide sequences are used, a single expression construct may be used to target CRISPR activity to multiple different, corresponding target sequences within a cell. For example, a single vector may comprise about or more than about 1, 2, 3, 4, 5, 6, 7, 8, 9, 10, 15, 20, or more guide sequences. In some embodiments, about or more than about 1, 2, 3, 4, 5, 6, 7, 8, 9, 10, or more such guide-sequence-containing vectors may be provided, and optionally delivered to a cell.

[00458] In some embodiments, a vector comprises a regulatory element operably linked to an enzyme-coding sequence encoding a CRISPR enzyme, such as a Cas protein. Non-limiting examples of Cas proteins include Cas1, Cas1B, Cas2, Cas3, Cas4, Cas5, Cas6, Cas7, Cas8, Cas9 (also known as Csn1 and Csx12), Cas10, Csy1, Csy2, Csy3, Cse1, Cse2, Csc1, Csc2, Csa5, Csn2, Csm2, Csm3, Csm4, Csm5, Csm6, Cmr1, Cmr3, Cmr4, Cmr5, Cmr6, Csb1, Csb2, Csb3, Csx17, Csx14, Csx10, Csx16, CsaX, Csx3, Csx1, Csx15, Csf1, Csf2, Csf3, Csf4, homologues thereof, or modified versions thereof. In some embodiments, the unmodified CRISPR enzyme has DNA cleavage activity, such as Cas9. In some embodiments, the CRISPR enzyme directs cleavage of one or both strands at the location of a target sequence, such as within the target sequence and/or within the complement of the target sequence. In some embodiments, the CRISPR enzyme directs cleavage of one or both strands within about 1, 2, 3, 4, 5, 6, 7, 8, 9, 10, 15, 20, 25, 50, 100, 200, 500, or more base pairs from the first or last nucleotide of a target sequence. In some embodiments, a vector encodes a CRISPR enzyme that is mutated to with respect to a corresponding wild-type enzyme such that the mutated CRISPR enzyme lacks the ability to cleave one or both strands of a target polynucleotide containing a target sequence. For example, an aspartate-to-alanine substitution (D10A) in the RuvC I catalytic domain of Cas9 from S. pyogenes converts Cas9 from a nuclease that cleaves both strands to a nickase (cleaves a

single strand). Other examples of mutations that render Cas9 a nickase include, without limitation, H840A, N854A, and N863A. As a further example, two or more catalytic domains of Cas9 (RuvC I, RuvC II, and RuvC III or the HNH domain) may be mutated to produce a mutated Cas9 substantially lacking all DNA cleavage activity. In some embodiments, a D10A mutation is combined with one or more of H840A, N854A, or N863A mutations to produce a Cas9 enzyme substantially lacking all DNA cleavage activity. In some embodiments, a CRISPR enzyme is considered to substantially lack all DNA cleavage activity when the DNA cleavage activity of the mutated enzyme is less than about 25%, 10%, 5%, 1%, 0.1%, 0.01%, or lower with respect to its non-mutated form. Where the enzyme is not SpCas9, mutations may be made at any or all residues corresponding to positions 10, 762, 840, 854, 863 and/or 986 of SpCas9 (which may be ascertained for instance by standard sequence comparison tools . In particular, any or all of the following mutations are preferred in SpCas9: D10A, E762A, H840A, N854A, N863A and/or D986A; as well as conservative substitution for any of the replacement amino acids is also envisaged. The same (or conservative substitutions of these mutations) at corresponding positions in other Cas9s are also preferred. Particularly preferred are D10 and H840 in SpCas9 . However, in other Cas9s, residues corresponding to SpCas9 D10 and H840 are also preferred.

[00459] An aspartate-to-alanine substitution (D10A) in the RuvC I catalytic domain of SpCas9 was engineered to convert the nuclease into a nickase (SpCas9n) (see e.g. Sapranauskas et al., 2011, Nucleic Acis Research, 39: 9275; Gasiunas et al., 2012, Proc. Natl. Acad. Sci. USA, 109:E2579), such that nicked genomic DNA undergoes the high-fidelity homology-directed repair (HDR). Surveyor assay confirmed that SpCas9n does not generate indels at the EMX1 protospacer target. Co-expression of EMX1-targeting chimeric crRNA (having the tracrRNA component as well) with SpCas9 produced indels in the target site, whereas co-expression with SpCas9n did not (n=3). Moreover, sequencing of 327 amplicons did not detect any indels induced by SpCas9n. The same locus was selected to test CRISPR-mediated HR by co-transfecting HEK 293FT cells with the chimeric RNA targeting EMX1, hSpCas9 or hSpCas9n, as well as a HR template to introduce a pair of restriction sites (HindIII and NheI) near the protospacer.

[00460] Preferred orthologs are described herein. A Cas enzyme may be identified Cas9 as this can refer to the general class of enzymes that share homology to the biggest nuclease with

multiple nuclease domains from the type II CRISPR system. Most preferably, the Cas9 enzyme is from, or is derived from, spCas9 or saCas9. By derived, Applicants mean that the derived enzyme is largely based, in the sense of having a high degree of sequence homology with, a wildtype enzyme, but that it has been mutated (modified) in some way as described herein.

[00461] It will be appreciated that the terms Cas and CRISPR enzyme are generally used herein interchangeably, unless otherwise apparent. As mentioned above, many of the residue numberings used herein refer to the Cas9 enzyme from the type II CRISPR locus in *Streptococcus pyogenes*. However, it will be appreciated that this invention includes many more Cas9s from other species of microbes, such as SpCas9, SaCa9, St1Cas9 and so forth.

Codon optimization

[00462] An example of a codon optimized sequence, in this instance optimized for humans (i.e. being optimized for expression in humans) is provided herein, see the SaCas9 human codon optimized sequence. Whilst this is preferred, it will be appreciated that other examples are possible and codon optimization for a host species is known.

[00463] In some embodiments, an enzyme coding sequence encoding a CRISPR enzyme is codon optimized for expression in particular cells, such as eukaryotic cells. The eukaryotic cells may be those of or derived from a particular organism, such as a mammal, including but not limited to human, mouse, rat, rabbit, dog, or non-human mammal 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.

[00464] In general, codon optimization refers to a process of modifying a nucleic acid sequence for enhanced expression in the host cells of interest by replacing at least one codon (e.g. about or more than about 1, 2, 3, 4, 5, 10, 15, 20, 25, 50, or more codons) of the native sequence with codons that are more frequently or most frequently used in the genes of that host cell while maintaining the native amino acid sequence. Various species exhibit particular bias for certain codons of a particular amino acid. Codon bias (differences in codon usage between organisms) often correlates with the efficiency of translation of messenger RNA (mRNA), which is in turn believed to be dependent on, among other things, the properties of the codons being translated and the availability of particular transfer RNA (tRNA) molecules. The predominance

of selected tRNAs in a cell is generally a reflection of the codons used most frequently in peptide synthesis. Accordingly, genes can be tailored for optimal gene expression in a given organism based on codon optimization. Codon usage tables are readily available, for example, at the "Codon Usage Database" available at www.kazusa.orjp/codon/ (visited Jul. 9, 2002), and these tables can be adapted in a number of ways. See Nakamura, Y., et al. "Codon usage tabulated from the international DNA sequence databases: status for the year 2000" Nucl. Acids Res. 28:292 (2000). Computer algorithms for codon optimizing a particular sequence for expression in a particular host cell are also available, such as Gene Forge (Aptagen; Jacobus, PA), are also available. In some embodiments, one or more codons (e.g. 1, 2, 3, 4, 5, 10, 15, 20, 25, 50, or more, or all codons) in a sequence encoding a CRISPR enzyme correspond to the most frequently used codon for a particular amino acid.

Nuclear localization sequences (NLSs)

[00465] In some embodiments, a vector encodes a CRISPR enzyme comprising one or more nuclear localization sequences (NLSs), such as about or more than about 1, 2, 3, 4, 5, 6, 7, 8, 9, 10, or more NLSs. In some embodiments, the CRISPR enzyme comprises about or more than about 1, 2, 3, 4, 5, 6, 7, 8, 9, 10, or more NLSs at or near the amino-terminus, about or more than about 1, 2, 3, 4, 5, 6, 7, 8, 9, 10, or more NLSs at or near the carboxy-terminus, or a combination of these (e.g. one or more NLS at the amino-terminus and one or more NLS at the carboxy terminus). When more than one NLS is present, each may be selected independently of the others, such that a single NLS may be present in more than one copy and/or in combination with one or more other NLSs present in one or more copies. In a preferred embodiment of the invention, the CRISPR enzyme comprises at most 6 NLSs. In some embodiments, an NLS is considered near the N- or C-terminus when the nearest amino acid of the NLS is within about 1, 2, 3, 4, 5, 10, 15, 20, 25, 30, 40, 50, or more amino acids along the polypeptide chain from the N- or C-terminus. Non-limiting examples of NLSs include an NLS sequence derived from: the NLS of the SV40 virus large T-antigen, having the amino acid sequence PKKKRKV; the NLS from nucleoplasmin (e.g. the nucleoplasmin bipartite NLS with the sequence KRPAATKKAGQAKKKK); the c-myc NLS having the amino acid sequence PAAKRVKLD or RQRRNELKRSP; the hRNPA1 M9 NLS having the sequence NQSSNFGPMKGGNFGGRSSGPYGGGGQYFAKPRNQGGY; the sequence RMRIZFKNKGKDTAELRRRRVEVSVELRKAKKDEQILKRRNV of the IBB domain from

importin-alpha; the sequences VSRKRPRP and PPKKARED of the myoma T protein; the sequence POPKKKPL of human p53; the sequence SALIKKKKKMAP of mouse c-abl IV; the sequences DRLRR and PKQKKRK of the influenza virus NS1; the sequence RKLKKKIKKL of the Hepatitis virus delta antigen; the sequence REKKKFLKRR of the mouse Mx1 protein; the sequence KRKGDEVDGVDEVAKKKSKK of the human poly(ADP-ribose) polymerase; and the sequence RKCLQAGMNLEARKTKK of the steroid hormone receptors (human) glucocorticoid.

[00466] In general, the one or more NLSs are of sufficient strength to drive accumulation of the CRISPR enzyme in a detectable amount in the nucleus of a eukaryotic cell. In general, strength of nuclear localization activity may derive from the number of NLSs in the CRISPR enzyme, the particular NLS(s) used, or a combination of these factors. Detection of accumulation in the nucleus may be performed by any suitable technique. For example, a detectable marker may be fused to the CRISPR enzyme, such that location within a cell may be visualized, such as in combination with a means for detecting the location of the nucleus (e.g. a stain specific for the nucleus such as DAPI). Cell nuclei may also be isolated from cells, the contents of which may then be analyzed by any suitable process for detecting protein, such as immunohistochemistry, Western blot, or enzyme activity assay. Accumulation in the nucleus may also be determined indirectly, such as by an assay for the effect of CRISPR complex formation (e.g. assay for DNA cleavage or mutation at the target sequence, or assay for altered gene expression activity affected by CRISPR complex formation and/or CRISPR enzyme activity), as compared to a control no exposed to the CRISPR enzyme or complex, or exposed to a CRISPR enzyme lacking the one or more NLSs.

Guide sequence

[00467] In general, a guide sequence is any polynucleotide sequence having sufficient complementarity with a target polynucleotide sequence to hybridize with the target sequence and direct sequence-specific binding of a CRISPR complex to the target sequence. In some embodiments, the degree of complementarity between a guide sequence and its corresponding target sequence, when optimally aligned using a suitable alignment algorithm, is about or more than about 50%, 60%, 75%, 80%, 85%, 90%, 95%, 97.5%, 99%, or more. Optimal alignment may be determined with the use of any suitable algorithm for aligning sequences, non-limiting example of which include the Smith-Waterman algorithm, the Needleman-Wunsch algorithm,

algorithms based on the Burrows-Wheeler Transform (e.g. the Burrows Wheeler Aligner), ClustalW, Clustal X, BLAT, Novoalign (Novocraft Technologies; available at www.novocraft.com), ELAND (Illumina, San Diego, CA), SOAP (available at soap.genomics.org.cn), and Maq (available at maq.sourceforge.net). In some embodiments, a guide sequence is about or more than about 5, 10, 11, 12, 13, 14, 15, 16, 17, 18, 19, 20, 21, 22, 23, 24, 25, 26, 27, 28, 29, 30, 35, 40, 45, 50, 75, or more nucleotides in length. In some embodiments, a guide sequence is less than about 75, 50, 45, 40, 35, 30, 25, 20, 15, 12, or fewer nucleotides in length. The ability of a guide sequence to direct sequence-specific binding of a CRISPR complex to a target sequence may be assessed by any suitable assay. For example, the components of a CRISPR system sufficient to form a CRISPR complex, including the guide sequence to be tested, may be provided to a host cell having the corresponding target sequence, such as by transfection with vectors encoding the components of the CRISPR sequence, followed by an assessment of preferential cleavage within the target sequence, such as by Surveyor assay as described herein. Similarly, cleavage of a target polynucleotide sequence may be evaluated in a test tube by providing the target sequence, components of a CRISPR complex, including the guide sequence to be tested and a control guide sequence different from the test guide sequence, and comparing binding or rate of cleavage at the target sequence between the test and control guide sequence reactions. Other assays are possible, and will occur to those skilled in the art.

[00468] A guide sequence may be selected to target any target sequence. In some embodiments, the target sequence is a sequence within a genome of a cell. Exemplary target sequences include those that are unique in the target genome. For example, for the *S. pyogenes* Cas9, a unique target sequence in a genome may include a Cas9 target site of the form MMMMMMMMMNNNNNNNNNNNNXGG where NNNNNNNNNNNNXGG (N is A, G, T, or C; and X can be anything) has a single occurrence in the genome. A unique target sequence in a genome may include an *S. pyogenes* Cas9 target site of the form MMMMMMMMMNNNNNNNNNNNNXGG where NNNNNNNNNNNNXGG (N is A, G, T, or C; and X can be anything) has a single occurrence in the genome. For the *S. thermophilus* CRISPR1 Cas9, a unique target sequence in a genome may include a Cas9 target site of the form MMMMMMMMMNNNNNNNNNNNNXXAGAAW where NNNNNNNNNNNNXXAGAAW (N is A, G, T, or C; X can be anything; and W is A or T) has a single occurrence in the genome. A unique target sequence in a genome may include an *S. thermophilus* CRISPR1 Cas9 target site

of the form MMMMMMMMMNNNNNNNNNNNNXXAGAAW where NNNNNNNNNNNNXXAGAAW (N is A, G, T, or C; X can be anything; and W is A or T) has a single occurrence in the genome. For the *S. pyogenes* Cas9, a unique target sequence in a genome may include a Cas9 target site of the form MMMMMMMMMNNNNNNNNNNNNXGGXG where NNNNNNNNNNNNXGGXG (N is A, G, T, or C; and X can be anything) has a single occurrence in the genome. A unique target sequence in a genome may include an *S. pyogenes* Cas9 target site of the form MMMMMMMMMNNNNNNNNNNNNXGGXG where NNNNNNNNNNNNXGGXG (N is A, G, T, or C; and X can be anything) has a single occurrence in the genome. In each of these sequences "M" may be A, G, T, or C, and need not be considered in identifying a sequence as unique.

[00469] In some embodiments, a guide sequence is selected to reduce the degree secondary structure within the guide sequence. In some embodiments, about or less than about 75%, 50%, 40%, 30%, 25%, 20%, 15%, 10%, 5%, 1%, or fewer of the nucleotides of the guide sequence participate in self-complementary base pairing when optimally folded. Optimal folding may be determined by any suitable polynucleotide folding algorithm. Some programs are based on calculating the minimal Gibbs free energy. An example of one such algorithm is mFold, as described by Zuker and Stiegler (Nucleic Acids Res. 9 (1981), 133-148). Another example folding algorithm is the online webserver RNAfold, developed at Institute for Theoretical Chemistry at the University of Vienna, using the centroid structure prediction algorithm (see e.g. A.R. Gruber *et al.*, 2008, *Cell* 106(1): 23-24; and PA Carr and GM Church, 2009, *Nature Biotechnology* 27(12): 1151-62).

Tracr mate sequence

[00470] In general, a tracr mate sequence includes any sequence that has sufficient complementarity with a tracr sequence to promote one or more of: (1) excision of a guide sequence flanked by tracr mate sequences in a cell containing the corresponding tracr sequence; and (2) formation of a CRISPR complex at a target sequence, wherein the CRISPR complex comprises the tracr mate sequence hybridized to the tracr sequence. In general, degree of complementarity is with reference to the optimal alignment of the tracr mate sequence and tracr sequence, along the length of the shorter of the two sequences. Optimal alignment may be determined by any suitable alignment algorithm, and may further account for secondary

structures, such as self-complementarity within either the tracr sequence or tracr mate sequence. In some embodiments, the degree of complementarity between the tracr sequence and tracr mate sequence along the length of the shorter of the two when optimally aligned is about or more than about 25%, 30%, 40%, 50%, 60%, 70%, 80%, 90%, 95%, 97.5%, 99%, or higher. In some embodiments, the tracr sequence is about or more than about 5, 6, 7, 8, 9, 10, 11, 12, 13, 14, 15, 16, 17, 18, 19, 20, 25, 30, 40, 50, or more nucleotides in length. In some embodiments, the tracr sequence and tracr mate sequence are contained within a single transcript, such that hybridization between the two produces a transcript having a secondary structure, such as a hairpin. In an embodiment of the invention, the transcript or transcribed polynucleotide sequence has at least two or more hairpins. In preferred embodiments, the transcript has two, three, four or five hairpins. In a further embodiment of the invention, the transcript has at most five hairpins. In a hairpin structure the portion of the sequence 5' of the final "N" and upstream of the loop corresponds to the tracr mate sequence, and the portion of the sequence 3' of the loop corresponds to the tracr sequence Further non-limiting examples of single polynucleotides comprising a guide sequence, a tracr mate sequence, and a tracr sequence are as follows (listed 5' to 3'), where "N" represents a base of a guide sequence, the first block of lower case letters represent the tracr mate sequence, and the second block of lower case letters represent the tracr sequence, and the final poly-T sequence represents the transcription terminator: (1) NNNNNNNNNNNNNNNNNNNNgttttgtactctcaagatttaGAAAtaaatcttgcagaagctacaaagataaggcttcatgccgaaatcaacaccctgtcattttatggcagggtgttttcgttatttaaTTTTTT; (2) NNNNNNNNNNNNNNNNNNNNgttttgtactctcaGAAAtgcagaagctacaaagataaggcttcatgccgaaatcaacaccctgtcattttatggcagggtgttttcgttatttaaTTTTTT; (3) NNNNNNNNNNNNNNNNNNNNgttttgtactctcaGAAAtgcagaagctacaaagataaggcttcatgccgaaatcaacaccctgtcattttatggcagggtgtTTTTTT; (4) NNNNNNNNNNNNNNNNNNNNgttttagagctaGAAAtagcaagttaaaataaggctagtccgttatcaacttgaaaaagtggcaccgagtcggtgcTTTTTT; (5) NNNNNNNNNNNNNNNNNNNNgttttagagctaGAAATAGcaagttaaaataaggctagtccgttatcaacttgaaaaagtgTTTTTTT; and (6) NNNNNNNNNNNNNNNNNNNNgttttagagctagAAATAGcaagttaaaataaggctagtccgttatcaTTTTTTTT. In some embodiments, sequences (1) to (3) are used in combination with Cas9 from *S. thermophilus* CRISPR1. In some embodiments, sequences (4) to (6) are used in combination

with Cas9 from *S. pyogenes*. In some embodiments, the tracr sequence is a separate transcript from a transcript comprising the tracr mate sequence.

Recombination template

[00471] In some embodiments, a recombination template is also provided. A recombination template may be a component of another vector as described herein, contained in a separate vector, or provided as a separate polynucleotide. In some embodiments, a recombination template is designed to serve as a template in homologous recombination, such as within or near a target sequence nicked or cleaved by a CRISPR enzyme as a part of a CRISPR complex. A template polynucleotide may be of any suitable length, such as about or more than about 10, 15, 20, 25, 50, 75, 100, 150, 200, 500, 1000, or more nucleotides in length. In some embodiments, the template polynucleotide is complementary to a portion of a polynucleotide comprising the target sequence. When optimally aligned, a template polynucleotide might overlap with one or more nucleotides of a target sequences (e.g. about or more than about 1, 5, 10, 15, 20, or more nucleotides). In some embodiments, when a template sequence and a polynucleotide comprising a target sequence are optimally aligned, the nearest nucleotide of the template polynucleotide is within about 1, 5, 10, 15, 20, 25, 50, 75, 100, 200, 300, 400, 500, 1000, 5000, 10000, or more nucleotides from the target sequence.

Fusion protein

[00472] In some embodiments, the CRISPR enzyme is part of a fusion protein comprising one or more heterologous protein domains (e.g. about or more than about 1, 2, 3, 4, 5, 6, 7, 8, 9, 10, or more domains in addition to the CRISPR enzyme). A CRISPR enzyme fusion protein may comprise any additional protein sequence, and optionally a linker sequence between any two domains. Examples of protein domains that may be fused to a CRISPR enzyme include, without limitation, epitope tags, reporter gene sequences, and protein domains having one or more of the following activities: methylase activity, demethylase activity, transcription activation activity, transcription repression activity, transcription release factor activity, histone modification activity, RNA cleavage activity and nucleic acid binding activity. Non-limiting examples of epitope tags include histidine (His) tags, V5 tags, FLAG tags, influenza hemagglutinin (HA) tags, Myc tags, VSV-G tags, and thioredoxin (Trx) tags. Examples of reporter genes include, but are not limited to, glutathione-S-transferase (GST), horseradish peroxidase (HRP), chloramphenicol acetyltransferase (CAT) beta-galactosidase, beta-glucuronidase, luciferase,

green fluorescent protein (GFP), HcRed, DsRed, cyan fluorescent protein (CFP), yellow fluorescent protein (YFP), and autofluorescent proteins including blue fluorescent protein (BFP). A CRISPR enzyme may be fused to a gene sequence encoding a protein or a fragment of a protein that bind DNA molecules or bind other cellular molecules, including but not limited to maltose binding protein (MBP), S-tag, Lex A DNA binding domain (DBD) fusions, GAL4 DNA binding domain fusions, and herpes simplex virus (HSV) BP16 protein fusions. Additional domains that may form part of a fusion protein comprising a CRISPR enzyme are described in US20110059502, incorporated herein by reference. In some embodiments, a tagged CRISPR enzyme is used to identify the location of a target sequence.

Inducible system

[00473] In some embodiments, a CRISPR enzyme may form a component of an inducible system. The inducible nature of the system would allow for spatiotemporal control of gene editing or gene expression using a form of energy. The form of energy may include but is not limited to electromagnetic radiation, sound energy, chemical energy and thermal energy. Examples of inducible system include tetracycline inducible promoters (Tet-On or Tet-Off), small molecule two-hybrid transcription activations systems (FKBP, ABA, etc), or light inducible systems (Phytochrome, LOV domains, or cryptochrome).In one embodiment, the CRISPR enzyme may be a part of a Light Inducible Transcriptional Effector (LITE) to direct changes in transcriptional activity in a sequence-specific manner. The components of a light may include a CRISPR enzyme, a light-responsive cytochrome heterodimer (e.g. from Arabidopsis thaliana), and a transcriptional activation/repression domain. Further examples of inducible DNA binding proteins and methods for their use are provided in US 61/736465 and US 61/721,283, which is hereby incorporated by reference in its entirety.

Delivery

[00474] In some aspects, the invention provides methods comprising delivering one or more polynucleotides, such as or one or more vectors as described herein, one or more transcripts thereof, and/or one or proteins transcribed therefrom, to a host cell. In some aspects, the invention further provides cells produced by such methods, and animals comprising or produced from such cells. In some embodiments, a CRISPR enzyme in combination with (and optionally complexed with) a guide sequence is delivered to a cell. Conventional viral and non-viral based gene transfer methods can be used to introduce nucleic acids in mammalian cells or target

tissues. Such methods can be used to administer nucleic acids encoding components of a CRISPR system to cells in culture, or in a host organism. Non-viral vector delivery systems include DNA plasmids, RNA (e.g. a transcript of a vector described herein), naked nucleic acid, and nucleic acid complexed with a delivery vehicle, such as a liposome. Viral vector delivery systems include DNA and RNA viruses, which have either episomal or integrated genomes after delivery to the cell. For a review of gene therapy procedures, see Anderson, Science 256:808-813 (1992); Nabel & Felgner, TIBTECH 11:211-217 (1993); Mitani & Caskey, TIBTECH 11:162-166 (1993); Dillon, TIBTECH 11:167-175 (1993); Miller, Nature 357:455-460 (1992); Van Brunt, Biotechnology 6(10):1149-1154 (1988); Vigne, Restorative Neurology and Neuroscience 8:35-36 (1995); Kremer & Perricaudet, British Medical Bulletin 51(1):31-44 (1995); Haddada et al., in Current Topics in Microbiology and Immunology Doerfler and Böhm (eds) (1995); and Yu et al., Gene Therapy 1:13-26 (1994).

[00475] Methods of non-viral delivery of nucleic acids include lipofection, microinjection, biolistics, virosomes, liposomes, immunoliposomes, polycation or lipid:nucleic acid conjugates, naked DNA, artificial virions, and agent-enhanced uptake of DNA. Lipofection is described in e.g., U.S. Pat. Nos. 5,049,386, 4,946,787; and 4,897,355) and lipofection reagents are sold commercially (e.g., Transfectam™ and Lipofectin™). Cationic and neutral lipids that are suitable for efficient receptor-recognition lipofection of polynucleotides include those of Felgner, WO 91/17424; WO 91/16024. Delivery can be to cells (e.g. in vitro or ex vivo administration) or target tissues (e.g. in vivo administration).

[00476] The preparation of lipid:nucleic acid complexes, including targeted liposomes such as immunolipid complexes, is well known to one of skill in the art (see, e.g., Crystal, Science 270:404-410 (1995); Blaese et al., Cancer Gene Ther. 2:291-297 (1995); Behr et al., Bioconjugate Chem. 5:382-389 (1994); Remy et al., Bioconjugate Chem. 5:647-654 (1994); Gao et al., Gene Therapy 2:710-722 (1995); Ahmad et al., Cancer Res. 52:4817-4820 (1992); U.S. Pat. Nos. 4,186,183, 4,217,344, 4,235,871, 4,261,975, 4,485,054, 4,501,728, 4,774,085, 4,837,028, and 4,946,787).

[00477] The use of RNA or DNA viral based systems for the delivery of nucleic acids take advantage of highly evolved processes for targeting a virus to specific cells in the body and trafficking the viral payload to the nucleus. Viral vectors can be administered directly to patients (in vivo) or they can be used to treat cells in vitro, and the modified cells may optionally be

administered to patients (ex vivo). Conventional viral based systems could include retroviral, lentivirus, adenoviral, adeno-associated and herpes simplex virus vectors for gene transfer. Integration in the host genome is possible with the retrovirus, lentivirus, and adeno-associated virus gene transfer methods, often resulting in long term expression of the inserted transgene. Additionally, high transduction efficiencies have been observed in many different cell types and target tissues.

[00478] The tropism of a retrovirus can be altered by incorporating foreign envelope proteins, expanding the potential target population of target cells. Lentiviral vectors are retroviral vectors that are able to transduce or infect non-dividing cells and typically produce high viral titers. Selection of a retroviral gene transfer system would therefore depend on the target tissue. Retroviral vectors are comprised of cis-acting long terminal repeats with packaging capacity for up to 6-10 kb of foreign sequence. The minimum cis-acting LTRs are sufficient for replication and packaging of the vectors, which are then used to integrate the therapeutic gene into the target cell to provide permanent transgene expression. Widely used retroviral vectors include those based upon murine leukemia virus (MuLV), gibbon ape leukemia virus (GaLV), Simian Immuno deficiency virus (SIV), human immuno deficiency virus (HIV), and combinations thereof (see, e.g., Buchscher et al., J. Virol. 66:2731-2739 (1992); Johann et al., J. Virol. 66:1635-1640 (1992); Sommnerfelt et al., Virol. 176:58-59 (1990); Wilson et al., J. Virol. 63:2374-2378 (1989); Miller et al., J. Virol. 65:2220-2224 (1991); PCT/US94/05700).

[00479] In another embodiment, Cocal vesiculovirus envelope pseudotyped retroviral vector particles are contemplated (see, e.g., US Patent Publication No. 20120164118 assigned to the Fred Hutchinson Cancer Research Center). Cocal virus is in the Vesiculovirus genus, and is a causative agent of vesicular stomatitis in mammals. Cocal virus was originally isolated from mites in Trinidad (Jonkers et al., Am. J. Vet. Res. 25:236-242 (1964)), and infections have been identified in Trinidad, Brazil, and Argentina from insects, cattle, and horses. Many of the vesiculoviruses that infect mammals have been isolated from naturally infected arthropods, suggesting that they are vector-borne. Antibodies to vesiculoviruses are common among people living in rural areas where the viruses are endemic and laboratory-acquired; infections in humans usually result in influenza-like symptoms. The Cocal virus envelope glycoprotein shares 71.5% identity at the amino acid level with VSV-G Indiana, and phylogenetic comparison of the envelope gene of vesiculoviruses shows that Cocal virus is serologically distinct from, but most

closely related to, VSV-G Indiana strains among the vesiculoviruses. Jonkers et al., Am. J. Vet. Res. 25:236-242 (1964) and Travassos da Rosa et al., Am. J. Tropical Med. & Hygiene 33:999-1006 (1984). The Cocal vesiculovirus envelope pseudotyped retroviral vector particles may include for example, lentiviral, alpharetroviral, betaretroviral, gammaretroviral, deltaretroviral, and epsilonretroviral vector particles that may comprise retroviral Gag, Pol, and/or one or more accessory protein(s) and a Cocal vesiculovirus envelope protein. Within certain aspects of these embodiments, the Gag, Pol, and accessory proteins are lentiviral and/or gammaretroviral.

[00480] In applications where transient expression is preferred, adenoviral based systems may be used. Adenoviral based vectors are capable of very high transduction efficiency in many cell types and do not require cell division. With such vectors, high titer and levels of expression have been obtained. This vector can be produced in large quantities in a relatively simple system.

[00481] Adeno-associated virus ("AAV") vectors may also be used to transduce cells with target nucleic acids, e.g., in the in vitro production of nucleic acids and peptides, and for in vivo and ex vivo gene therapy procedures (see, e.g., West et al., Virology 160:38-47 (1987); U.S. Pat. No. 4,797,368; WO 93/24641; Kotin, Human Gene Therapy 5:793-801 (1994); Muzyczka, J. Clin. Invest. 94:1351 (1994). Construction of recombinant AAV vectors are described in a number of publications, including U.S. Pat. No. 5,173,414; Tratschin et al., Mol. Cell. Biol. 5:3251-3260 (1985); Tratschin, et al., Mol. Cell. Biol. 4:2072-2081 (1984); Hermonat & Muzyczka, PNAS 81:6466-6470 (1984); and Samulski et al., J. Virol. 63:03822-3828 (1989).

[00482] Packaging cells are typically used to form virus particles that are capable of infecting a host cell. Such cells include 293 cells, which package adenovirus, and ψ2 cells or PA317 cells, which package retrovirus. Viral vectors used in gene therapy are usually generated by producer a cell line that packages a nucleic acid vector into a viral particle. The vectors typically contain the minimal viral sequences required for packaging and subsequent integration into a host, other viral sequences being replaced by an expression cassette for the polynucleotide(s) to be expressed. The missing viral functions are typically supplied in trans by the packaging cell line. For example, AAV vectors used in gene therapy typically only possess ITR sequences from the AAV genome which are required for packaging and integration into the host genome. Viral DNA is packaged in a cell line, which contains a helper plasmid encoding the other AAV genes, namely rep and cap, but lacking ITR sequences. The cell line may also infected with adenovirus as a helper. The helper virus promotes replication of the AAV vector and expression of AAV

genes from the helper plasmid. The helper plasmid is not packaged in significant amounts due to a lack of ITR sequences. Contamination with adenovirus can be reduced by, e.g., heat treatment to which adenovirus is more sensitive than AAV.

[00483] Accordingly, AAV is considered an ideal candidate for use as a transducing vector. Such AAV transducing vectors can comprise sufficient cis-acting functions to replicate in the presence of adenovirus or herpesvirus or poxvirus (e.g., vaccinia virus) helper functions provided in trans. Recombinant AAV (rAAV) can be used to carry exogenous genes into cells of a variety of lineages. In these vectors, the AAV cap and/or rep genes are deleted from the viral genome and replaced with a DNA segment of choice. Current AAV vectors may accommodate up to 4300 bases of inserted DNA.

[00484] There are a number of ways to produce rAAV, and the invention provides rAAV and methods for preparing rAAV. For example, plasmid(s) containing or consisting essentially of the desired viral construct are transfected into AAV-infected cells. In addition, a second or additional helper plasmid is cotransfected into these cells to provide the AAV rep and/or cap genes which are obligatory for replication and packaging of the recombinant viral construct. Under these conditions, the rep and/or cap proteins of AAV act in trans to stimulate replication and packaging of the rAAV construct. Two to Three days after transfection, rAAV is harvested. Traditionally rAAV is harvested from the cells along with adenovirus. The contaminating adenovirus is then inactivated by heat treatment. In the instant invention, rAAV is advantageously harvested not from the cells themselves, but from cell supernatant. Accordingly, in an initial aspect the invention provides for preparing rAAV, and in addition to the foregoing, rAAV can be prepared by a method that comprises or consists essentially of: infecting susceptible cells with a rAAV containing exogenous DNA including DNA for expression, and helper virus (e.g., adenovirus, herpesvirus, poxvirus such as vaccinia virus) wherein the rAAV lacks functioning cap and/or rep (and the helper virus (e.g., adenovirus, herpesvirus, poxvirus such as vaccinia virus) provides the cap and/or rev function that the rAAV lacks); or infecting susceptible cells with a rAAV containing exogenous DNA including DNA for expression, wherein the recombinant lacks functioning cap and/or rep, and transfecting said cells with a plasmid supplying cap and/or rep function that the rAAV lacks; or infecting susceptible cells with a rAAV containing exogenous DNA including DNA for expression, wherein the recombinant lacks functioning cap and/or rep, wherein said cells supply cap and/or rep function

that the recombinant lacks; or transfecting the susceptible cells with an AAV lacking functioning cap and/or rep and plasmids for inserting exogenous DNA into the recombinant so that the exogenous DNA is expressed by the recombinant and for supplying rep and/or cap functions whereby transfection results in an rAAV containing the exogenous DNA including DNA for expression that lacks functioning cap and/or rep.

[00485] The rAAV can be from an AAV as herein described, and advantageously can be an rAAV1, rAAV2, AAV5 or rAAV having hybrid or capsid which may comprise AAV1, AAV2, AAV5 or any combination thereof. One can select the AAV of the rAAV with regard to the cells to be targeted by the rAAV; e.g., one can select AAV serotypes 1, 2, 5 or a hybrid or capsid AAV1, AAV2, AAV5 or any combination thereof for targeting brain or neuronal cells; and one can select AAV4 for targeting cardiac tissue.

[00486] In addition to 293 cells, other cells that can be used in the practice of the invention and the relative infectivity of certain AAV serotypes *in vitro* as to these cells (see Grimm, D. et al, J. Virol. 82: 5887-5911 (2008)) are as follows:

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

[00487] The invention provides rAAV that contains or consists essentially of an exogenous nucleic acid molecule encoding a CRISPR (Clustered Regularly Interspaced Short Palindromic Repeats) system, e.g., a plurality of cassettes comprising or consisting a first cassette comprising or consisting essentially of a promoter, a nucleic acid molecule encoding a CRISPR-associated

(Cas) protein (putative nuclease or helicase proteins), e.g., Cas9 and a terminator, and a two, or more, advantageously up to the packaging size limit of the vector, e.g., in total (including the first cassette) five, cassettes comprising or consisting essentially of a promoter, nucleic acid molecule encoding guide RNA (gRNA) and a terminator (e.g., each cassette schematically represented as Promoter-gRNA1-terminator, Promoter-gRNA2-terminator ... Promoter-gRNA(N)-terminator (where N is a number that can be inserted that is at an upper limit of the packaging size limit of the vector), or two or more individual rAAVs, each containing one or more than one cassette of a CRISPR system, e.g., a first rAAV containing the first cassette comprising or consisting essentially of a promoter, a nucleic acid molecule encoding Cas, e.g., Cas9 and a terminator, and a second rAAV containing a plurality, four, cassettes comprising or consisting essentially of a promoter, nucleic acid molecule encoding guide RNA (gRNA) and a terminator (e.g., each cassette schematically represented as Promoter-gRNA1-terminator, Promoter-gRNA2-terminator ... Promoter-gRNA(N)-terminator (where N is a number that can be inserted that is at an upper limit of the packaging size limit of the vector). As rAAV is a DNA virus, the nucleic acid molecules in the herein discussion concerning AAV or rAAV are advantageously DNA. The promoter is in some embodiments advantageously human Synapsin I promoter (hSyn).

[00488] Additional methods for the delivery of nucleic acids to cells are known to those skilled in the art. See, for example, US20030087817, incorporated herein by reference.

[00489] In some embodiments, a host cell is transiently or non-transiently transfected with one or more vectors described herein. In some embodiments, a cell is transfected as it naturally occurs in a subject. In some embodiments, a cell that is transfected is taken from a subject. In some embodiments, the cell is derived from cells taken from a subject, such as a cell line. A wide variety of cell lines for tissue culture are known in the art. Examples of cell lines include, but are not limited to, C8161, CCRF-CEM, MOLT, mIMCD-3, NHDF, HeLa-S3, Huh1, Huh4, Huh7, HUVEC, HASMC, HEKn, HEKa, MiaPaCell, Panc1, PC-3, TF1, CTLL-2, C1R, Rat6, CV1, RPTE, A10, T24, J82, A375, ARH-77, Calu1, SW480, SW620, SKOV3, SK-UT, CaCo2, P388D1, SEM-K2, WEHI-231, HB56, TIB55, Jurkat, J45.01, LRMB, Bcl-1, BC-3, IC21, DLD2, Raw264.7, NRK, NRK-52E, MRC5, MEF, Hep G2, HeLa B, HeLa T4, COS, COS-1, COS-6, COS-M6A, BS-C-1 monkey kidney epithelial, BALB/ 3T3 mouse embryo fibroblast, 3T3 Swiss, 3T3-L1, 132-d5 human fetal fibroblasts; 10.1 mouse fibroblasts, 293-T, 3T3, 721, 9L, A2780,

A2780ADR, A2780cis, A172, A20, A253, A431, A-549, ALC, B16, B35, BCP-1 cells, BEAS-2B, bEnd.3, BHK-21, BR 293, BxPC3, C3H-10T1/2, C6/36, Cal-27, CHO, CHO-7, CHO-IR, CHO-K1, CHO-K2, CHO-T, CHO Dhfr -/-, COR-L23, COR-L23/CPR, COR-L23/5010, COR-L23/R23, COS-7, COV-434, CML T1, CMT, CT26, D17, DH82, DU145, DuCaP, EL4, EM2, EM3, EMT6/AR1, EMT6/AR10.0, FM3, H1299, H69, HB54, HB55, HCA2, HEK-293, HeLa, Hepa1c1c7, HL-60, HMEC, HT-29, Jurkat, JY cells, K562 cells, Ku812, KCL22, KG1, KYO1, LNCap, Ma-Mel 1-48, MC-38, MCF-7, MCF-10A, MDA-MB-231, MDA-MB-468, MDA-MB-435, MDCK II, MDCK II, MOR/0.2R, MONO-MAC 6, MTD-1A, MyEnd, NCI-H69/CPR, NCI-H69/LX10, NCI-H69/LX20, NCI-H69/LX4, NIH-3T3, NALM-1, NW-145, OPCN / OPCT cell lines, Peer, PNT-1A / PNT 2, RenCa, RIN-5F, RMA/RMAS, Saos-2 cells, Sf-9, SkBr3, T2, T-47D, T84, THP1 cell line, U373, U87, U937, VCaP, Vero cells, WM39, WT-49, X63, YAC-1, YAR, and transgenic varieties thereof. Cell lines are available from a variety of sources known to those with skill in the art (see, e.g., the American Type Culture Collection (ATCC) (Manassus, Va.)). In some embodiments, a cell transfected with one or more vectors described herein is used to establish a new cell line comprising one or more vector-derived sequences. In some embodiments, a cell transiently transfected with the components of a CRISPR system as described herein (such as by transient transfection of one or more vectors, or transfection with RNA), and modified through the activity of a CRISPR complex, is used to establish a new cell line comprising cells containing the modification but lacking any other exogenous sequence. In some embodiments, cells transiently or non-transiently transfected with one or more vectors described herein, or cell lines derived from such cells are used in assessing one or more test compounds.

[00490] In some embodiments, one or more vectors described herein are used to produce a non-human transgenic animal or transgenic plant. In some embodiments, the transgenic animal is a mammal, such as a mouse, rat, or rabbit. Methods for producing transgenic animals and plants are known in the art, and generally begin with a method of cell transfection, such as described herein.

[00491] In another embodiment, a fluid delivery device with an array of needles (see, e.g., US Patent Publication No. 20110230839 assigned to the Fred Hutchinson Cancer Research Center) may be contemplated for delivery of CRISPR Cas to solid tissue. A device of US Patent Publication No. 20110230839 for delivery of a fluid to a solid tissue may comprise a plurality of

needles arranged in an array; a plurality of reservoirs, each in fluid communication with a respective one of the plurality of needles; and a plurality of actuators operatively coupled to respective ones of the plurality of reservoirs and configured to control a fluid pressure within the reservoir. In certain embodiments each of the plurality of actuators may comprise one of a plurality of plungers, a first end of each of the plurality of plungers being received in a respective one of the plurality of reservoirs, and in certain further embodiments the plungers of the plurality of plungers are operatively coupled together at respective second ends so as to be simultaneously depressable. Certain still further embodiments may comprise a plunger driver configured to depress all of the plurality of plungers at a selectively variable rate. In other embodiments each of the plurality of actuators may comprise one of a plurality of fluid transmission lines having first and second ends, a first end of each of the plurality of fluid transmission lines being coupled to a respective one of the plurality of reservoirs. In other embodiments the device may comprise a fluid pressure source, and each of the plurality of actuators comprises a fluid coupling between the fluid pressure source and a respective one of the plurality of reservoirs. In further embodiments the fluid pressure source may comprise at least one of a compressor, a vacuum accumulator, a peristaltic pump, a master cylinder, a microfluidic pump, and a valve. In another embodiment, each of the plurality of needles may comprise a plurality of ports distributed along its length.

Modifying a target

[00492] In one aspect, the invention provides for methods of modifying a target polynucleotide in a eukaryotic cell, which may be *in vivo, ex vivo* or *in vitro*. In some embodiments, the method comprises sampling a cell or population of cells from a human or non-human animal, or a plant, and modifying the cell or cells. Culturing may occur at any stage *ex vivo*. The cell or cells may even be re-introduced into the non-human animal or plant. For re-introduced cells it is particularly preferred that the cells are stem cells.

[00493] In some embodiments, the method comprises allowing a CRISPR complex to bind to the target polynucleotide to effect cleavage of said target polynucleotide thereby modifying the target polynucleotide, wherein the CRISPR complex comprises a CRISPR enzyme complexed with a guide sequence hybridized to a target sequence within said target polynucleotide, wherein said guide sequence is linked to a tracr mate sequence which in turn hybridizes to a tracr sequence.

[00494] In one aspect, the invention provides a method of modifying expression of a polynucleotide in a eukaryotic cell. In some embodiments, the method comprises allowing a CRISPR complex to bind to the polynucleotide such that said binding results in increased or decreased expression of said polynucleotide; wherein the CRISPR complex comprises a CRISPR enzyme complexed with a guide sequence hybridized to a target sequence within said polynucleotide, wherein said guide sequence is linked to a tracr mate sequence which in turn hybridizes to a tracr sequence. Similar considerations and conditions apply as above for methods of modifying a target polynucleotide. In fact, these sampling, culturing and re-introduction options apply across the aspects of the present invention.

[00495] Indeed, in any aspect of the invention, the CRISPR complex may comprise a CRISPR enzyme complexed with a guide sequence hybridized to a target sequence, wherein said guide sequence may be linked to a tracr mate sequence which in turn may hybridize to a tracr sequence. Similar considerations and conditions apply as above for methods of modifying a target polynucleotide.

Kits

[00496] In one aspect, the invention provides kits containing any one or more of the elements disclosed in the above methods and compositions. Elements may be provided individually or in combinations, and may be provided in any suitable container, such as a vial, a bottle, or a tube. In some embodiments, the kit includes instructions in one or more languages, for example in more than one language.

[00497] In some embodiments, a kit comprises one or more reagents for use in a process utilizing one or more of the elements described herein. Reagents may be provided in any suitable container. For example, a kit may provide one or more reaction or storage buffers. Reagents may be provided in a form that is usable in a particular assay, or in a form that requires addition of one or more other components before use (e.g. in concentrate or lyophilized form). A buffer can be any buffer, including but not limited to a sodium carbonate buffer, a sodium bicarbonate buffer, a borate buffer, a Tris buffer, a MOPS buffer, a HEPES buffer, and combinations thereof. In some embodiments, the buffer is alkaline. In some embodiments, the buffer has a pH from about 7 to about 10. In some embodiments, the kit comprises one or more oligonucleotides corresponding to a guide sequence for insertion into a vector so as to operably link the guide sequence and a regulatory element. In some embodiments, the kit comprises a

homologous recombination template polynucleotide. In some embodiments, the kit comprises one or more of the vectors and/or one or more of the polynucleotides described herein. The kit may advantageously allows to provide all elements of the systems of the invention.

CRISPR complex

[00498] In one aspect, the invention provides methods for using one or more elements of a CRISPR system. The CRISPR complex of the invention provides an effective means for modifying a target polynucleotide. The CRISPR complex of the invention has a wide variety of utility including modifying (e.g., deleting, inserting, translocating, inactivating, activating) a target polynucleotide in a multiplicity of cell types. As such the CRISPR complex of the invention has a broad spectrum of applications in, e.g., gene therapy, drug screening, disease diagnosis, and prognosis. An exemplary CRISPR complex comprises a CRISPR enzyme complexed with a guide sequence hybridized to a target sequence within the target polynucleotide. The guide sequence is linked to a tracr mate sequence, which in turn hybridizes to a tracr sequence.

[00499] In one embodiment, this invention provides a method of cleaving a target polynucleotide. The method comprises modifying a target polynucleotide using a CRISPR complex that binds to the target polynucleotide and effect cleavage of said target polynucleotide. Typically, the CRISPR complex of the invention, when introduced into a cell, creates a break (e.g., a single or a double strand break) in the genome sequence. For example, the method can be used to cleave a disease gene in a cell.

[00500] The break created by the CRISPR complex can be repaired by a repair processes such as the error prone non-homologous end joining (NHEJ) pathway or the high fidelity homology-directed repair (HDR) (Fig. 29). During these repair process, an exogenous polynucleotide template can be introduced into the genome sequence. In some methods, the HDR process is used modify genome sequence. For example, an exogenous polynucleotide template comprising a sequence to be integrated flanked by an upstream sequence and a downstream sequence is introduced into a cell. The upstream and downstream sequences share sequence similarity with either side of the site of integration in the chromosome.

[00501] Where desired, a donor polynucleotide can be DNA, e.g., a DNA plasmid, a bacterial artificial chromosome (BAC), a yeast artificial chromosome (YAC), a viral vector, a linear piece

of DNA, a PCR fragment, a naked nucleic acid, or a nucleic acid complexed with a delivery vehicle such as a liposome or poloxamer.

[00502] The exogenous polynucleotide template comprises a sequence to be integrated (e.g., a mutated gene). The sequence for integration may be a sequence endogenous or exogenous to the cell. Examples of a sequence to be integrated include polynucleotides encoding a protein or a non-coding RNA (e.g., a microRNA). Thus, the sequence for integration may be operably linked to an appropriate control sequence or sequences. Alternatively, the sequence to be integrated may provide a regulatory function.

[00503] The upstream and downstream sequences in the exogenous polynucleotide template are selected to promote recombination between the chromosomal sequence of interest and the donor polynucleotide. The upstream sequence is a nucleic acid sequence that shares sequence similarity with the genome sequence upstream of the targeted site for integration. Similarly, the downstream sequence is a nucleic acid sequence that shares sequence similarity with the chromosomal sequence downstream of the targeted site of integration. The upstream and downstream sequences in the exogenous polynucleotide template can have 75%, 80%, 85%, 90%, 95%, or 100% sequence identity with the targeted genome sequence. Preferably, the upstream and downstream sequences in the exogenous polynucleotide template have about 95%, 96%, 97%, 98%, 99%, or 100% sequence identity with the targeted genome sequence. In some methods, the upstream and downstream sequences in the exogenous polynucleotide template have about 99% or 100% sequence identity with the targeted genome sequence.

[00504] An upstream or downstream sequence may comprise from about 20 bp to about 2500 bp, for example, about 50, 100, 200, 300, 400, 500, 600, 700, 800, 900, 1000, 1100, 1200, 1300, 1400, 1500, 1600, 1700, 1800, 1900, 2000, 2100, 2200, 2300, 2400, or 2500 bp. In some methods, the exemplary upstream or downstream sequence have about 200 bp to about 2000 bp, about 600 bp to about 1000 bp, or more particularly about 700 bp to about 1000 bp.

[00505] In some methods, the exogenous polynucleotide template may further comprise a marker. Such a marker may make it easy to screen for targeted integrations. Examples of suitable markers include restriction sites, fluorescent proteins, or selectable markers. The exogenous polynucleotide template of the invention can be constructed using recombinant techniques (see, for example, Sambrook et al., 2001 and Ausubel et al., 1996).

[00506] In an exemplary method for modifying a target polynucleotide by integrating an exogenous polynucleotide template, a double stranded break is introduced into the genome sequence by the CRISPR complex, the break is repaired via homologous recombination an exogenous polynucleotide template such that the template is integrated into the genome. The presence of a double-stranded break facilitates integration of the template.

[00507] In other embodiments, this invention provides a method of modifying expression of a polynucleotide in a eukaryotic cell. The method comprises increasing or decreasing expression of a target polynucleotide by using a CRISPR complex that binds to the polynucleotide.

[00508] In some methods, a target polynucleotide can be inactivated to effect the modification of the expression in a cell. For example, upon the binding of a CRISPR complex to a target sequence in a cell, the target polynucleotide is inactivated such that the sequence is not transcribed, the coded protein is not produced, or the sequence does not function as the wild-type sequence does. For example, a protein or microRNA coding sequence may be inactivated such that the protein is not produced.

[00509] In some methods, a control sequence can be inactivated such that it no longer functions as a control sequence. As used herein, "control sequence" refers to any nucleic acid sequence that effects the transcription, translation, or accessibility of a nucleic acid sequence. Examples of a control sequence include, a promoter, a transcription terminator, and an enhancer are control sequences.

[00510] The inactivated target sequence may include a deletion mutation (i.e., deletion of one or more nucleotides), an insertion mutation (i.e., insertion of one or more nucleotides), or a nonsense mutation (i.e., substitution of a single nucleotide for another nucleotide such that a stop codon is introduced). In some methods, the inactivation of a target sequence results in "knock-out" of the target sequence.

Disease models

[00511] A method of the invention may be used to create a plant, an animal or cell that may be used as a disease model. 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.

[00512] 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.

[00513] 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.

[00514] 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 a CRISPR enzyme, a guide sequence linked to a tracr mate sequence, and a tracr sequence; 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.

[00515] 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 CRISPR complex of the invention 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.

[00516] 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.

[00517] 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.

[00518] 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.

[00519] For purpose of this invention, amplification means any method employing a primer and a polymerase capable of replicating a target sequence with reasonable fidelity. Amplification may be carried out by natural or recombinant DNA polymerases such as TaqGold™, T7 DNA polymerase, Klenow fragment of E.coli DNA polymerase, and reverse transcriptase. A preferred amplification method is PCR. In particular, the isolated RNA can be subjected to a reverse transcription assay that is coupled with a quantitative polymerase chain reaction (RT-PCR) in order to quantify the expression level of a sequence associated with a signaling biochemical pathway.

[00520] Detection of the gene expression level can be conducted in real time in an amplification assay. In one aspect, the amplified products can be directly visualized with fluorescent DNA-binding agents including but not limited to DNA intercalators and DNA groove binders. Because the amount of the intercalators incorporated into the double-stranded DNA molecules is typically proportional to the amount of the amplified DNA products, one can conveniently determine the amount of the amplified products by quantifying the fluorescence of the intercalated dye using conventional optical systems in the art. DNA-binding dye suitable for this application include SYBR green, SYBR blue, DAPI, propidium iodine, Hoeste, SYBR gold, ethidium bromide, acridines, proflavine, acridine orange, acriflavine, fluorcoumanin, ellipticine, daunomycin, chloroquine, distamycin D, chromomycin, homidium, mithramycin, ruthenium polypyridyls, anthramycin, and the like.

[00521] In another aspect, other fluorescent labels such as sequence specific probes can be employed in the amplification reaction to facilitate the detection and quantification of the amplified products. Probe-based quantitative amplification relies on the sequence-specific detection of a desired amplified product. It utilizes fluorescent, target-specific probes (e.g., TaqMan® probes) resulting in increased specificity and sensitivity. Methods for performing probe-based quantitative amplification are well established in the art and are taught in U.S. Patent No. 5,210,015.

[00522] In yet another aspect, conventional hybridization assays using hybridization probes that share sequence homology with sequences associated with a signaling biochemical pathway can be performed. Typically, probes are allowed to form stable complexes with the sequences associated with a signaling biochemical pathway contained within the biological sample derived from the test subject in a hybridization reaction. It will be appreciated by one of skill in the art that where antisense is used as the probe nucleic acid, the target polynucleotides provided in the sample are chosen to be complementary to sequences of the antisense nucleic acids. Conversely, where the nucleotide probe is a sense nucleic acid, the target polynucleotide is selected to be complementary to sequences of the sense nucleic acid.

[00523] Hybridization can be performed under conditions of various stringency. Suitable hybridization conditions for the practice of the present invention are such that the recognition interaction between the probe and sequences associated with a signaling biochemical pathway is both sufficiently specific and sufficiently stable. Conditions that increase the stringency of a

hybridization reaction are widely known and published in the art. See, for example, (Sambrook, et al., (1989); Nonradioactive In Situ Hybridization Application Manual, Boehringer Mannheim, second edition). The hybridization assay can be formed using probes immobilized on any solid support, including but are not limited to nitrocellulose, glass, silicon, and a variety of gene arrays. A preferred hybridization assay is conducted on high-density gene chips as described in U.S. Patent No. 5,445,934.

[00524] For a convenient detection of the probe-target complexes formed during the hybridization assay, the nucleotide probes are conjugated to a detectable label. Detectable labels suitable for use in the present invention include any composition detectable by photochemical, biochemical, spectroscopic, immunochemical, electrical, optical or chemical means. A wide variety of appropriate detectable labels are known in the art, which include fluorescent or chemiluminescent labels, radioactive isotope labels, enzymatic or other ligands. In preferred embodiments, one will likely desire to employ a fluorescent label or an enzyme tag, such as digoxigenin, ß-galactosidase, urease, alkaline phosphatase or peroxidase, avidin/biotin complex.

[00525] The detection methods used to detect or quantify the hybridization intensity will typically depend upon the label selected above. For example, radiolabels may be detected using photographic film or a phosphoimager. Fluorescent markers may be detected and quantified using a photodetector to detect emitted light. Enzymatic labels are typically detected by providing the enzyme with a substrate and measuring the reaction product produced by the action of the enzyme on the substrate; and finally colorimetric labels are detected by simply visualizing the colored label.

[00526] An agent-induced change in expression of sequences associated with a signaling biochemical pathway can also be determined by examining the corresponding gene products. Determining the protein level typically involves a) contacting the protein contained in a biological sample with an agent that specifically bind to a protein associated with a signaling biochemical pathway; and (b) identifying any agent:protein complex so formed. In one aspect of this embodiment, the agent that specifically binds a protein associated with a signaling biochemical pathway is an antibody, preferably a monoclonal antibody.

[00527] The reaction is performed by contacting the agent with a sample of the proteins associated with a signaling biochemical pathway derived from the test samples under conditions that will allow a complex to form between the agent and the proteins associated with a signaling

biochemical pathway. The formation of the complex can be detected directly or indirectly according to standard procedures in the art. In the direct detection method, the agents are supplied with a detectable label and unreacted agents may be removed from the complex; the amount of remaining label thereby indicating the amount of complex formed. For such method, it is preferable to select labels that remain attached to the agents even during stringent washing conditions. It is preferable that the label does not interfere with the binding reaction. In the alternative, an indirect detection procedure may use an agent that contains a label introduced either chemically or enzymatically. A desirable label generally does not interfere with binding or the stability of the resulting agent:polypeptide complex. However, the label is typically designed to be accessible to an antibody for an effective binding and hence generating a detectable signal.

[00528] A wide variety of labels suitable for detecting protein levels are known in the art. Non-limiting examples include radioisotopes, enzymes, colloidal metals, fluorescent compounds, bioluminescent compounds, and chemiluminescent compounds.

[00529] The amount of agent:polypeptide complexes formed during the binding reaction can be quantified by standard quantitative assays. As illustrated above, the formation of agent:polypeptide complex can be measured directly by the amount of label remained at the site of binding. In an alternative, the protein associated with a signaling biochemical pathway is tested for its ability to compete with a labeled analog for binding sites on the specific agent. In this competitive assay, the amount of label captured is inversely proportional to the amount of protein sequences associated with a signaling biochemical pathway present in a test sample.

[00530] A number of techniques for protein analysis based on the general principles outlined above are available in the art. They include but are not limited to radioimmunoassays, ELISA (enzyme linked immunoradiometric assays), "sandwich" immunoassays, immunoradiometric assays, in situ immunoassays (using e.g., colloidal gold, enzyme or radioisotope labels), western blot analysis, immunoprecipitation assays, immunofluorescent assays, and SDS-PAGE.

[00531] Antibodies that specifically recognize or bind to proteins associated with a signaling biochemical pathway are preferable for conducting the aforementioned protein analyses. Where desired, antibodies that recognize a specific type of post-translational modifications (e.g., signaling biochemical pathway inducible modifications) can be used. Post-translational modifications include but are not limited to glycosylation, lipidation, acetylation, and

phosphorylation. These antibodies may be purchased from commercial vendors. For example, anti-phosphotyrosine antibodies that specifically recognize tyrosine-phosphorylated proteins are available from a number of vendors including Invitrogen and Perkin Elmer. Anti-phosphotyrosine antibodies are particularly useful in detecting proteins that are differentially phosphorylated on their tyrosine residues in response to an ER stress. Such proteins include but are not limited to eukaryotic translation initiation factor 2 alpha (eIF-2α). Alternatively, these antibodies can be generated using conventional polyclonal or monoclonal antibody technologies by immunizing a host animal or an antibody-producing cell with a target protein that exhibits the desired post-translational modification.

[00532] In practicing the subject method, it may be desirable to discern the expression pattern of an protein associated with a signaling biochemical pathway in different bodily tissue, in different cell types, and/or in different subcellular structures. These studies can be performed with the use of tissue-specific, cell-specific or subcellular structure specific antibodies capable of binding to protein markers that are preferentially expressed in certain tissues, cell types, or subcellular structures.

[00533] An altered expression of a gene associated with a signaling biochemical pathway can also be determined by examining a change in activity of the gene product relative to a control cell. The assay for an agent-induced change in the activity of a protein associated with a signaling biochemical pathway will dependent on the biological activity and/or the signal transduction pathway that is under investigation. For example, where the protein is a kinase, a change in its ability to phosphorylate the downstream substrate(s) can be determined by a variety of assays known in the art. Representative assays include but are not limited to immunoblotting and immunoprecipitation with antibodies such as anti-phosphotyrosine antibodies that recognize phosphorylated proteins. In addition, kinase activity can be detected by high throughput chemiluminescent assays such as AlphaScreen™ (available from Perkin Elmer) and eTag™ assay (Chan-Hui, et al. (2003) Clinical Immunology 111: 162-174).

[00534] Where the protein associated with a signaling biochemical pathway is part of a signaling cascade leading to a fluctuation of intracellular pH condition, pH sensitive molecules such as fluorescent pH dyes can be used as the reporter molecules. In another example where the protein associated with a signaling biochemical pathway is an ion channel, fluctuations in membrane potential and/or intracellular ion concentration can be monitored. A number of

commercial kits and high-throughput devices are particularly suited for a rapid and robust screening for modulators of ion channels. Representative instruments include FLIPRTM (Molecular Devices, Inc.) and VIPR (Aurora Biosciences). These instruments are capable of detecting reactions in over 1000 sample wells of a microplate simultaneously, and providing real-time measurement and functional data within a second or even a minisecond.

[00535] 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.

[00536] 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).

[00537] 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.

[00538] The target polynucleotide of a CRISPR complex can be any polynucleotide endogenous or exogenous to the eukaryotic cell. For example, the target polynucleotide can be a polynucleotide residing in the nucleus of the eukaryotic cell. The target polynucleotide can be a sequence coding a gene product (e.g., a protein) or a non-coding sequence (e.g., a regulatory polynucleotide or a junk DNA). Without wishing to be bound by theory, it is believed that the target sequence should be associated with a PAM (protospacer adjacent motif); that is, a short sequence recognized by the CRISPR complex. The precise sequence and length requirements for the PAM differ depending on the CRISPR enzyme used, but PAMs are typically 2-5 base pair sequences adjacent the protospacer (that is, the target sequence) Examples of PAM sequences are given in the examples section below, and the skilled person will be able to identify further PAM sequences for use with a given CRISPR enzyme.

[00539] The target polynucleotide of a CRISPR complex may include a number of disease-associated genes and polynucleotides as well as signaling biochemical pathway-associated genes and polynucleotides as listed in US provisional patent applications 61/736,527 and 61/748,427 having Broad reference BI-2011/008/WSGR Docket No. 44063-701.101 and BI-2011/008/WSGR Docket No. 44063-701.102 respectively, both entitled SYSTEMS METHODS AND COMPOSITIONS FOR SEQUENCE MANIPULATION filed on December 12, 2012 and January 2, 2013, respectively, the contents of all of which are herein incorporated by reference in their entirety.

[00540] 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.

[00541] Examples of disease-associated genes and polynucleotides are listed in Tables A and B. Disease specific information is available from McKusick-Nathans Institute of Genetic Medicine, Johns Hopkins University (Baltimore, Md.) and National Center for Biotechnology Information, National Library of Medicine (Bethesda, Md.), available on the World Wide Web. Examples of signaling biochemical pathway-associated genes and polynucleotides are listed in Table C.

[00542] Mutations in these genes and pathways can result in production of improper proteins or proteins in improper amounts which affect function. Further examples of genes, diseases and proteins are hereby incorporated by reference from US Provisional application 61/736,527 filed December 12, 2012. Such genes, proteins and pathways may be the target polynucleotide of a CRISPR complex.

Table A

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

	(Alzheimer's); Atxn7; Atxn10
Fragile X Syndrome	FMR2; FXR1; FXR2; mGLUR5
Secretase Related	APH-1 (alpha and beta); Presenilin (Psen1); nicastrin
Disorders	(Ncstn); PEN-2
Others	Nos1; Parp1; Nat1; Nat2
Prion - related disorders	Prp
ALS	SOD1; ALS2; STEX; FUS; TARDBP; VEGF (VEGF-a;
	VEGF-b; VEGF-c)
Drug addiction	Prkce (alcohol); Drd2; Drd4; ABAT (alcohol); GRIA2;
	Grm5; Grin1; Htr1b; Grin2a; Drd3; Pdyn; Gria1 (alcohol)
Autism	Mecp2; BZRAP1; MDGA2; Sema5A; Neurexin 1; Fragile X
	(FMR2 (AFF2); FXR1; FXR2; Mglur5)
Alzheimer's Disease	E1; CHIP; UCH; UBB; Tau; LRP; PICALM; Clusterin; PS1;
	SORL1; CR1; Vldlr; Uba1; Uba3; CHIP28 (Aqp1,
	Aquaporin 1); Uchl1; Uchl3; APP
Inflammation	IL-10; IL-1 (IL-1a; IL-1b); IL-13; IL-17 (IL-17a (CTLA8); IL-
	17b; IL-17c; IL-17d; IL-17f); Il-23; Cx3cr1; ptpn22; TNFa;
	NOD2/CARD15 for IBD; IL-6; IL-12 (IL-12a; IL-12b);
	CTLA4; Cx3cl1
Parkinson's Disease	x-Synuclein; DJ-1; LRRK2; Parkin; PINK1

Table B:

Blood and coagulation diseases and disorders	Anemia (CDAN1, CDA1, RPS19, DBA, PKLR, PK1, NT5C3, UMPH1, PSN1, RHAG, RH50A, NRAMP2, SPTB, ALAS2, ANH1, ASB, ABCB7, ABC7, ASAT); Bare lymphocyte syndrome (TAPBP, TPSN, TAP2, ABCB3, PSF2, RING11, MHC2TA, C2TA, RFX5, RFXAP, RFX5), Bleeding disorders (TBXA2R, P2RX1, P2X1); Factor H and factor H-like 1 (HF1, CFH, HUS); Factor V and factor VIII (MCFD2); Factor VII deficiency (F7); Factor X deficiency (F10); Factor XI deficiency (F11); Factor XII deficiency (F12, HAF); Factor XIIIA deficiency (F13A1, F13A); Factor XIIIB deficiency (F13B); Fanconi anemia (FANCA, FACA, FA1, FA, FAA, FAAP95, FAAP90, FLJ34064, FANCB, FANCC, FACC, BRCA2, FANCD1, FANCD2, FANCD, FACD, FAD, FANCE, FACE, FANCF, XRCC9, FANCG, BRIP1, BACH1, FANCJ, PHF9, FANCL, FANCM, KIAA1596); Hemophagocytic lymphohistiocytosis disorders (PRF1, HPLH2, UNC13D, MUNC13-4, HPLH3, HLH3, FHL3); Hemophilia A (F8, F8C, HEMA); Hemophilia B (F9, HEMB), Hemorrhagic disorders (PI, ATT, F5); Leukocyde deficiencies and disorders (ITGB2, CD18, LCAMB, LAD, EIF2B1, EIF2BA, EIF2B2, EIF2B3, EIF2B5, LVWM, CACH, CLE, EIF2B4); Sickle cell anemia (HBB); Thalassemia (HBA2, HBB, HBD, LCRB, HBA1).
Cell dysregulation	B-cell non-Hodgkin lymphoma (BCL7A, BCL7); Leukemia (TAL1,

and oncology diseases and disorders	TCL5, SCL, TAL2, FLT3, NBS1, NBS, ZNFN1A1, IK1, LYF1, HOXD4, HOX4B, BCR, CML, PHL, ALL, ARNT, KRAS2, RASK2, GMPS, AF10, ARHGEF12, LARG, KIAA0382, CALM, CLTH, CEBPA, CEBP, CHIC2, BTL, FLT3, KIT, PBT, LPP, NPM1, NUP214, D9S46E, CAN, CAIN, RUNX1, CBFA2, AML1, WHSC1L1, NSD3, FLT3, AF1Q, NPM1, NUMA1, ZNF145, PLZF, PML, MYL, STAT5B, AF10, CALM, CLTH, ARL11, ARLTS1, P2RX7, P2X7, BCR, CML, PHL, ALL, GRAF, NF1, VRNF, WSS, NFNS, PTPN11, PTP2C, SHP2, NS1, BCL2, CCND1, PRAD1, BCL1, TCRA, GATA1, GF1, ERYF1, NFE1, ABL1, NQO1, DIA4, NMOR1, NUP214, D9S46E, CAN, CAIN).
Inflammation and immune related diseases and disorders	AIDS (KIR3DL1, NKAT3, NKB1, AMB11, KIR3DS1, IFNG, CXCL12, SDF1); Autoimmune lymphoproliferative syndrome (TNFRSF6, APT1, FAS, CD95, ALPS1A); Combined immunodeficiency, (IL2RG, SCIDX1, SCIDX, IMD4); HIV-1 (CCL5, SCYA5, D17S136E, TCP228), HIV susceptibility or infection (IL10, CSIF, CMKBR2, CCR2, CMKBR5, CCCKR5 (CCR5)); Immunodeficiencies (CD3E, CD3G, AICDA, AID, HIGM2, TNFRSF5, CD40, UNG, DGU, HIGM4, TNFSF5, CD40LG, HIGM1, IGM, FOXP3, IPEX, AIID, XPID, PIDX, TNFRSF14B, TACI); Inflammation (IL-10, IL-1 (IL-1a, IL-1b), IL-13, IL-17 (IL-17a (CTLA8), IL-17b, IL-17c, IL-17d, IL-17f), Il-23, Cx3cr1, ptpn22, TNFa, NOD2/CARD15 for IBD, IL-6, IL-12 (IL-12a, IL-12b), CTLA4, Cx3cl1); Severe combined immunodeficiencies (SCIDs)(JAK3, JAKL, DCLRE1C, ARTEMIS, SCIDA, RAG1, RAG2, ADA, PTPRC, CD45, LCA, IL7R, CD3D, T3D, IL2RG, SCIDX1, SCIDX, IMD4).
Metabolic, liver, kidney and protein diseases and disorders	Amyloid neuropathy (TTR, PALB); Amyloidosis (APOA1, APP, AAA, CVAP, AD1, GSN, FGA, LYZ, TTR, PALB); Cirrhosis (KRT18, KRT8, CIRH1A, NAIC, TEX292, KIAA1988); Cystic fibrosis (CFTR, ABCC7, CF, MRP7); Glycogen storage diseases (SLC2A2, GLUT2, G6PC, G6PT, G6PT1, GAA, LAMP2, LAMPB, AGL, GDE, GBE1, GYS2, PYGL, PFKM); Hepatic adenoma, 142330 (TCF1, HNF1A, MODY3), Hepatic failure, early onset, and neurologic disorder (SCOD1, SCO1), Hepatic lipase deficiency (LIPC), Hepatoblastoma, cancer and carcinomas (CTNNB1, PDGFRL, PDGRL, PRLTS, AXIN1, AXIN, CTNNB1, TP53, P53, LFS1, IGF2R, MPRI, MET, CASP8, MCH5; Medullary cystic kidney disease (UMOD, HNFJ, FJHN, MCKD2, ADMCKD2); Phenylketonuria (PAH, PKU1, QDPR, DHPR, PTS); Polycystic kidney and hepatic disease (FCYT, PKHD1, ARPKD, PKD1, PKD2, PKD4, PKDTS, PRKCSH, G19P1, PCLD, SEC63).
Muscular / Skeletal diseases and disorders	Becker muscular dystrophy (DMD, BMD, MYF6), Duchenne Muscular Dystrophy (DMD, BMD); Emery-Dreifuss muscular dystrophy (LMNA, LMN1, EMD2, FPLD, CMD1A, HGPS, LGMD1B, LMNA, LMN1, EMD2, FPLD, CMD1A); Facioscapulohumeral muscular dystrophy (FSHMD1A, FSHD1A); Muscular dystrophy (FKRP, MDC1C,

	LGMD2I, LAMA2, LAMM, LARGE, KIAA0609, MDC1D, FCMD, TTID, MYOT, CAPN3, CANP3, DYSF, LGMD2B, SGCG, LGMD2C, DMDA1, SCG3, SGCA, ADL, DAG2, LGMD2D, DMDA2, SGCB, LGMD2E, SGCD, SGD, LGMD2F, CMD1L, TCAP, LGMD2G, CMD1N, TRIM32, HT2A, LGMD2H, FKRP, MDC1C, LGMD2I, TTN, CMD1G, TMD, LGMD2J, POMT1, CAV3, LGMD1C, SEPN1, SELN, RSMD1, PLEC1, PLTN, EBS1); Osteopetrosis (LRP5, BMND1, LRP7, LR3, OPPG, VBCH2, CLCN7, CLC7, OPTA2, OSTM1, GL, TCIRG1, TIRC7, OC116, OPTB1); Muscular atrophy (VAPB, VAPC, ALS8, SMN1, SMA1, SMA2, SMA3, SMA4, BSCL2, SPG17, GARS, SMAD1, CMT2D, HEXB, IGHMBP2, SMUBP2, CATF1, SMARD1).
Neurological and neuronal diseases and disorders	ALS (SOD1, ALS2, STEX, FUS, TARDBP, VEGF (VEGF-a, VEGF-b, VEGF-c); Alzheimer disease (APP, AAA, CVAP, AD1, APOE, AD2, PSEN2, AD4, STM2, APBB2, FE65L1, NOS3, PLAU, URK, ACE, DCP1, ACE1, MPO, PACIP1, PAXIP1L, PTIP, A2M, BLMH, BMH, PSEN1, AD3); Autism (Mecp2, BZRAP1, MDGA2, Sema5A, Neurexin 1, GLO1, MECP2, RTT, PPMX, MRX16, MRX79, NLGN3, NLGN4, KIAA1260, AUTSX2); Fragile X Syndrome (FMR2, FXR1, FXR2, mGLUR5); Huntington's disease and disease like disorders (HD, IT15, PRNP, PRIP, JPH3, JP3, HDL2, TBP, SCA17); Parkinson disease (NR4A2, NURR1, NOT, TINUR, SNCAIP, TBP, SCA17, SNCA, NACP, PARK1, PARK4, DJ1, PARK7, LRRK2, PARK8, PINK1, PARK6, UCHL1, PARK5, SNCA, NACP, PARK1, PARK4, PRKN, PARK2, PDJ, DBH, NDUFV2); Rett syndrome (MECP2, RTT, PPMX, MRX16, MRX79, CDKL5, STK9, MECP2, RTT, PPMX, MRX16, MRX79, x-Synuclein, DJ-1); Schizophrenia (Neuregulin1 (Nrg1), Erb4 (receptor for Neuregulin), Complexin1 (Cplx1), Tph1 Tryptophan hydroxylase, Tph2, Tryptophan hydroxylase 2, Neurexin 1, GSK3, GSK3a, GSK3b, 5-HTT (Slc6a4), COMT, DRD (Drd1a), SLC6A3, DAOA, DTNBP1, Dao (Dao1)); Secretase Related Disorders (APH-1 (alpha and beta), Presenilin (Psen1), nicastrin, (Ncstn), PEN-2, Nos1, Parp1, Nat1, Nat2); Trinucleotide Repeat Disorders (HTT (Huntington's Dx), SBMA/SMAX1/AR (Kennedy's Dx), FXN/X25 (Friedrich's Ataxia), ATX3 (Machado- Joseph's Dx), ATXN1 and ATXN2 (spinocerebellar ataxias), DMPK (myotonic dystrophy), Atrophin-1 and Atn1 (DRPLA Dx), CBP (Creb-BP - global instability), VLDLR (Alzheimer's), Atxn7, Atxn10).
Occular diseases and disorders	Age-related macular degeneration (Aber, 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,

DEMANDE OU BREVET VOLUMINEUX

LA PRÉSENTE PARTIE DE CETTE DEMANDE OU CE BREVET COMPREND PLUS D'UN TOME.

CECI EST LE TOME 1 DE 2
CONTENANT LES PAGES 1 À 179

NOTE : Pour les tomes additionels, veuillez contacter le Bureau canadien des brevets

JUMBO APPLICATIONS/PATENTS

THIS SECTION OF THE APPLICATION/PATENT CONTAINS MORE THAN ONE VOLUME

THIS IS VOLUME 1 OF 2
CONTAINING PAGES 1 TO 179

NOTE: For additional volumes, please contact the Canadian Patent Office

NOM DU FICHIER / FILE NAME :

NOTE POUR LE TOME / VOLUME NOTE:

WHAT IS CLAIMED IS:

1. A method of modifying an organism or a non-human organism by manipulation of a target sequence in a genomic locus of interest comprising

delivering a non-naturally occurring or engineered composition comprising :

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

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

(b) a tracr mate sequence, and

(c) a tracr sequence, and

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

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

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

wherein the CRISPR complex comprises the CRISPR enzyme complexed with (1) the guide sequence that is hybridized to the target sequence, and (2) the tracr mate sequence that is hybridized to the tracr sequence and the polynucleotide sequence encoding a CRISPR enzyme is DNA or RNA,

or

(B) I. polynucleotides comprising:

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

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

II. a polynucleotide sequence encoding a CRISPR enzyme, and

III. a polynucleotide sequence comprising a tracr sequence,

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

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

hybridized to the tracr sequence, and the polynucleotide sequence encoding a CRISPR enzyme is DNA or RNA.

2. The method of claim 1, wherein any or all of the polynucleotide sequence encoding a CRISPR enzyme, guide sequence, tracr mate sequence or tracr sequence, is/are RNA.

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

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

5. A method of modifying an organism or a non-human organism by manipulation of a target sequence in a genomic locus of interest comprising

delivering a non-naturally occurring or engineered composition comprising a viral vector system comprising one or more viral vectors operably encoding a composition for expression thereof, wherein the composition comprises:

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

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

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

(b) a tracr mate sequence, and

(c) a tracr sequence, and

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

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

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

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

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

or

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

I. a first regulatory element operably linked to

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

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

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

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

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

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

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

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

7. A method of treating or inhibiting a condition caused by a defect in a target sequence in a genomic locus of interest in a subject or a non-human subject in need thereof comprising modifying the subject or a non-human subject by manipulation of the target sequence and wherein the condition is susceptible to treatment or inhibition by manipulation of the target sequence comprising providing treatment comprising:

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

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

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

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

(b) a tracr mate sequence, and

(c) a tracr sequence, and

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

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

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

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

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

or

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

I. a first regulatory element operably linked to

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

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

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

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

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

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

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

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

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

10. The method of any preceding claim wherein the organism or subject is a eukaryote.

11. The method of claim 10 wherein the organism or subject is a non-human eukaryote.

12. The method of any of claims 1 to 11 wherein the organism or subject is a mammal or a non-human mammal.

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

14. The method according to any preceding claim wherein the CRISPR enzyme is a Cas9.

15. The method according to any preceding claim wherein expression of the guide sequence is under the control of the T7 promoter and is driven by the expression of T7 polymerase.

16. A method of delivering a CRISPR enzyme of any preceding claim, comprising delivering to a cell mRNA encoding the CRISPR enzyme.

17. The method of any one of claims 1 to 16, wherein the polynucleotide or enzyme coding sequence encoding the CRISPR enzyme is delivered to the cell by delivering mRNA encoding the CRISPR enzyme to the cell.

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

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

20. The method of claim 18 or 19 wherein the AAV or lentivirus rep and/or cap obligatory for replication and packaging of the AAV or lentivirus are supplied by transfecting the cells with helper plasmid(s) or helper virus(es).

21. The method of claim 20 wherein the helper virus is a poxvirus, adenovirus, lentivirus, herpesvirus or baculovirus.

22. The method of claim 21 wherein the poxvirus is a vaccinia virus.

23. The method of any of claims 18 to 22 wherein the cells are mammalian cells.

24. The method of any of claims 18 to 22 wherein the cells are insect cells and the helper virus (where present) is baculovirus.

25. The method of any of claims 1 to 15 wherein the target sequence is flanked at its 3' end or followed by 5'-NRG (where N is any Nucleotide), or where the CRISPR enzyme is (or is derived from) a genus belonging to the group consisting of *Corynebacter, Sutterella, Legionella, Treponema, Filifactor, Eubacterium, Streptococcus, Lactobacillus, Mycoplasma, Bacteroides, Flaviivola, Flavobacterium, Sphaerochaeta, Azospirillum, Gluconacetobacter, Neisseria, Roseburia, Parvibaculum, Staphylococcus, Nitratifractor, Mycoplasma* and *Campylobacter*..

26. A composition as defined in any of claims 1-25 for use in medicine or in therapy.

27. A composition as defined in any of claims 1-25 for use in a method of modifying an organism or a non-human organism by manipulation of a target sequence in a genomic locus of interest or in a method of treating or inhibiting a condition caused by a defect in a target sequence in a genomic locus of interest.

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

29. Use of a composition as defined in any of claims 1-25 in the manufacture of a medicament for *ex vivo* gene or genome editing or for use in a method of modifying an organism or a non-human organism by manipulation of a target sequence in a genomic locus of interest or in a method of treating or inhibiting a condition caused by a defect in a target sequence in a genomic locus of interest.

30. A composition comprising:

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

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

(b) a tracr mate sequence, and

(c) a tracr sequence, and

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

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

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

wherein the CRISPR complex comprises the CRISPR enzyme complexed with (1) the guide sequence that is hybridized to the target sequence, and (2) the tracr mate sequence that is hybridized to the tracr sequence and the polynucleotide sequence encoding a CRISPR enzyme is DNA or RNA,

or

(B) I. polynucleotides comprising:

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

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

II. a polynucleotide sequence encoding a CRISPR enzyme, and

III. a polynucleotide sequence comprising a tracr sequence,

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

wherein the CRISPR complex comprises the CRISPR enzyme complexed with (1) the guide sequence that is hybridized to the target sequence, and (2) the tracr mate sequence that is hybridized to the tracr sequence, and the polynucleotide sequence encoding a CRISPR enzyme is DNA or RNA;

for use in medicine or therapy; or for use in a method of modifying an organism or a non-human organism by manipulation of a target sequence in a genomic locus of interest; or for use in a method of treating or inhibiting a condition caused by a defect in a target sequence in a genomic locus of interest; or for use in *ex vivo* gene or genome editing.

31. The composition of claim 30, wherein the polynucleotides are comprised within a vector system comprising one or more vectors.

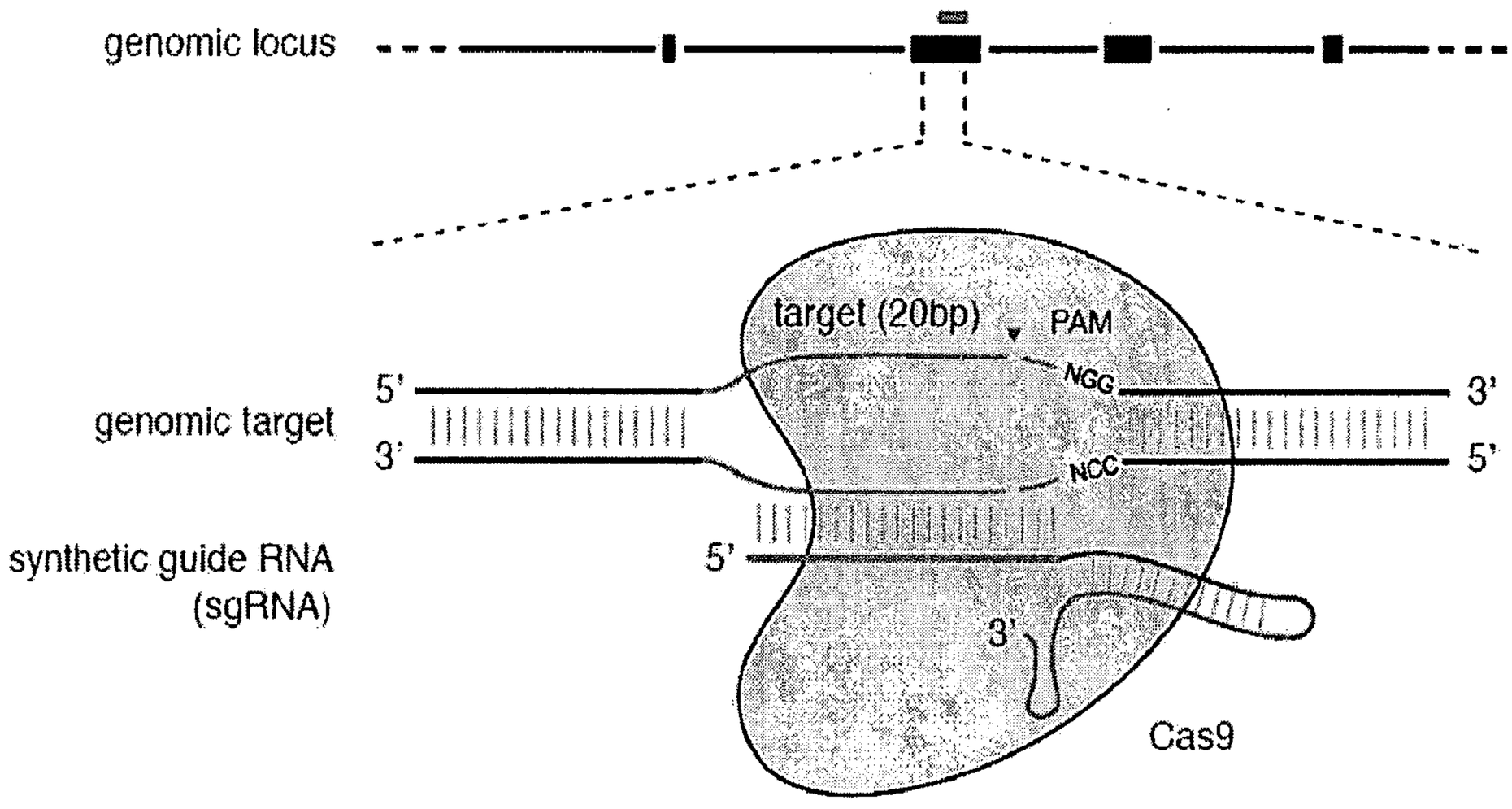

genomic locus
target (20bp)
PAM
NGG
NCC
5'
3'
genomic target
3'
5'
synthetic guide RNA
(sgRNA)
5'
3'
Cas9

FIG. 1

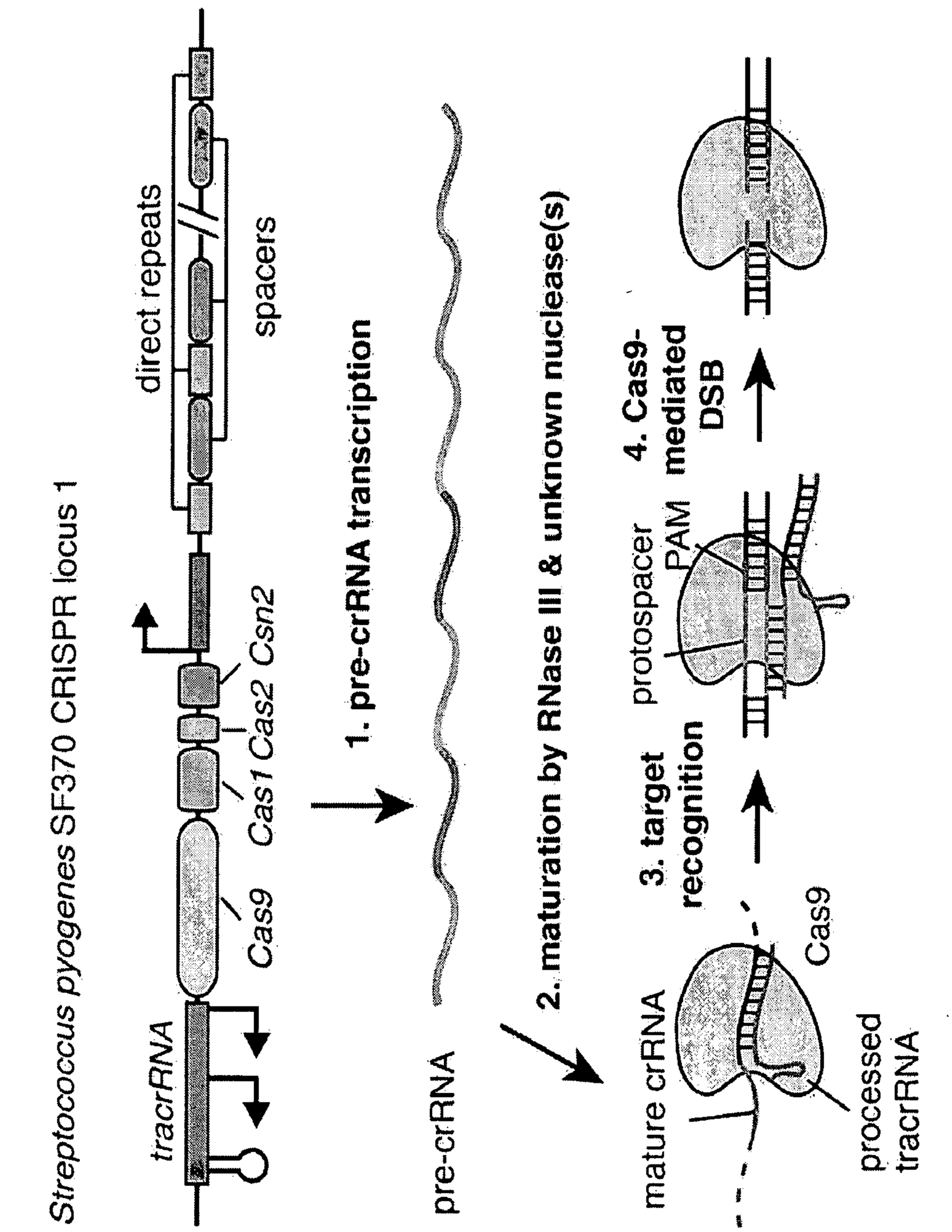

Streptococcus pyogenes SF370 CRISPR locus 1
direct repeats
spacers
tracrRNA
Cas9
Cas1
Cas2
Csn2
1. pre-crRNA transcription
pre-crRNA
2. maturation by RNase III & unknown nuclease(s)
mature crRNA
3. target recognition
protospacer
PAM
4. Cas9-mediated DSB
Cas9
processed tracrRNA

FIG. 2A

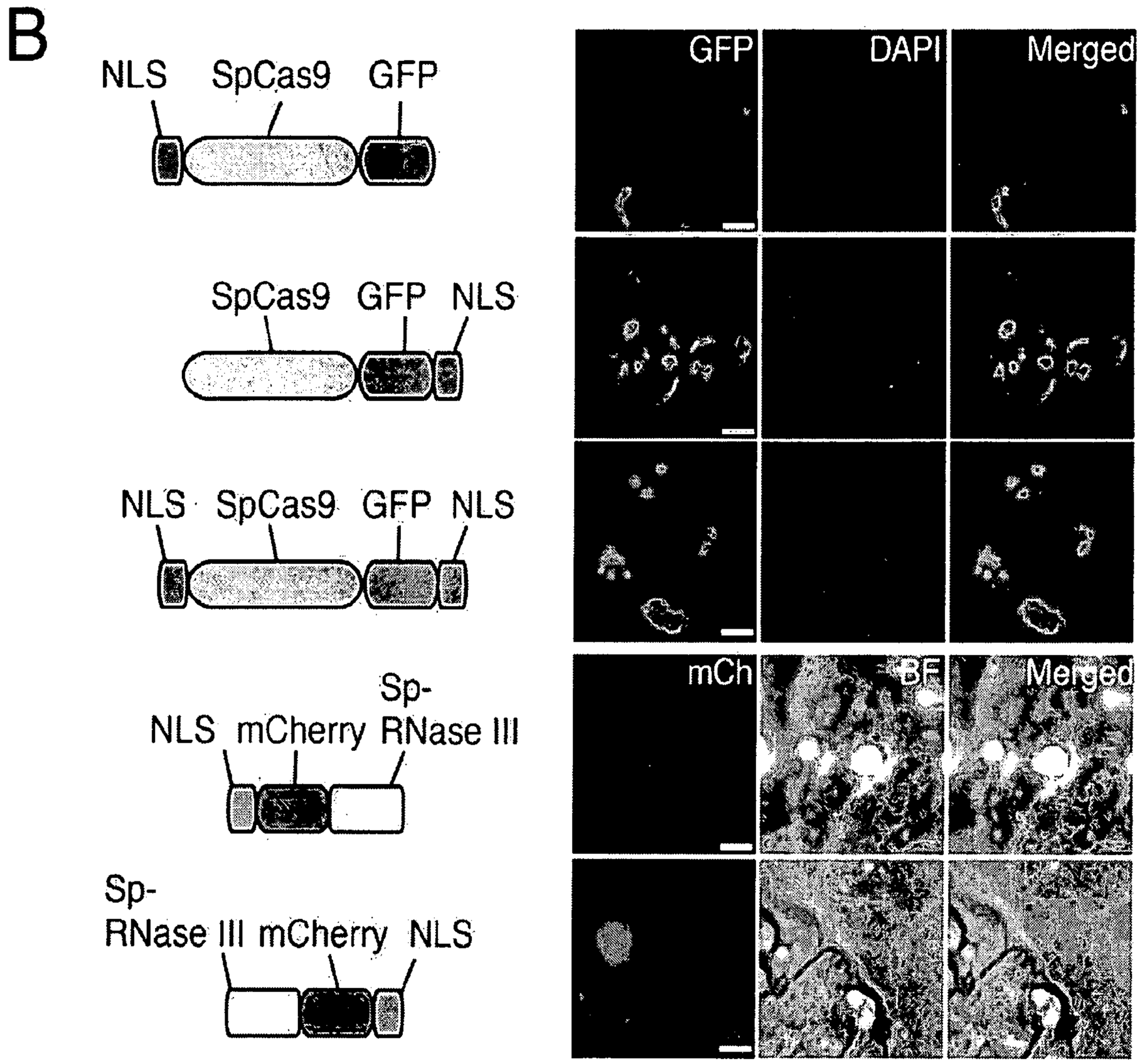

B
NLS SpCas9 GFP
SpCas9 GFP NLS
NLS SpCas9 GFP NLS
Sp-
NLS mCherry RNase III
Sp-
RNase III mCherry NLS
GFP
DAPI
Merged
mCh
BF
Merged

FIG. 2B

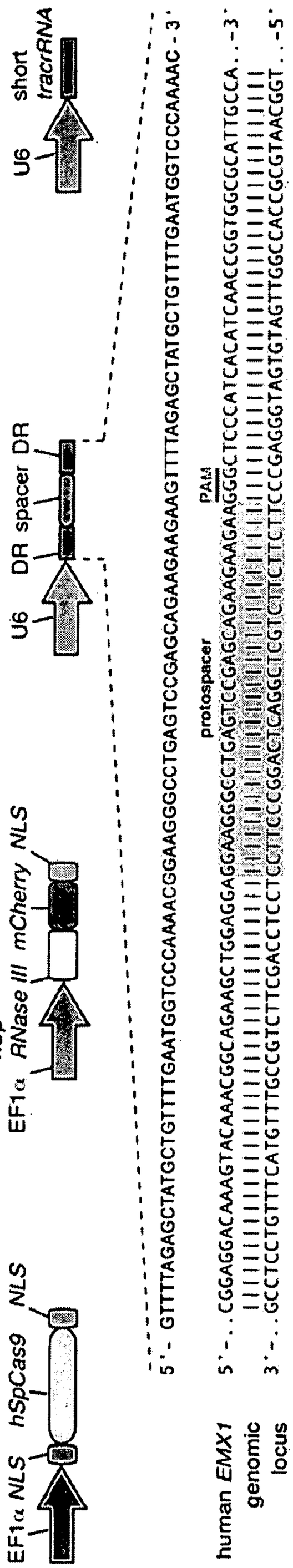

U6
short tracrRNA
5'- GTTTTAGAGCTATGCTGTTTTGAATGGTCCCAAAAC-3'
3'-...CGGAGGACAAAAGTACAAAACGGCAGAAGCTGGAGGAAGGGCCTGAGTCCGAGCAGAAGAAGAAGGGCTCCCATCACATCAACCGGTGGCGCATTGCCA...-3'
human EMX1 genomic locus
5'-...GCCTCCTGTTTTCATGTTTTGCCGTCTTCGACCTCCTCGTTCCCGAGGGTAGTGTAGTTGGCCACCGCGTAACGGT...-5'
protospacer
PAM
U6
DR spacer DR
5'- GTTTTAGAGCTATGCTGTTTTGAATGGTCCCAAAACGGAAGGGCCTGAGTCCGAGCAGAAGAAGAAGGGCTCCCATCACATCAACCGGTGGCGCATTGCCA...-3'
3'-...GCCTCCTGTTTTCATGTTTTGCCGTCTTCGACCTCCTCGTTCCCGAGGGTAGTGTAGTTGGCCACCGCGTAACGGT...-5'
hSp-
EF1α
RNase III
mCherry
NLS
5'- GTTTTAGAGCTATGCTGTTTTGAATGGTCCCAAAACGGAAGGGCCTGAGTCCGAGCAGAAGAAGAAGGGCTCCCATCACATCAACCGGTGGCGCATTGCCA...-3'
3'-...GCCTCCTGTTTTCATGTTTTGCCGTCTTCGACCTCCTCGTTCCCGAGGGTAGTGTAGTTGGCCACCGCGTAACGGT...-5'
C
EF1α NLS
hSpCas9
NLS
5'- GTTTTAGAGCTATGCTGTTTTGAATGGTCCCAAAACGGAAGGGCCTGAGTCCGAGCAGAAGAAGAAGGGCTCCCATCACATCAACCGGTGGCGCATTGCCA...-3'
3'-...GCCTCCTGTTTTCATGTTTTGCCGTCTTCGACCTCCTCGTTCCCGAGGGTAGTGTAGTTGGCCACCGCGTAACGGT...-5'
human EMX1 genomic locus

FIG. 2C

D

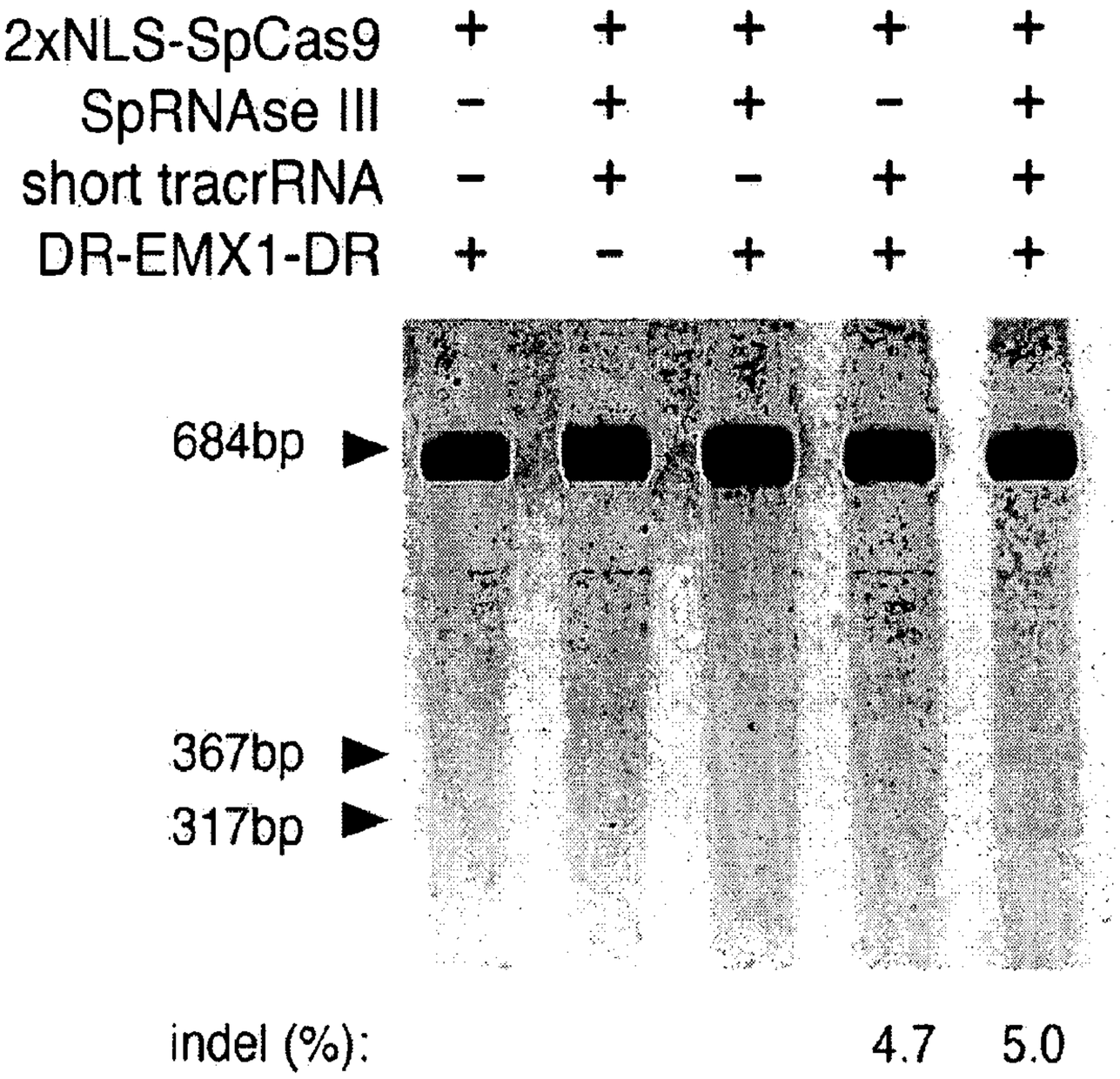

2xNLS-SpCas9 + + + + +
SpRNAse III - + + - +
short tracrRNA - + - + +
DR-EMX1-DR + - + + +
684bp
367bp
317bp
indel (%): 4.7 5.0

FIG. 2D

E

Target locus 5'-...AGCTGGAGGAGGAAGGGCCTGAGTCCGAGCAGAAGAAGGGCTCCCA...-3'

crRNA 3'-...TCGACCTCCTCCTTCCCGGACTCAGGCTCGTCTTCTTCCCGAGGGT...-5'

indel 5'- GAGUCCGAGCAGAAGAAGAAGUUUUAGAG...-3'

PAM

AGCTGGAGGAGGAAGGGCCTGAGTCCGAGCAGAAGAAGA–GAAGGGCTCCCA...-3'

F

EMX1 protospacer target (mutation in 5 of 43 sequenced clones = 11.6%)

human	5'-...CTGGAGGAGGAAGGGCCTGAGTCCGAGCAGAAGAAGGGCTCCCATCACAT...-3'
WT	CTGGAGGAGGAAGGGCCTGAGTCCGAGCAGAAGAAG--AGAAGGGCTCCCATCACAT
Δ1	CTGGAGGAGGAAGGGCCTGAGTCCGAGCAGAAGAAGAAGGGCTCCCATCACAT
+1	CTGGAGGAGGAAGGGCCTGAGTCCGAGCAGAAG----AAGGGCTCCCATCACAT
Δ3	CTGGAGGAGGAAGGGCCTGAGTCCGAGCAGAAG---------GGCTCCCATCACAT
m1, Δ6	CTGGAGGAGGAAGGGCCTGAGCCCGAGCAGAAG----------GGCTCCCATCACAT

FIG. 2E-F

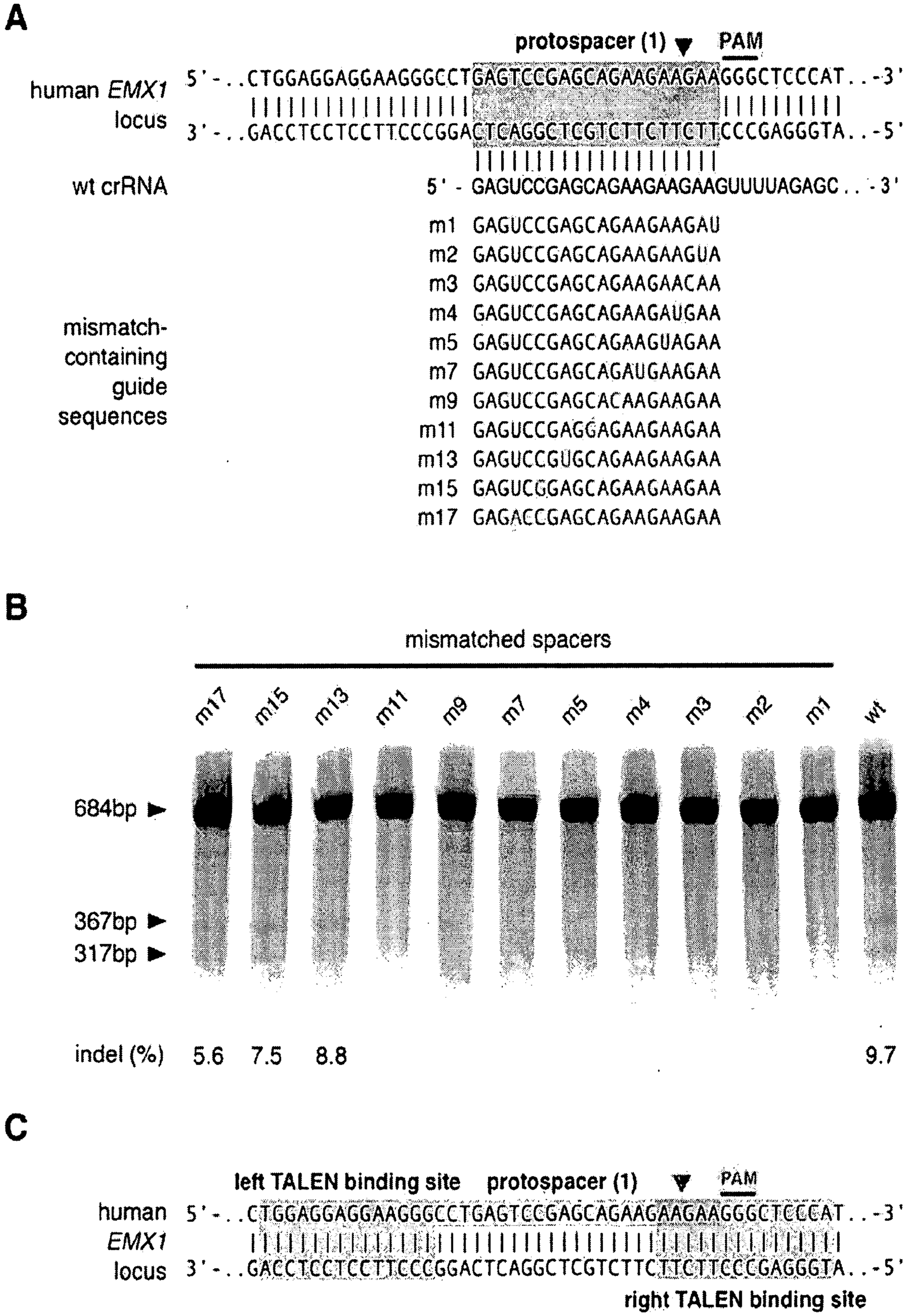

A
protospacer (1)
PAM
human EMX1 locus
5'-..CTGGAGGAGGAAGGGCCTGAGTCCGAGCAGAAGAAGAAGGGCTCCCAT..-3'
3'-..GACCTCCTCCTTCCCGGACTCAGGCTCGTCTTCTTCTTCCCGAGGGTA..-5'
wt crRNA
5' - GAGUCCGAGCAGAAGAAGAAGUUUUAGAGC..-3'
mismatch-containing guide sequences
m1 GAGUCCGAGCAGAAGAAGAU
m2 GAGUCCGAGCAGAAGAAGUA
m3 GAGUCCGAGCAGAAGAACAA
m4 GAGUCCGAGCAGAAGAUGAA
m5 GAGUCCGAGCAGAAGUAGAA
m7 GAGUCCGAGCAGAUGAAGAA
m9 GAGUCCGAGCACAAGAAGAA
m11 GAGUCCGAGGAGAAGAAGAA
m13 GAGUCCGUGCAGAAGAAGAA
m15 GAGUCGGAGCAGAAGAAGAA
m17 GAGACCGAGCAGAAGAAGAA
B
mismatched spacers
m17 m15 m13 m11 m9 m7 m5 m4 m3 m2 m1 wt
684bp
367bp
317bp
indel (%) 5.6 7.5 8.8 9.7
C
left TALEN binding site
protospacer (1)
PAM
human EMX1 locus
5'-..CTGGAGGAGGAAGGGCCTGAGTCCGAGCAGAAGAAGAAGGGCTCCCAT..-3'
3'-..GACCTCCTCCTTCCCGGACTCAGGCTCGTCTTCTTCTTCCCGAGGGTA..-5'
right TALEN binding site

FIG. 3A-C

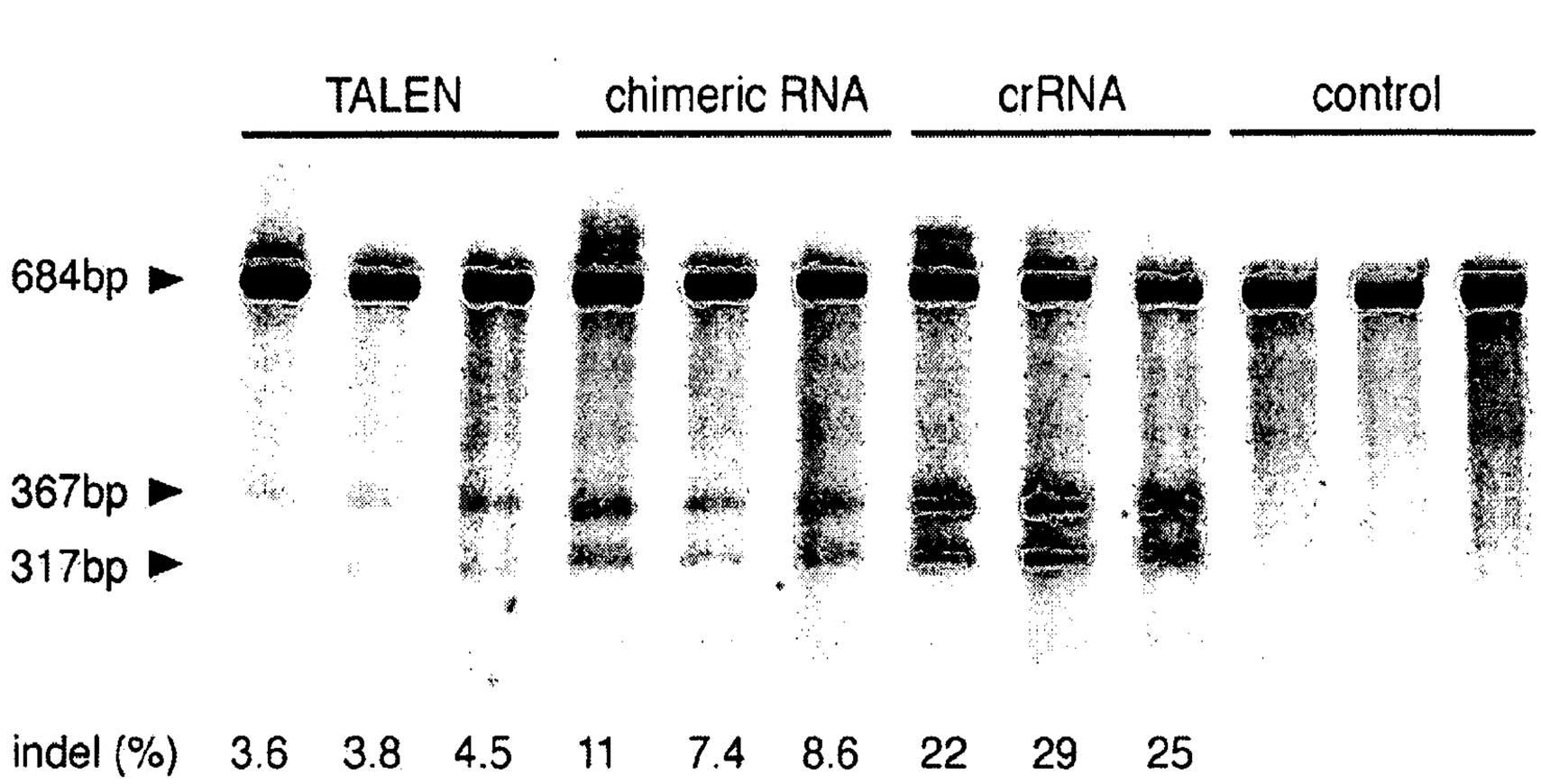
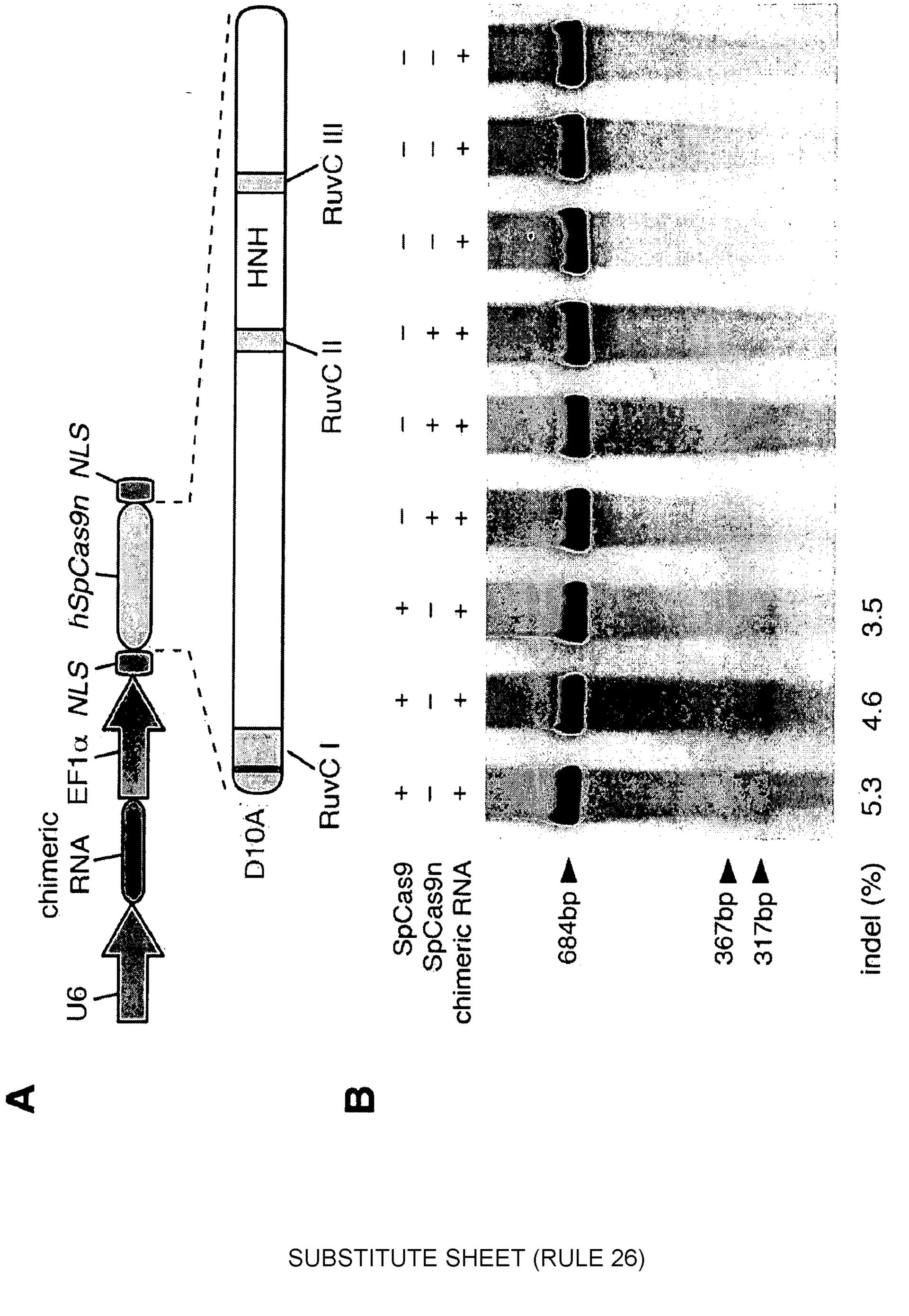

D
TALEN
chimeric RNA
crRNA
control
684bp
367bp
317bp
indel (%) 3.6 3.8 4.5 11 7.4 8.6 22 29 25

FIG. 3D

FIG. 4A-B


A
chimeric RNA
EF1α
NLS
hSpCas9n
NLS
U6
D10A
RuvC I
RuvC II
HNH
RuvC III
B
SpCas9
SpCas9n
chimeric RNA
684bp
367bp
317bp
indel (%)
5.3
4.6
3.5

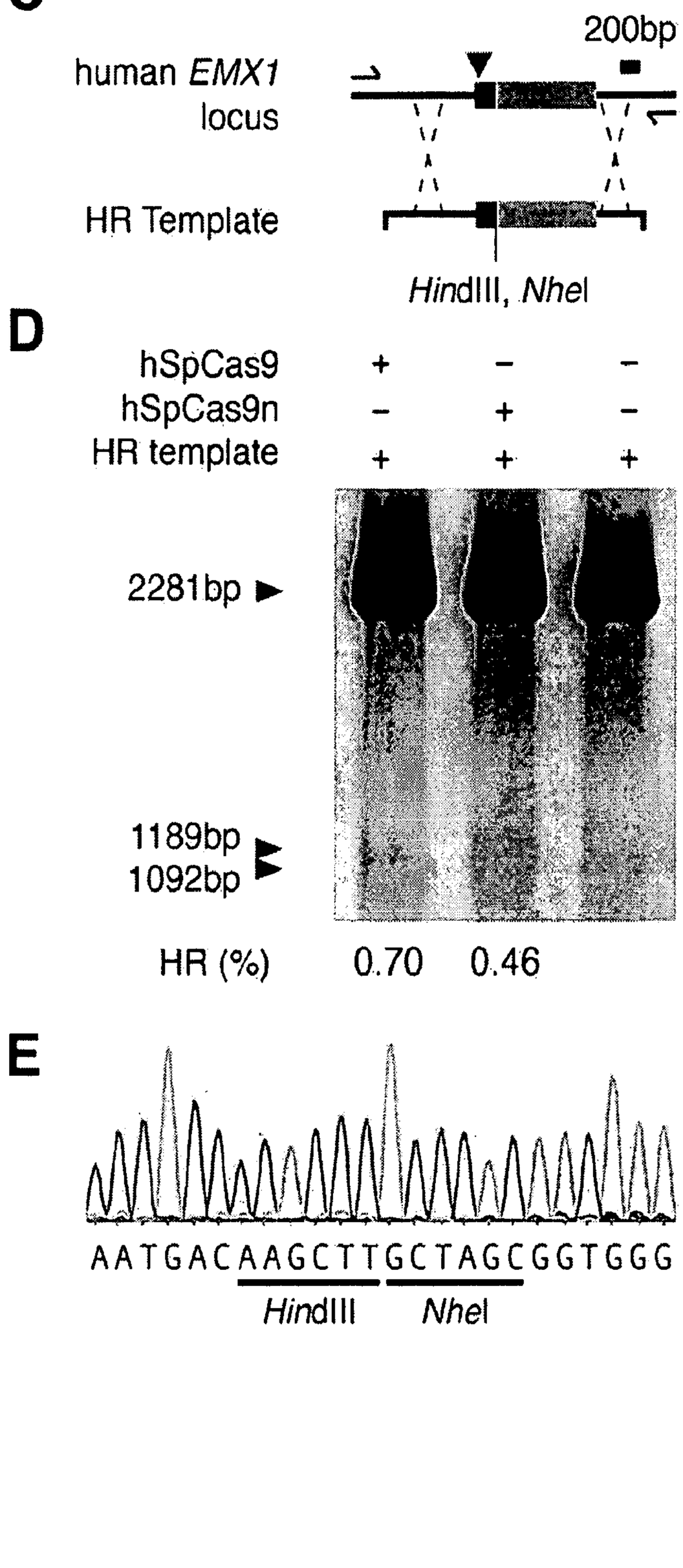

C
human EMX1 locus
200bp
HR Template
HindIII, NheI
D
hSpCas9 + − −
hSpCas9n − + −
HR template + + +
2281bp
1189bp
1092bp
HR (%) 0.70 0.46
E
AATGACAAGCTTGCTAGCGGTGGG
HindIII NheI

FIG. 4C-E

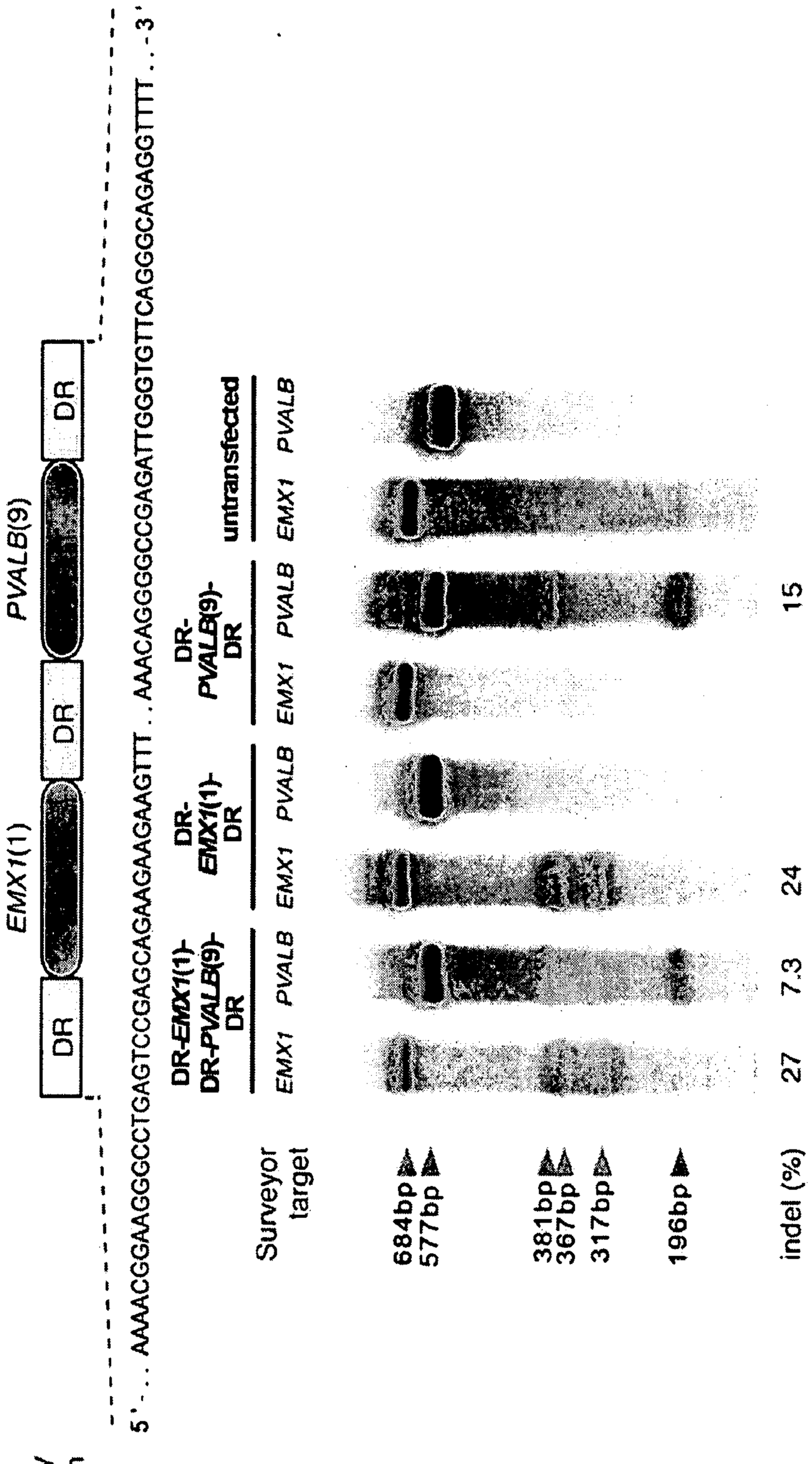

F
crRNA array design
5'
EMX1(1)
PVALB(9)
DR
DR
DR
DR
3'
AAAACGGAAGGGCCTGAGTCCGAGCAGAAGAAGTT . . AAACAGGGGCCGAGATTGGGTGTTCAGGCAGAGGTTTT . . -3'
DR-EMX1(1)-DR
DR-PVALB(9)-DR
DR-EMX1(1)-DR-PVALB(9)-DR
untransfected
EMX1 PVALB EMX1 PVALB EMX1 PVALB EMX1 PVALB
Surveyor target
684bp
577bp
381bp
367bp
317bp
196bp
indel (%) 27 7.3 24 15

FIG. 4F

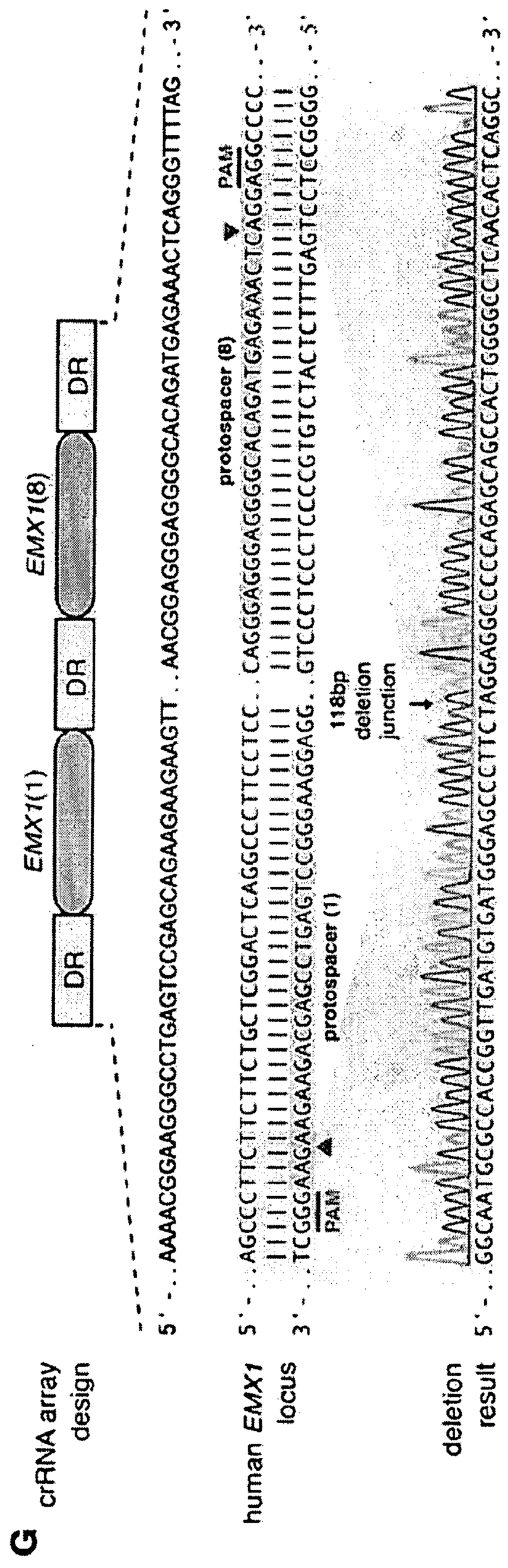

G
crRNA array design
human EMX1 locus
deletion result
EMX1(1)
EMX1(8)
DR
DR
DR
protospacer (8)
protospacer (1)
PAM
PAM
118bp deletion junction
5'
3'
5'
3'
5'
3'

FIG. 4G

Cas9	target species	gene	protospacer ID	protospacer sequence (5' to 3')	PAM	strand	cell line tested	% indel (pre-crRNA + tracrRNA)	% indel (chimeric RNA)
S. pyogenes SF370 type II CRISPR	*Homo sapiens*	*EMX1*	1	GGAAGGGCCTGAGTCCGAGCAGAAGAAGAA	GGG	+	293FT	20 ± 1.8	6.7 ± 0.62
		EMX1	2	CATTGGAGGTGACATCGATGTCCTCCCCAT	TGG	–	293FT	2.1 ± 0.31	N.D.
		EMX1	3	GGACATCGATGTCACCTCCAATGACTAGGG	TGG	+	293FT	14 ± 1.1	N.D.
		EMX1	4	CATCGATGTCCTCCCCATTGGCCTGCTTCG	TGG	–	293FT	11 ± 1.7	N.D.
		EMX1	5	TTCGTGGCAATGCGCCACCGGTTGATGTGA	TGG	–	293FT	4.3 ± 0.46	2.1 ± 0.51
		EMX1	6	TCGTGGCAATGCGCCACCGGTTGATGTGAT	GGG	–	293FT	4.0 ± 0.66	0.41 ± 0.25
		EMX1	7	TCCAGCTTCTGCCGTTTGTACTTTGTCCTC	CGG	–	293FT	1.5 ± 0.12	N.D.
		EMX1	8	GGAGGGAGGGGCACAGATGAGAAACTCAGG	AGG	–	293FT	7.8 ± 0.83	2.3 ± 1.2
	Homo sapiens	*PVALB*	9	AGGGGCCGAGATTGGGTGTTCAGGGCAGAG	AGG	+	293FT	21 ± 2.6	6.5 ± 0.32
		PVALB	10	ATGCAGGAGGGTGGCGAGAGGGGCCGAGAT	TGG	+	293FT	N.D.	N.D.
		PVALB	11	GGTGGCGAGAGGGGCCGAGATTGGGTGTTC	AGG	+	293FT	N.D.	N.D.
	Mus musculus	*Th*	12	CAAGCACTGAGTGCCATTAGCTAAATGCAT	AGG	–	Neuro2A	27 ± 4.3	4.1 ± 2.2
		Th	13	AATGCATAGGGTACCACCCACAGGTGCCAG	GGG	–	Neuro2A	4.8 ± 1.2	N.D.
		Th	14	ACACACATGGGAAAGCCTCTGGGCCAGGAA	AGG	+	Neuro2A	11.3 ± 1.3	N.D.
S. thermophilus LMD-9 CRISPR1	*Homo sapiens*	*EMX1*	15	GGAGGAGGTAGTATACAGAAACACAGAGAA	GTAGAAT	–	293FT	14 ± 0.88	N.T.
		EMX1	16	AGAATGTAGAGGAGTCACAGAAACTCAGCA	CTAGAAA	–	293FT	7.8 ± 0.77	N.T.

FIG. 5

SUBSTITUTE SHEET (RULE 26)

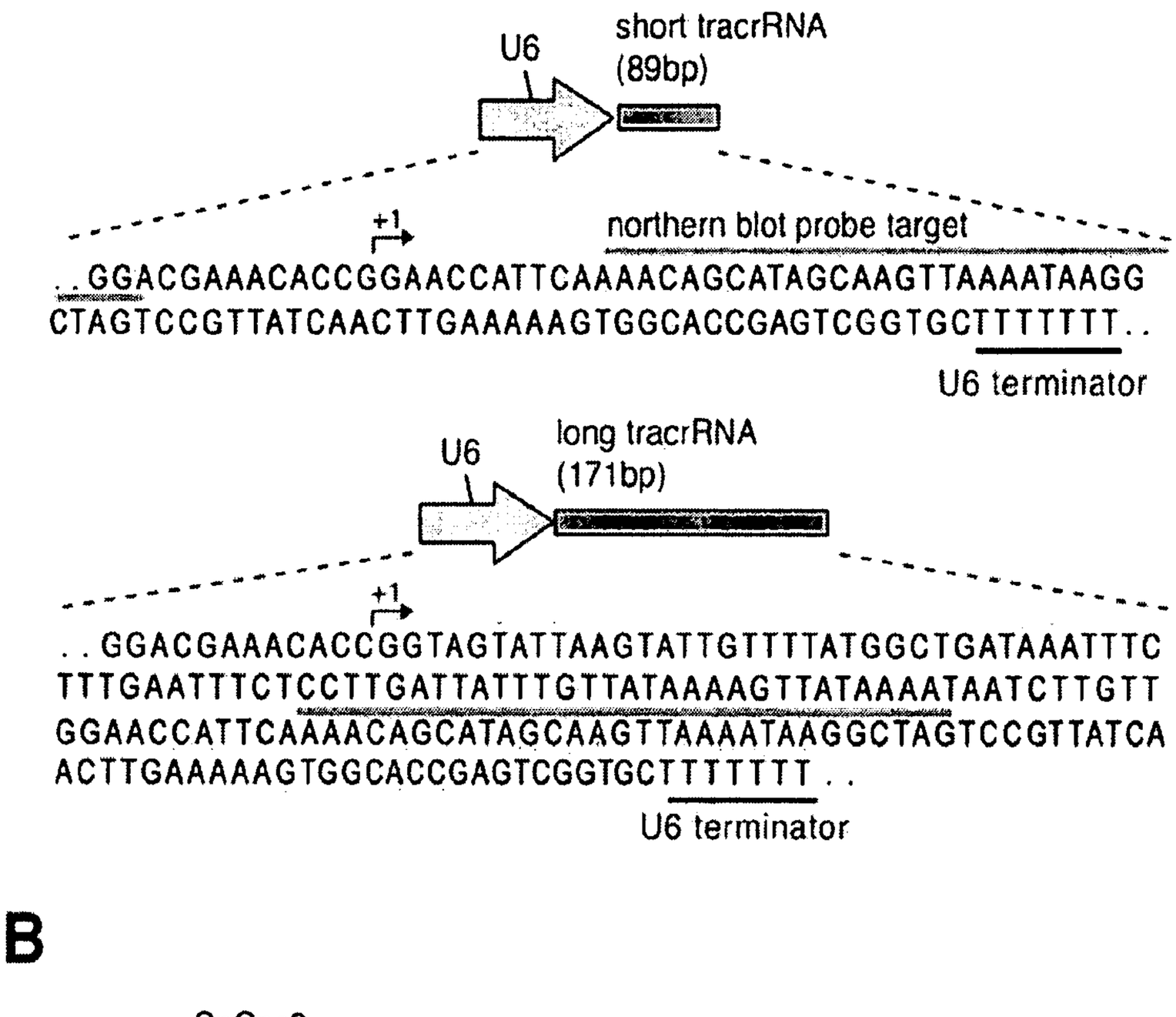

A
U6
short tracrRNA
(89bp)
+1
northern blot probe target
..GGACGAAACACCGGAACCATTCAAAACAGCATAGCAAGTTAAAATAAGG
CTAGTCCGTTATCAACTTGAAAAAGTGGCACCGAGTCGGTGCTTTTTTT..
U6 terminator
U6
long tracrRNA
(171bp)
+1
..GGACGAAACACCGGTAGTATTAAGTATTGTTTTATGGCTGATAAATTTC
TTTGAATTTCTCCTTGATTATTTGTTATAAAAGTTATAAAATAATCTTGTT
GGAACCATTCAAAACAGCATAGCAAGTTAAAATAAGGCTAGTCCGTTATCA
ACTTGAAAAAGTGGCACCGAGTCGGTGCTTTTTTT..
U6 terminator
B
SpCas9 + + + +
long tracrRNA + + – –
short tracrRNA – – + +
SpRNase III + + + +
DR-EMX1(1)-DR + + + +
684bp
367bp
317bp
indel (%) 0.60 14 11

FIG. 6A-B

C

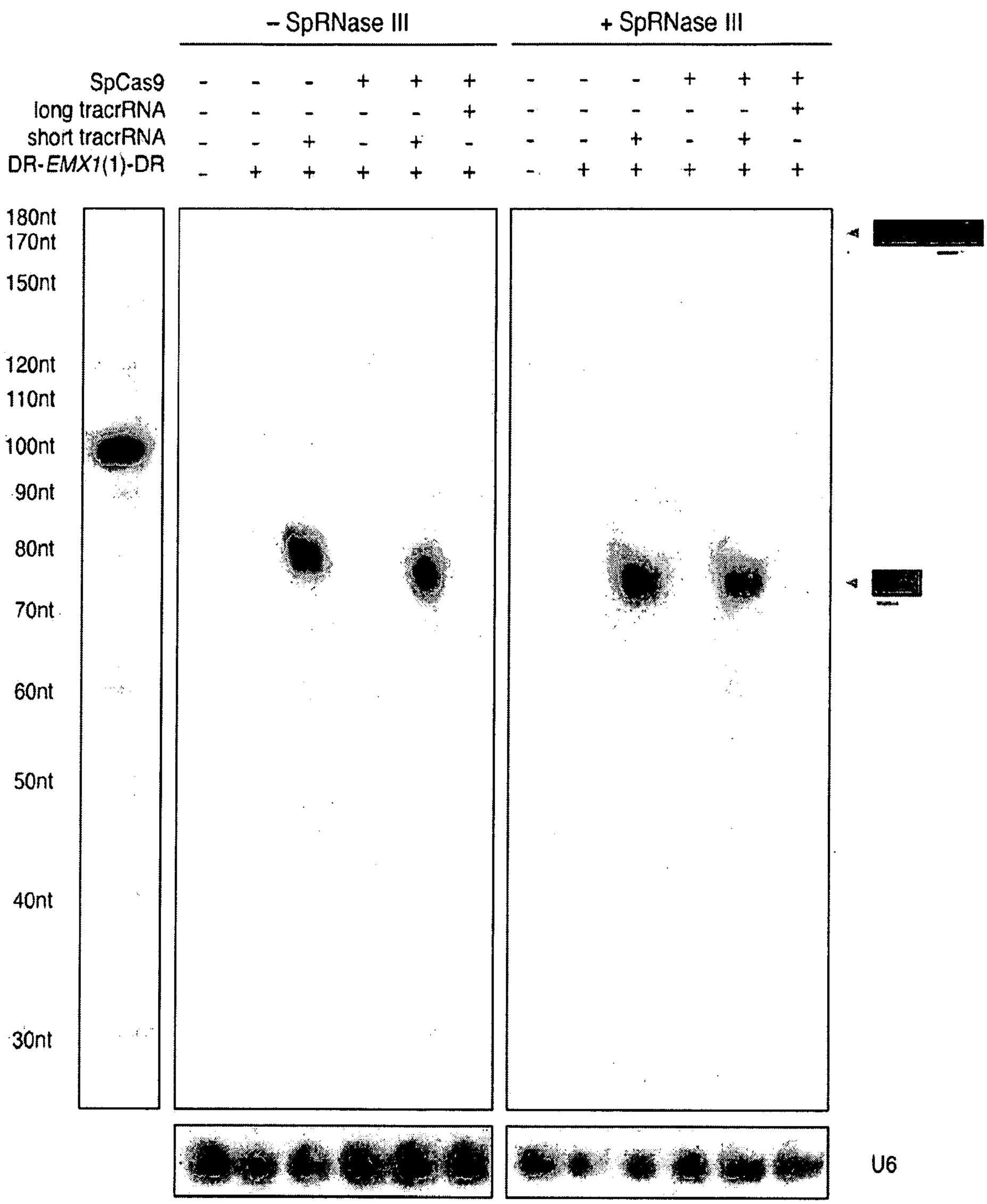

– SpRNase III
+ SpRNase III
SpCas9 - - - + + + - - - + + +
long tracrRNA - - - - - + - - - - - +
short tracrRNA - - + - + - - - + - + -
DR-EMX1(1)-DR - + + + + + - + + + + +
180nt
170nt
150nt
120nt
110nt
100nt
90nt
80nt
70nt
60nt
50nt
40nt
30nt
U6

FIG. 6C

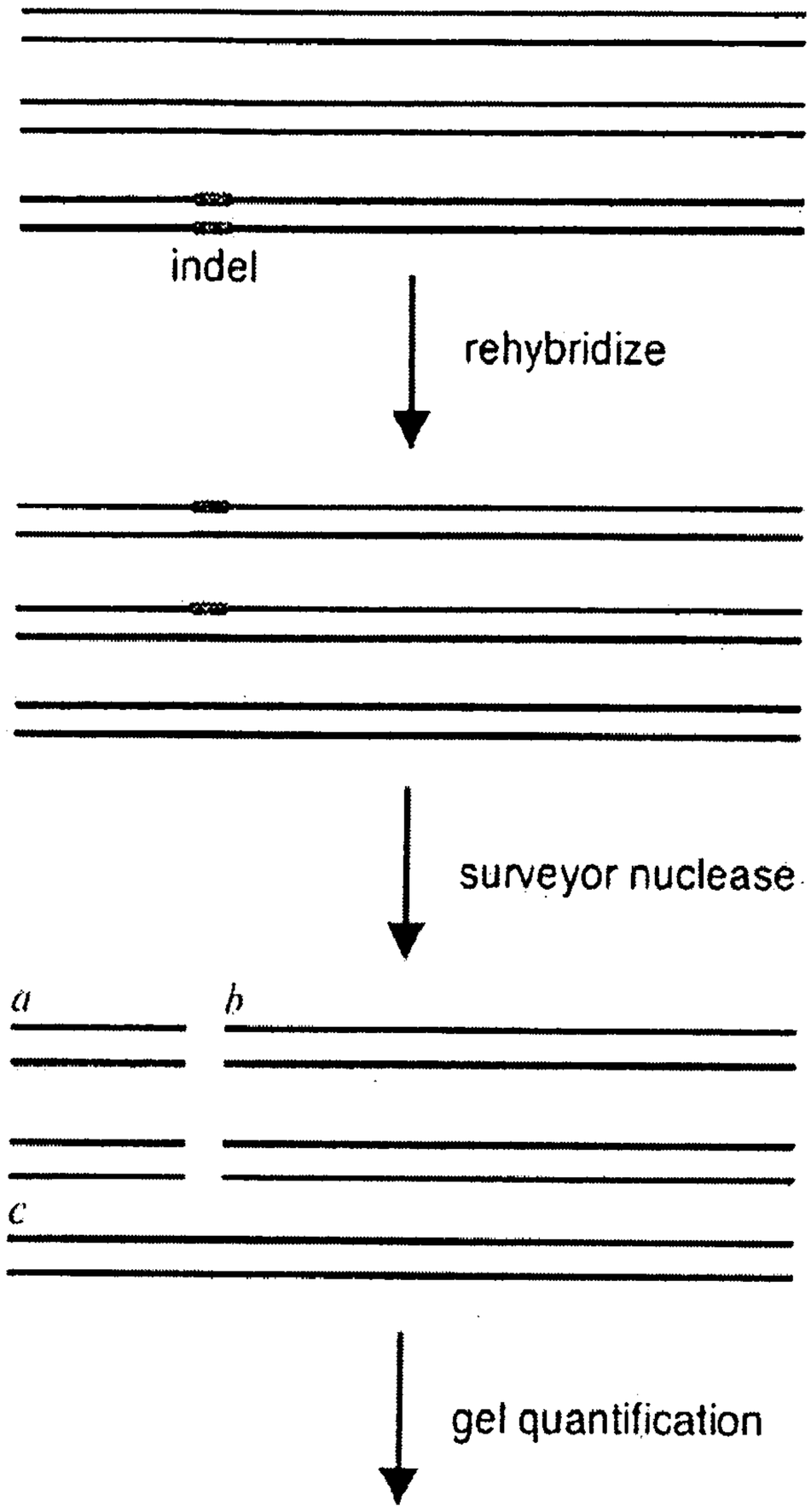

indel
rehybridize
surveyor nuclease
a
b
c
gel quantification
% indel = (1- √1 - (a + b)/(a + b + c)) *100

FIG. 7

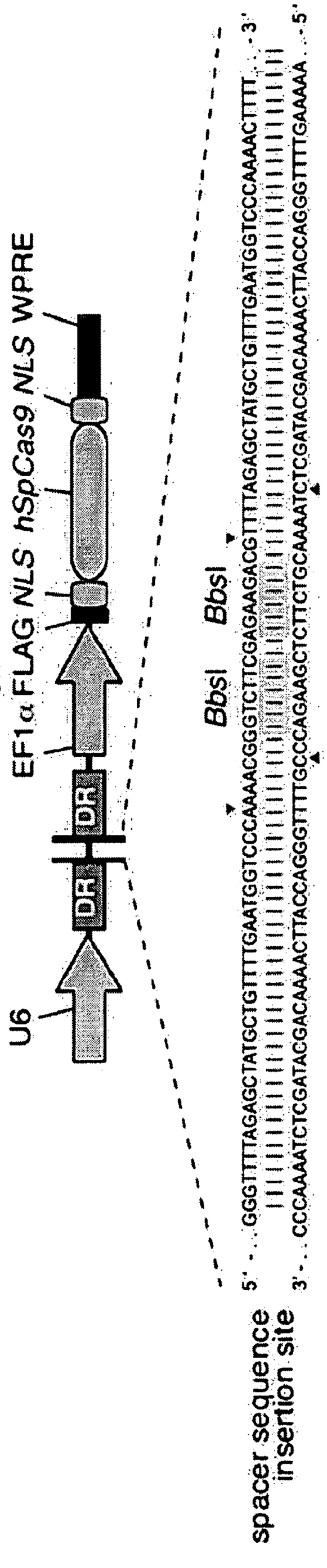
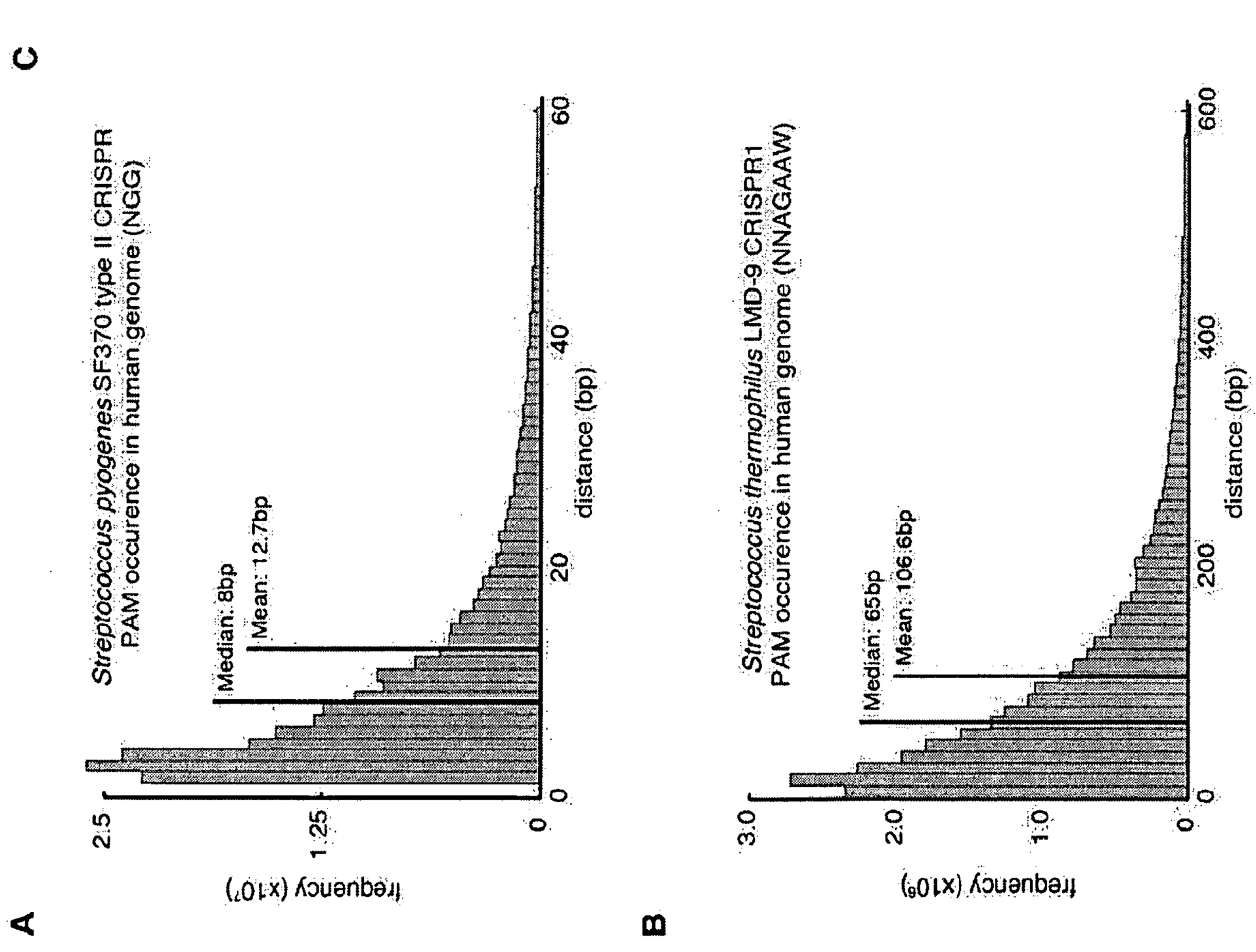
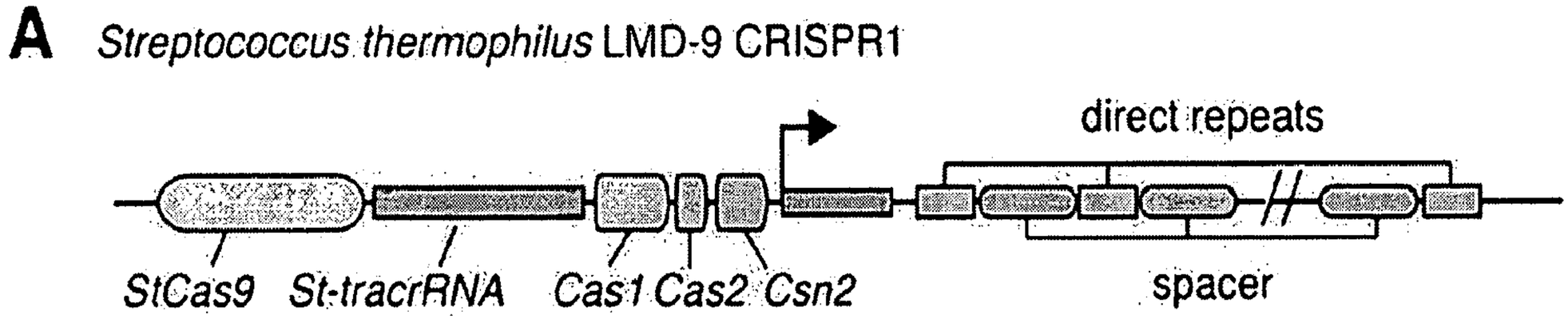

A
3x
EF1α FLAG NLS hSpCas9 NLS WPRE
U6
DR
DR
BbsI
BbsI
5' - . . . GGGTTTTAGAGCTATGCTGTTTTGAATGGTCCCAAAACGGGTCTTCGAGAAGACGTTTTGAATGGTCCCAAAACTTTT . . . - 3'
3' - . . . CCCAAAATCTCGATACGACAAAACTTACCAGGGTTTTGCCCAGAAGCTCTTCTGCAAAATCTCGATACGACAAAACTTACCAGGGTTTTGAAAAA . . . - 5'
spacer sequence insertion site
5' - AAACNNNNNNNNNNNNNNNNNNNNNNNNNNNNGT - 3'
3' - NNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNCAAAAT - 5'
spacer sequence insert
B
3x
EF1α FLAG NLS hSpCas9 NLS WPRE
U6
BbsI
BbsI
5' - . . . GTGGAAAGGACGAAACACCGGGTCTTCGAGAAGACCTGTTTTAGAGCTAGAAATAGCAAGTTAAAATAAGGCTAGTCCGTTTTT . . . - 3'
3' - . . . CACCTTTCCTGCTTTGTGGCCCAGAAGCTCTTCTGGACAAAATCTCGATCTTTATCGTTCAATTTTATTCCGATCAGGCAAAAA . . . - 5'
guide sequence insertion site
5' - CACCGNNNNNNNNNNNNNNNNNNN - 3'
3' - CNNNNNNNNNNNNNNNNNNNCAAA - 5'
guide sequence insert

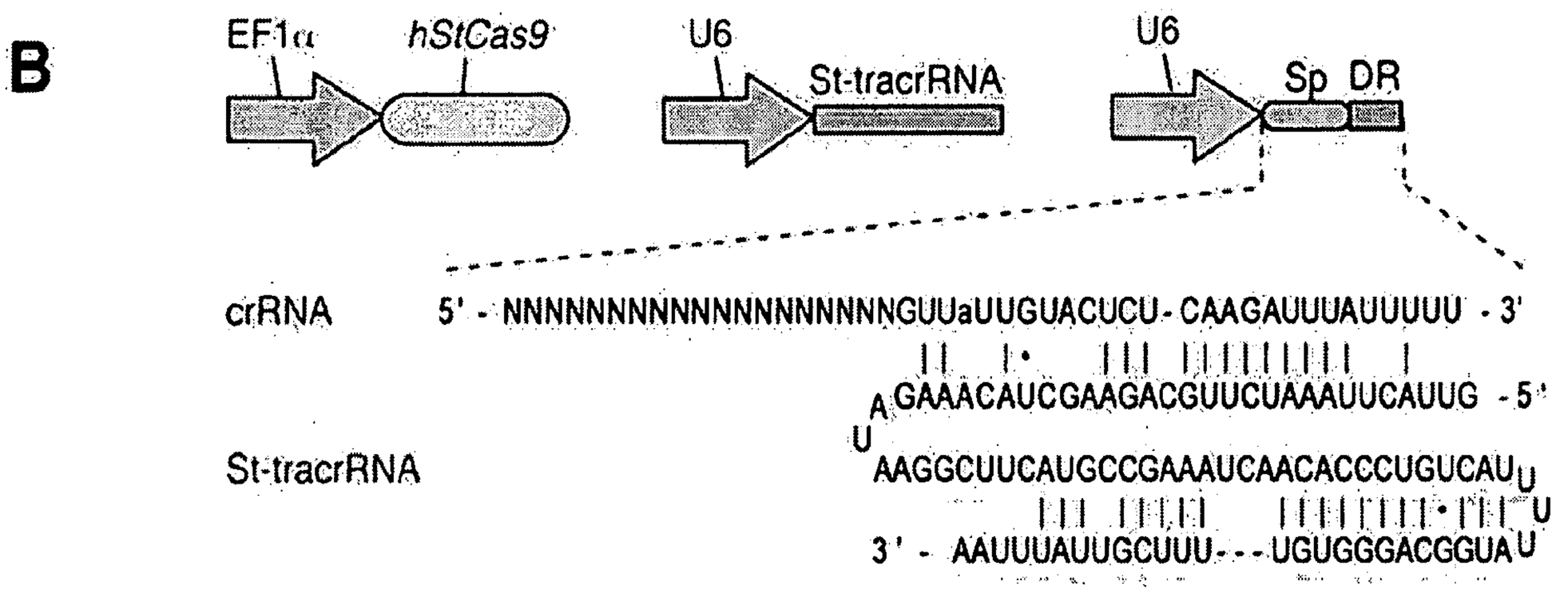
FIG. 8A-B

FIG. 9A-C

Chr	NGG median	NGG mean	NNAGAAW median	NNAGAAW mean
1	7	12.8	67	115.8
2	8	12.7	64	100.8
3	8	13.0	63	98.5
4	9	14.0	61	94.5
5	8	13.1	63	97.9
6	8	13.1	63	98.5
7	8	12.4	64	102.9
8	8	12.8	64	100.9
9	7	13.9	65	120.5
10	7	12.1	66	107.0
11	7	12.0	65	105.8
12	8	12.4	65	103.5
13	8	13.6	62	94.6
14	8	12.0	65	101.5
15	7	11.5	68	107.7
16	7	11.7	74	136.8
17	6	10.3	76	127.9
18	8	13.4	63	101.8
19	6	9.4	82	145.4
20	7	11.1	72	121.8
21	7	13.4	64	111.4
22	6	9.2	85	140.3
X	8	13.2	63	99.0
Y	8	29.2	62	223.7




A
Streptococcus thermophilus LMD-9 CRISPR1
direct repeats
StCas9
St-tracrRNA
Cas1
Cas2
Csn2
spacer


B
EF1α
hStCas9
U6
St-tracrRNA
U6
Sp
DR
crRNA
5' - NNNNNNNNNNNNNNNNNNNGUUaUUGUACUCU- CAAGAUUUAUUUUU - 3'
A GAAACAUCGAAGACGUUCUAAAUUCAUUG - 5'
U
St-tracrRNA
AAGGCUUCAUGCCGAAAUCAACACCCUGUCAU U
3' - AAUUUAUUGCUUU- - - UGUGGGACGGUA U U

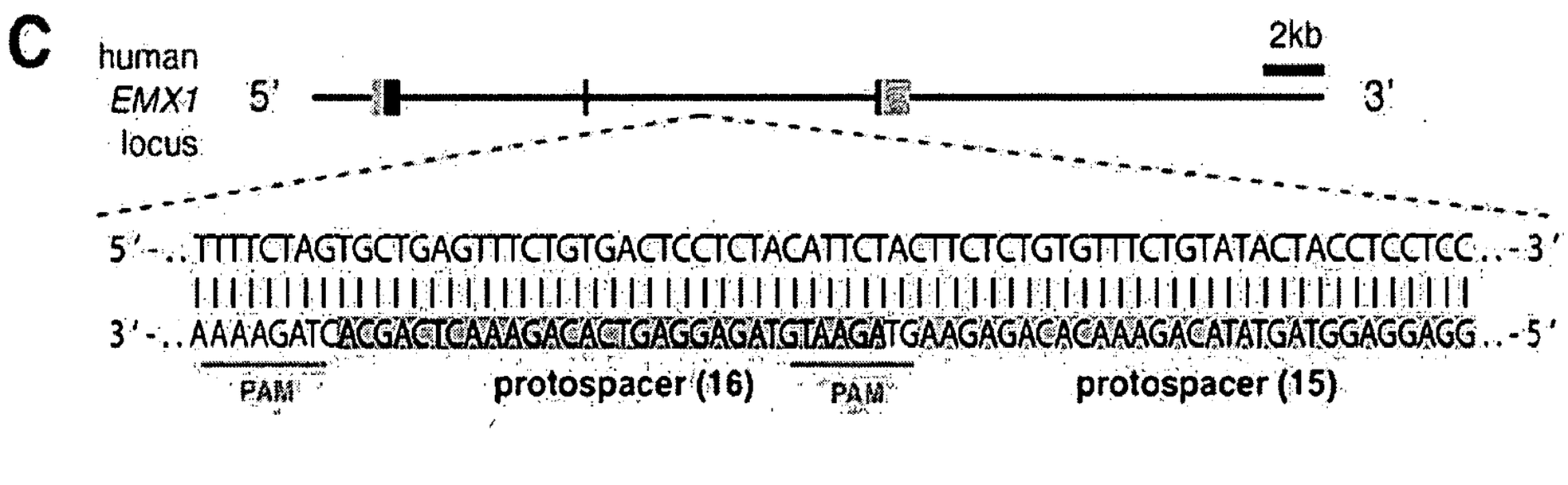

C
human
EMX1
locus
5'
2kb
3'
5'-..TTTTCTAGTGCTGAGTTTCTGTGACTCCTCTACATTCTACTTCTCTGTGTTTCTGTATACTACCTCCTCC..-3'
3'-..AAAAGATCACGACTCAAAGACACTGAGGAGATGTAAGATGAAGAGACACAAAGACATATGATGGAGGAGG..-5'
PAM
protospacer (16)
PAM
protospacer (15)

FIG. 10A-C

D

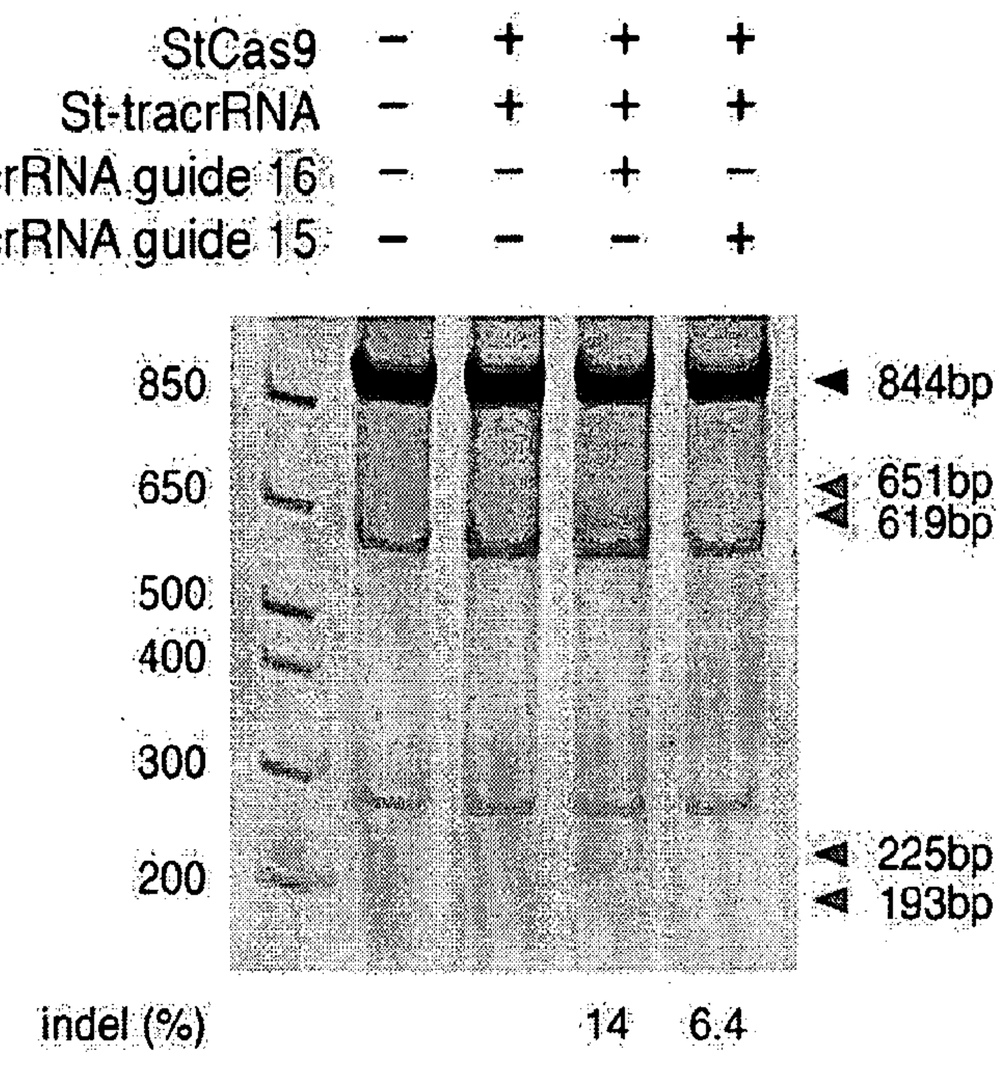

StCas9 − + + +
St-tracrRNA − + + +
crRNA guide 16 − − + −
crRNA guide 15 − − − +
850
650
500
400
300
200
844bp
651bp
619bp
225bp
193bp
indel (%) 14 6.4

FIG. 10D

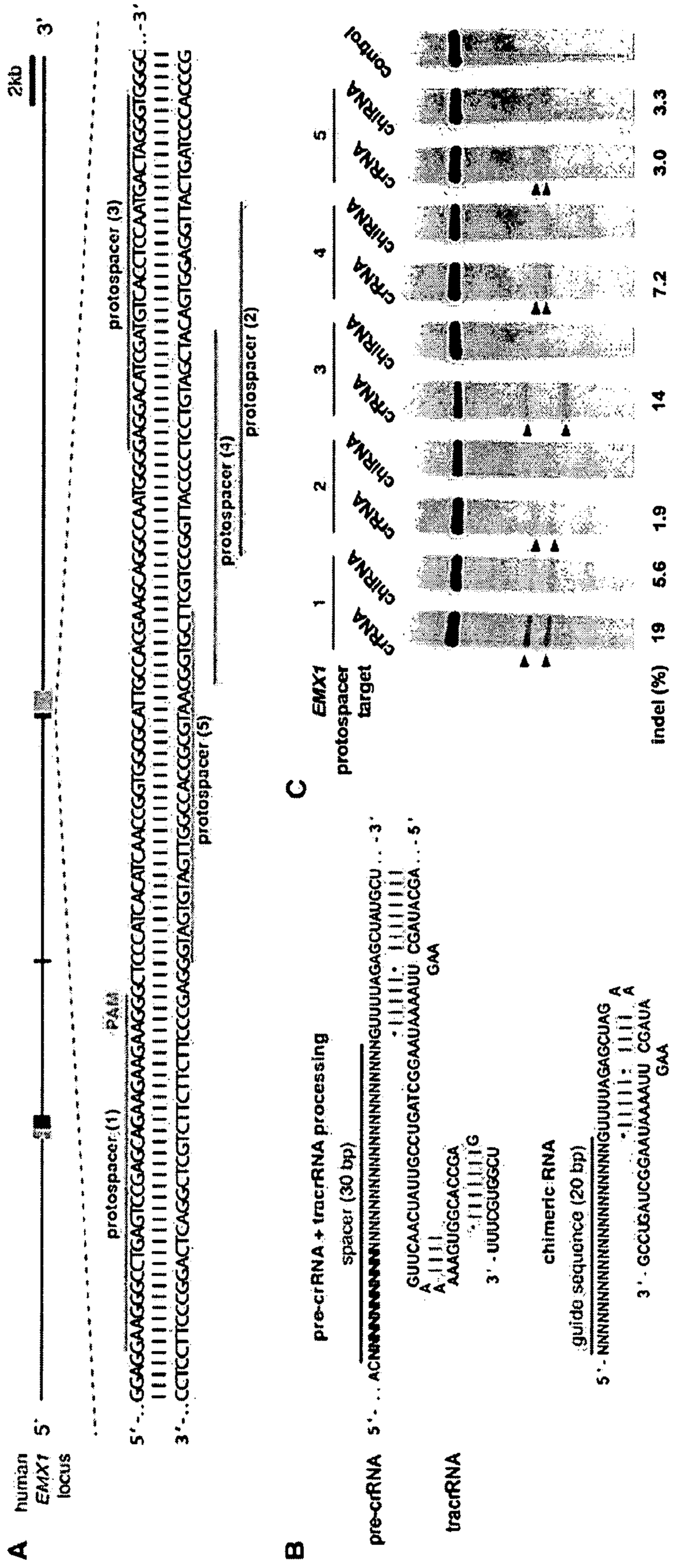
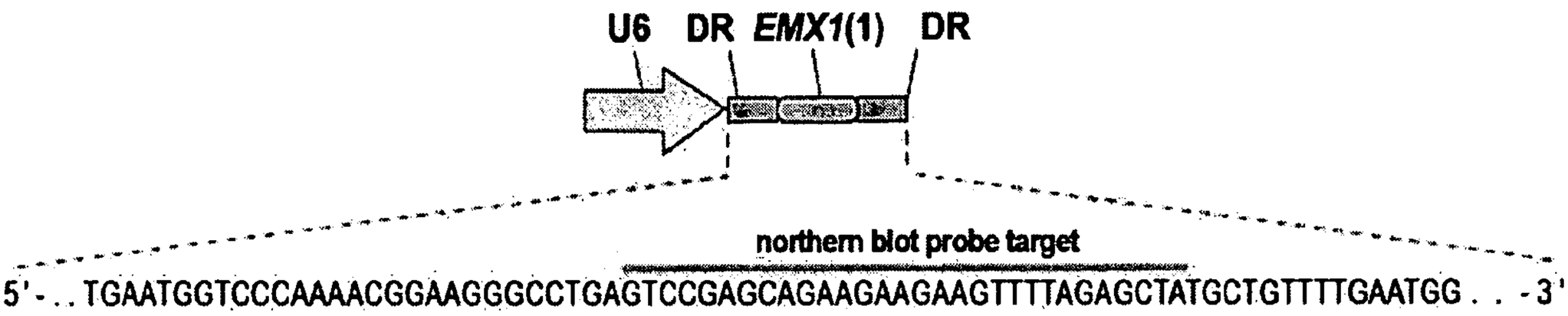

A
human EMX1 locus
5'
3'
2kb
protospacer (1)
PAM
protospacer (3)
protospacer (5)
protospacer (4)
protospacer (2)
B
pre-crRNA
tracrRNA
pre-crRNA + tracrRNA processing
spacer (30 bp)
chimeric RNA
guide sequence (20 bp)
C
EMX1 protospacer target
crRNA
tracrRNA
control
indel (%)
19
5.6
1.9
14
7.2
3.0
3.3

FIG. 11A-C

A


U6
DR
EMX1(1)
DR
northern blot probe target
5'- . . TGAATGGTCCCAAAACGGAAGGGCCTGAGTCCGAGCAGAAGAAGAAGTTTTAGAGCTATGCTGTTTTGAATGG . . -3'

B

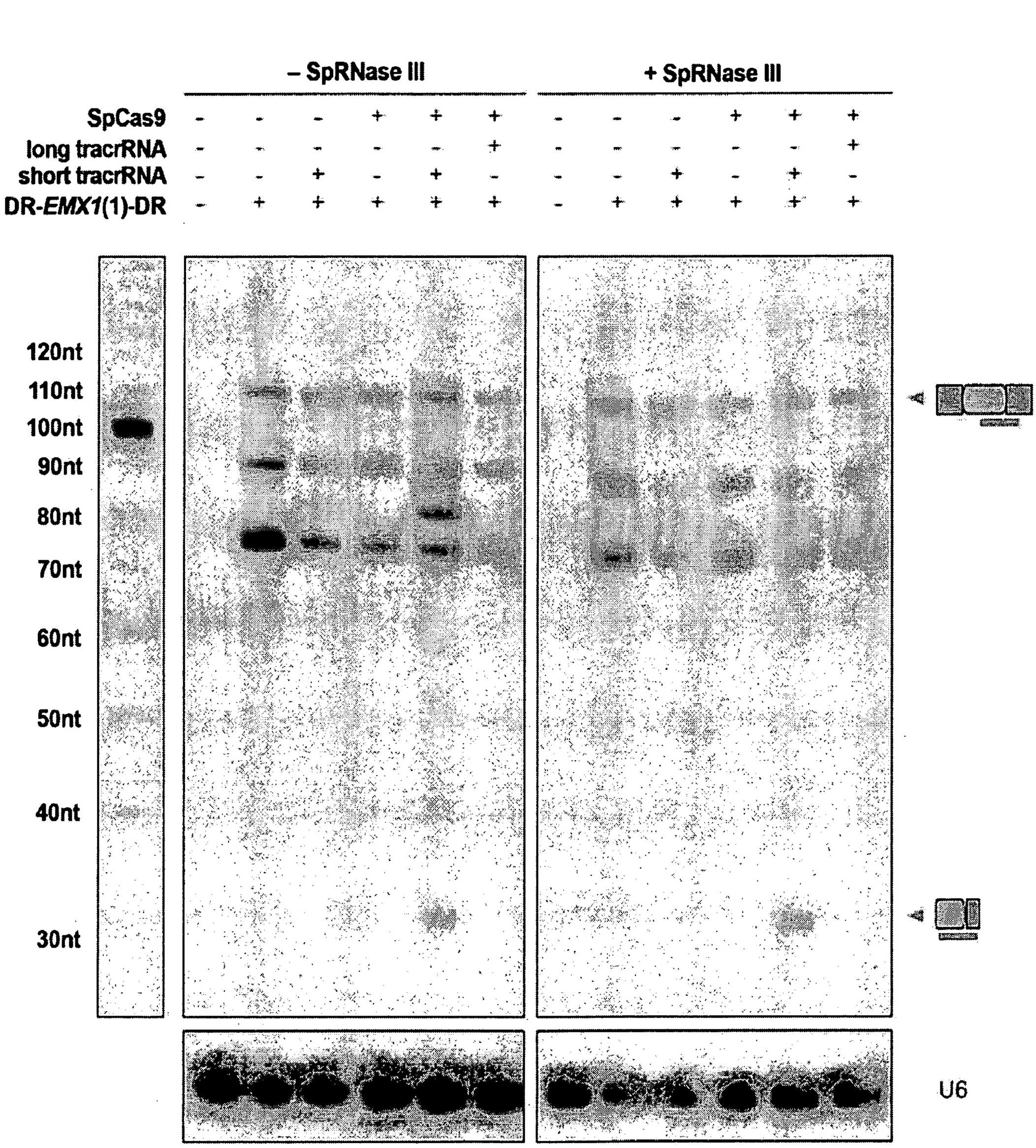

– SpRNase III
+ SpRNase III
SpCas9 - - - + + + - - - + + +
long tracrRNA - - - - - + - - - - - +
short tracrRNA - - + - + - - - + - + -
DR-EMX1(1)-DR - + + + + + - + + + + +
120nt
110nt
100nt
90nt
80nt
70nt
60nt
50nt
40nt
30nt
U6

FIG. 12A-B

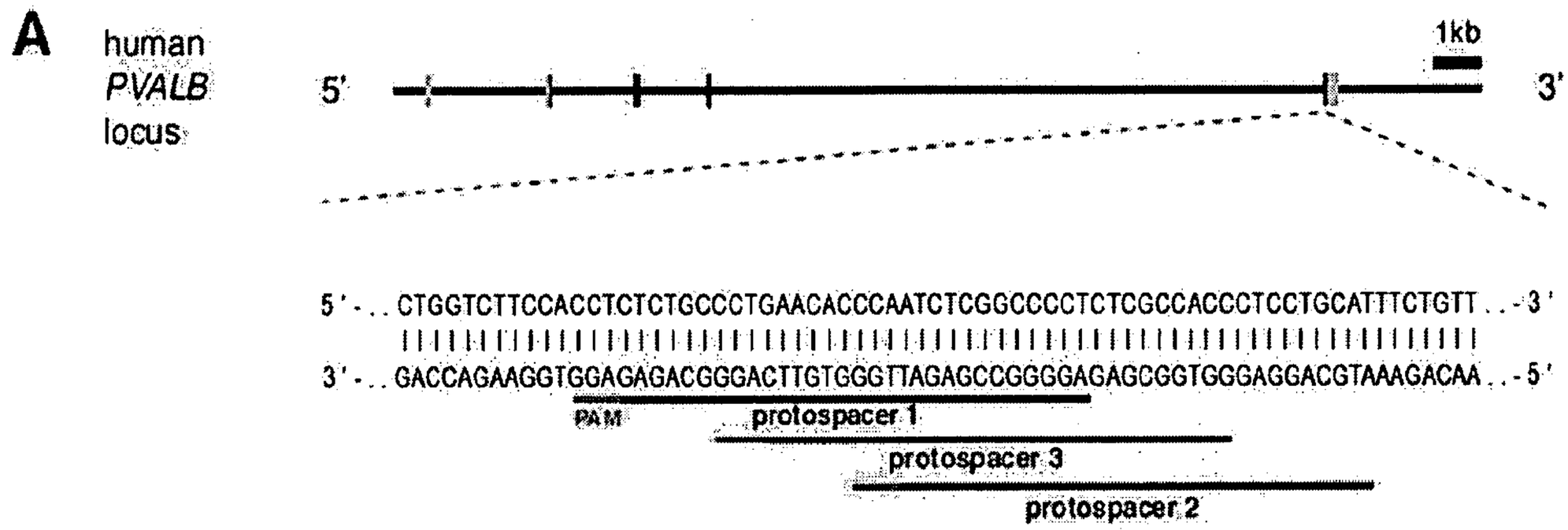

A
human
PVALB
locus
5'
1kb
3'
5' . . . CTGGTCTTCCACCTCTCTGCCCTGAACACCCAATCTCGGCCCCTCTCGCCACCCTCCTGCATTTCTGTT . . . 3'
3' . . . GACCAGAAGGTGGAGAGACGGGACTTGTGGGTTAGAGCCGGGGAGAGCGGTGGGAGGACGTAAAGACAA . . . 5'
PAM
protospacer 1
protospacer 3
protospacer 2
B
mouse
Th locus
5'
1kb
3'
protospacer 2
protospacer 1
PAM
5' . . . ACCCAAGCACTGAGTGCCATTAGCTAAATGCATAGGGTACCACCCACAGGTGCCAGGGGCCTTTCCCAA
3' . . . TGGGTTCGTGACTCACGGTAATCGATTTACGTATCCCATGGTGGGTGTCCACGGTCCCCGGAAAGGGTT
AGTTCCCAGCCCCTTCTCCAACCTTTCCTGGCCCAGAGGCTTTCCCATGTGTGTGGCTGACCCTTTGA . . . 3'
TCAAGGGTCGGGGAAGAGGTTGGAAAGGACCGGGTCTCCGAAAGGGTACACACACCGACCTGGGAAACT . . . 5'
protospacer 3

FIG. 13A-B

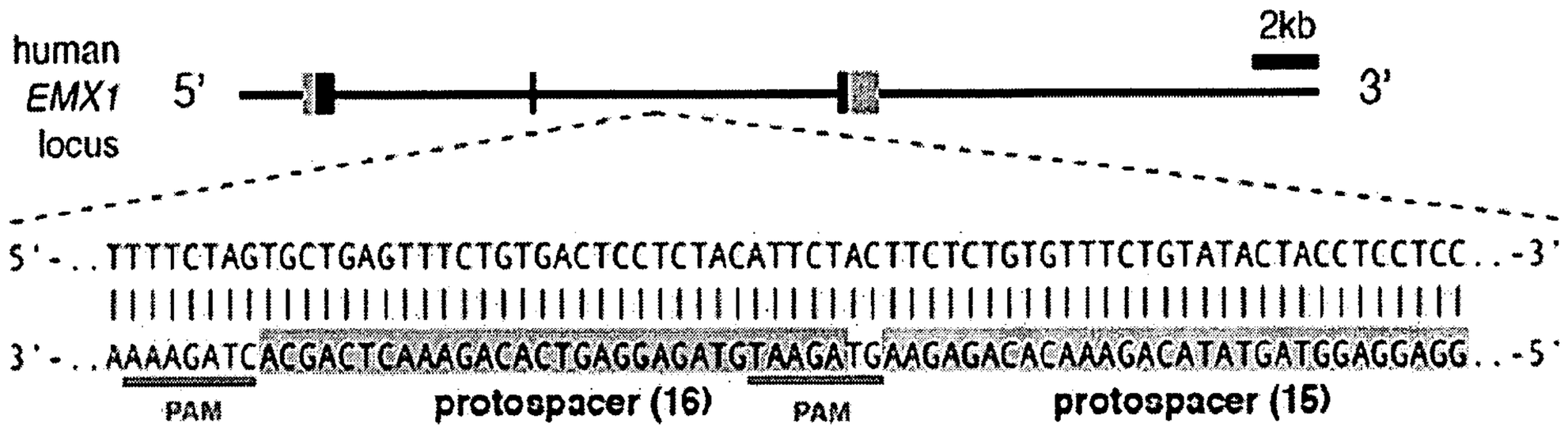

human
EMX1
locus
5'
2kb
3'
5'-..TTTTCTAGTGCTGAGTTTCTGTGACTCCTCTACATTCTACTTCTCTGTGTTTCTGTATACTACCTCCTCC..-3'
3'-..AAAAGATCACGACTCAAAGACACTGAGGAGATGTAAGATGAAGAGACACAAAGACATATGATGGAGGAGG..-5'
PAM
protospacer (16)
PAM
protospacer (15)

FIG. 14

Primer name	Assay	Genomic Target	Primer sequence
Sp-EMX1-F	SURVEYOR assay, sequencing	*EMX1*	AAAACCACCCTTCTCTCTGGC
Sp-EMX1-R	SURVEYOR assay, sequencing	*EMX1*	GGAGATTGGAGACACGGAGAG
Sp-PVALB-F	SURVEYOR assay, sequencing	*PVALB*	CTGGAAAGCCAATGCCTGAC
Sp-PVALB-R	SURVEYOR assay, sequencing	*PVALB*	GGCAGCAAACTCCTTGTCCT
Sp-Th-F	SURVEYOR assay, sequencing	*Th*	GTGCTTTGCAGAGGCCTACC
Sp-Th-R	SURVEYOR assay, sequencing	*Th*	CCTGGAGCGCATGCAGTAGT
St-EMX1-F	SURVEYOR assay, sequencing	*EMX1*	ACCTTCTGTGTTTCCACCATTC
St-EMX1-R	SURVEYOR assay, sequencing	*EMX1*	TTGGGGAGTGCACAGACTTC
Sp-EMX1-RFLP-F	RFLP, sequencing	*EMX1*	GGCTCCCTGGGTTCAAAGTA
Sp-EMX1-RFLP-R	RFLP, sequencing	*EMX1*	AGAGGGGTCTGGATGTCGTAA
Pb_EMX1_sp1	Northern Blot Probe	Not applicable	TAGCTCTAAAACTTCTTCTTCTGCTCGGAC
Pb_tracrRNA	Northern Blot Probe	Not applicable	CTAGCCTTATTTTAACTTGCTATGCTGTTT

FIG. 15

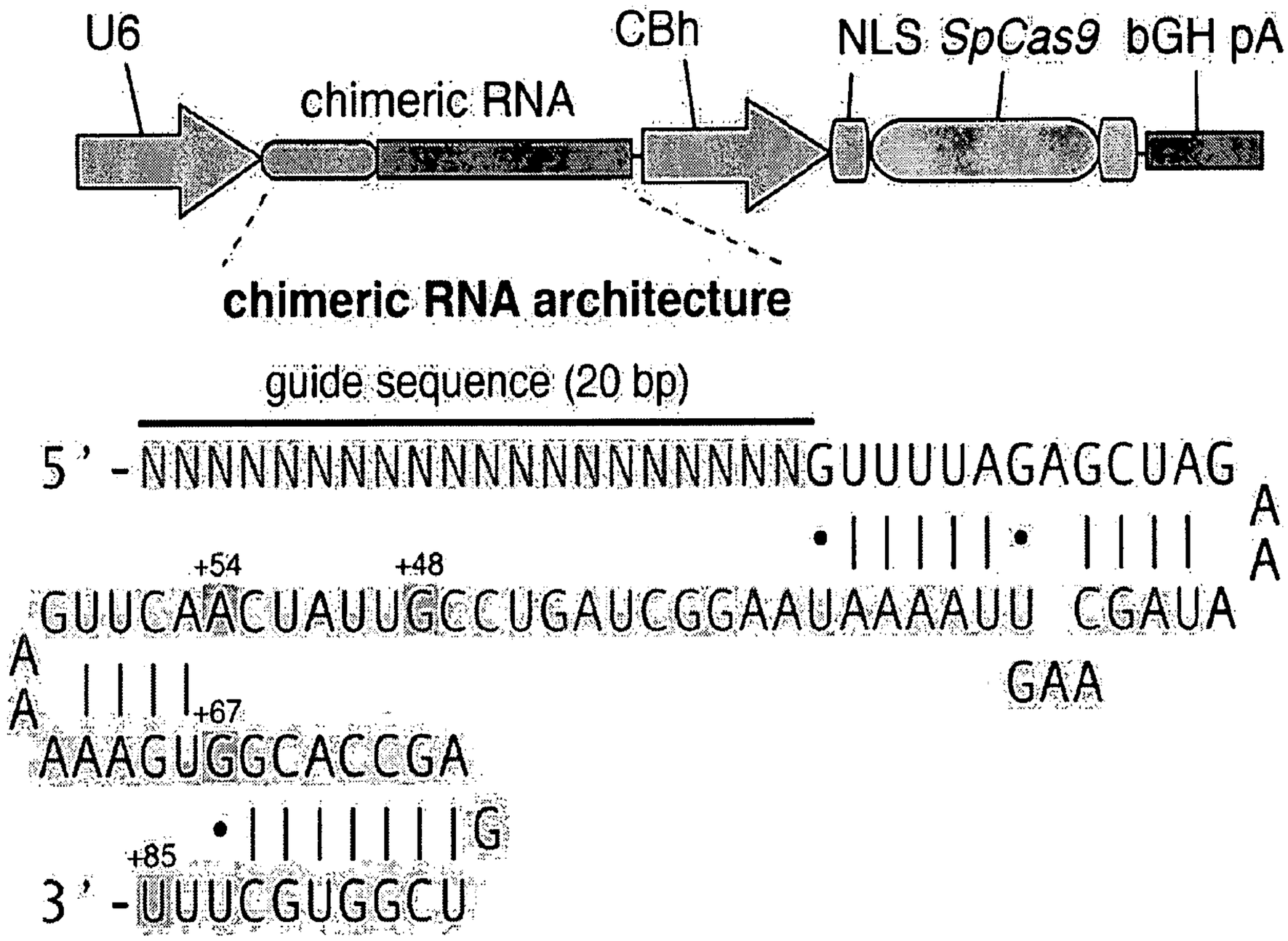

a
U6
chimeric RNA
CBh
NLS SpCas9 bGH pA
chimeric RNA architecture
guide sequence (20 bp)
5'-NNNNNNNNNNNNNNNNNNNNGUUUUAGAGCUAG
GUUCAACUAUUGCCUGAUCGGAAUAAAAUU CGAUA
AAAGUGGCACCGA
3'-UUUCGUGGCU
+54
+48
+67
+85

FIG. 16A

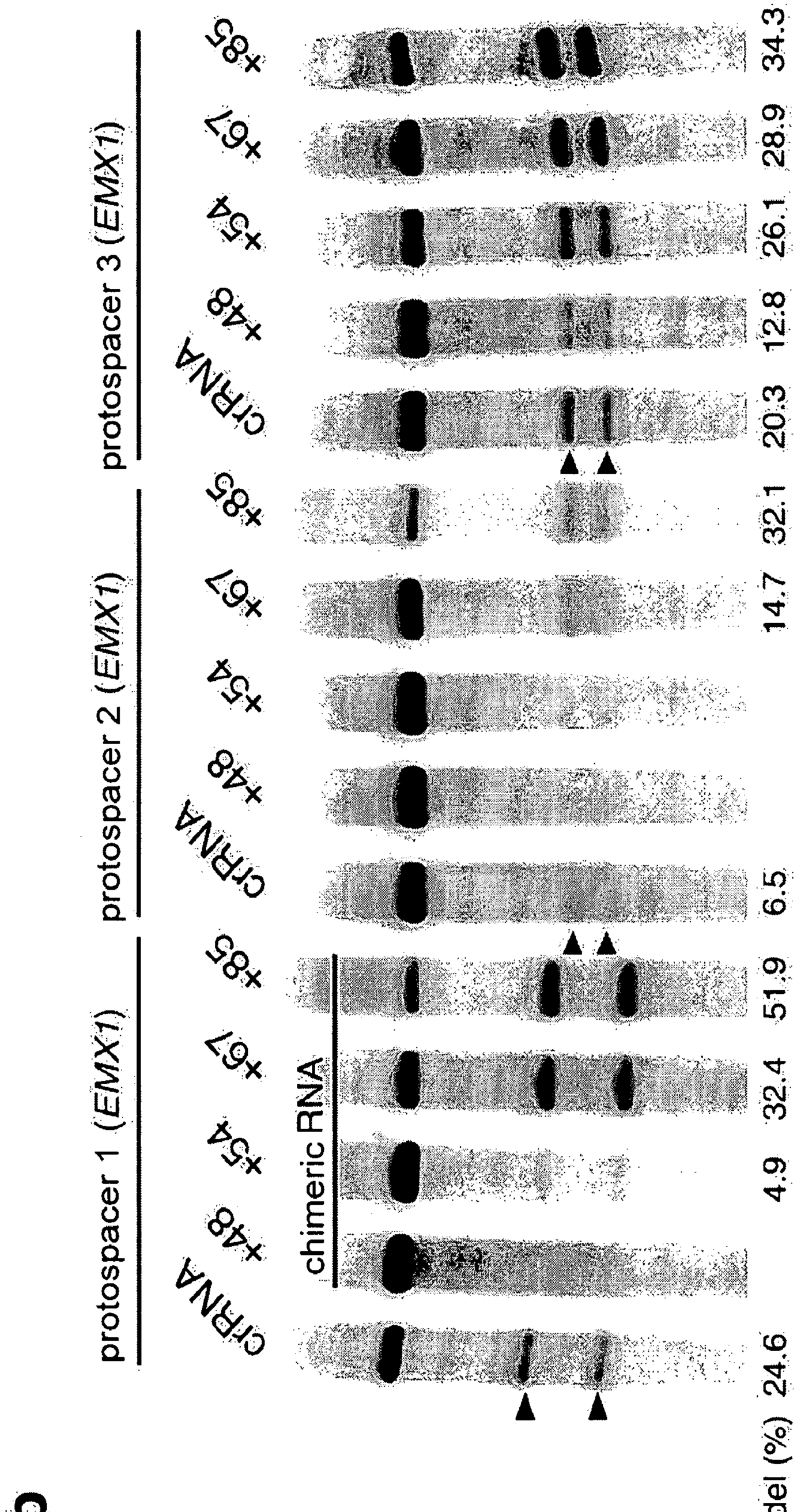
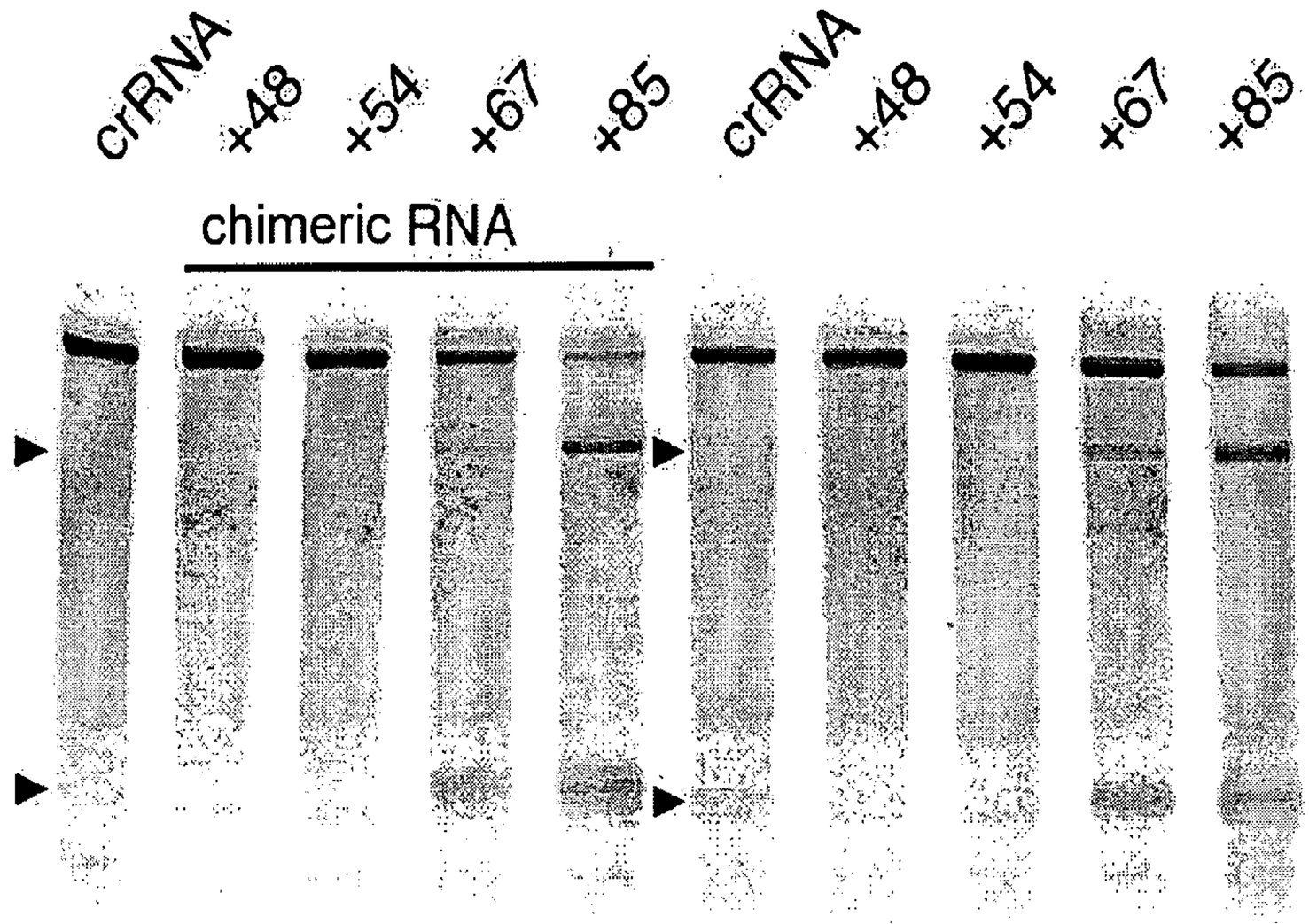

b
protospacer 1 (EMX1)
protospacer 2 (EMX1)
protospacer 3 (EMX1)
chimeric RNA
CRNA
+48
+54
+67
+85
4.9
32.4
51.9
6.5
14.7
32.1
20.3
12.8
26.1
28.9
34.3
indel (%) 24.6

FIG. 16B

C



3			18.3	49.9			25.1	39.7	

FIG. 16C

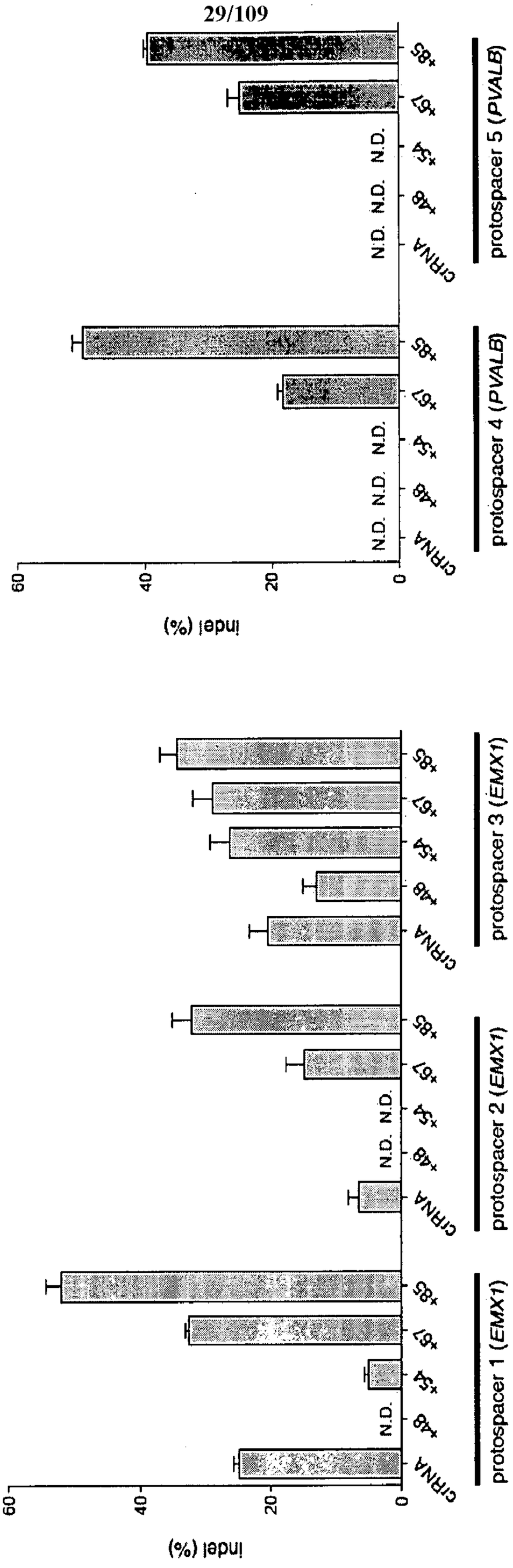
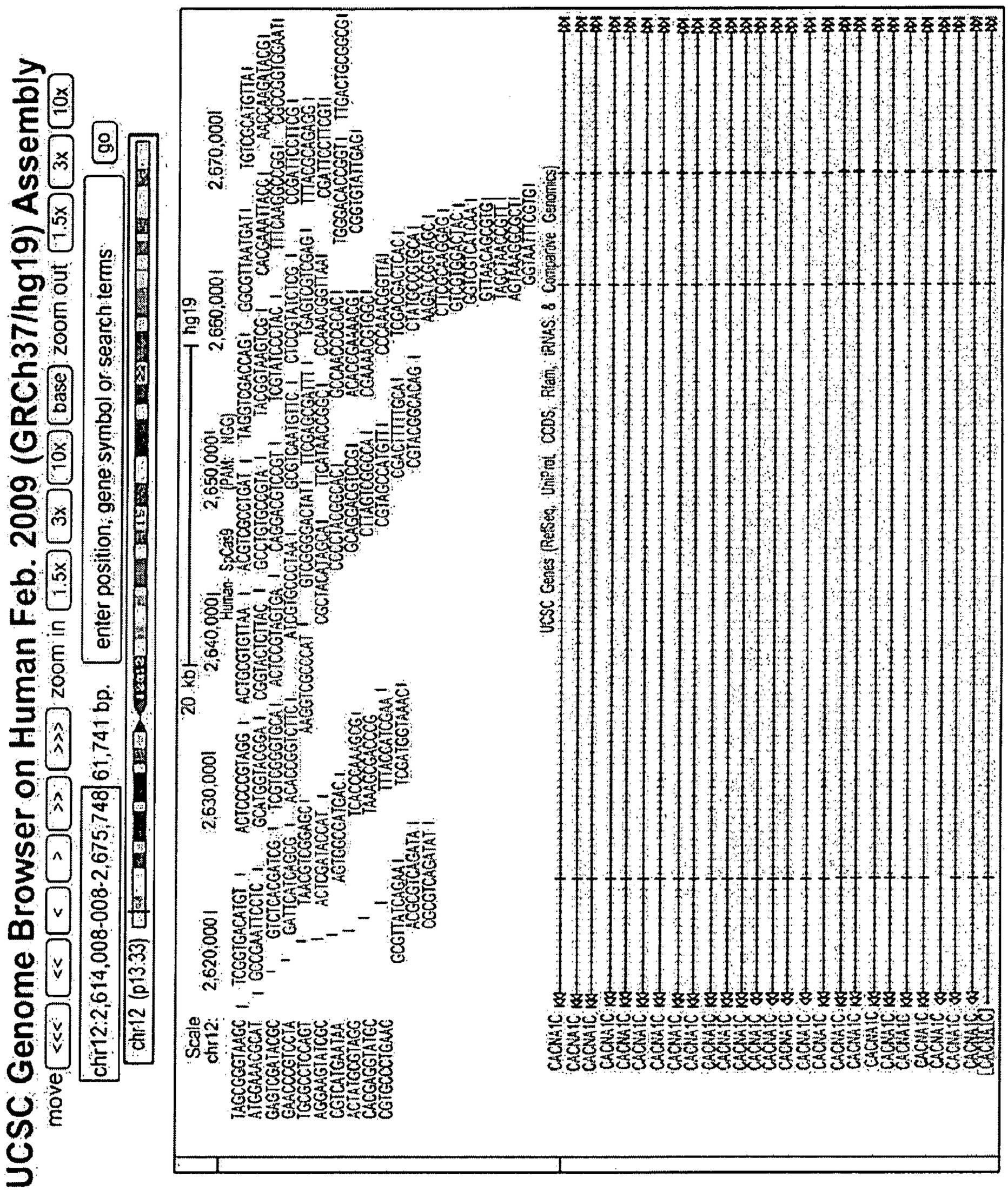
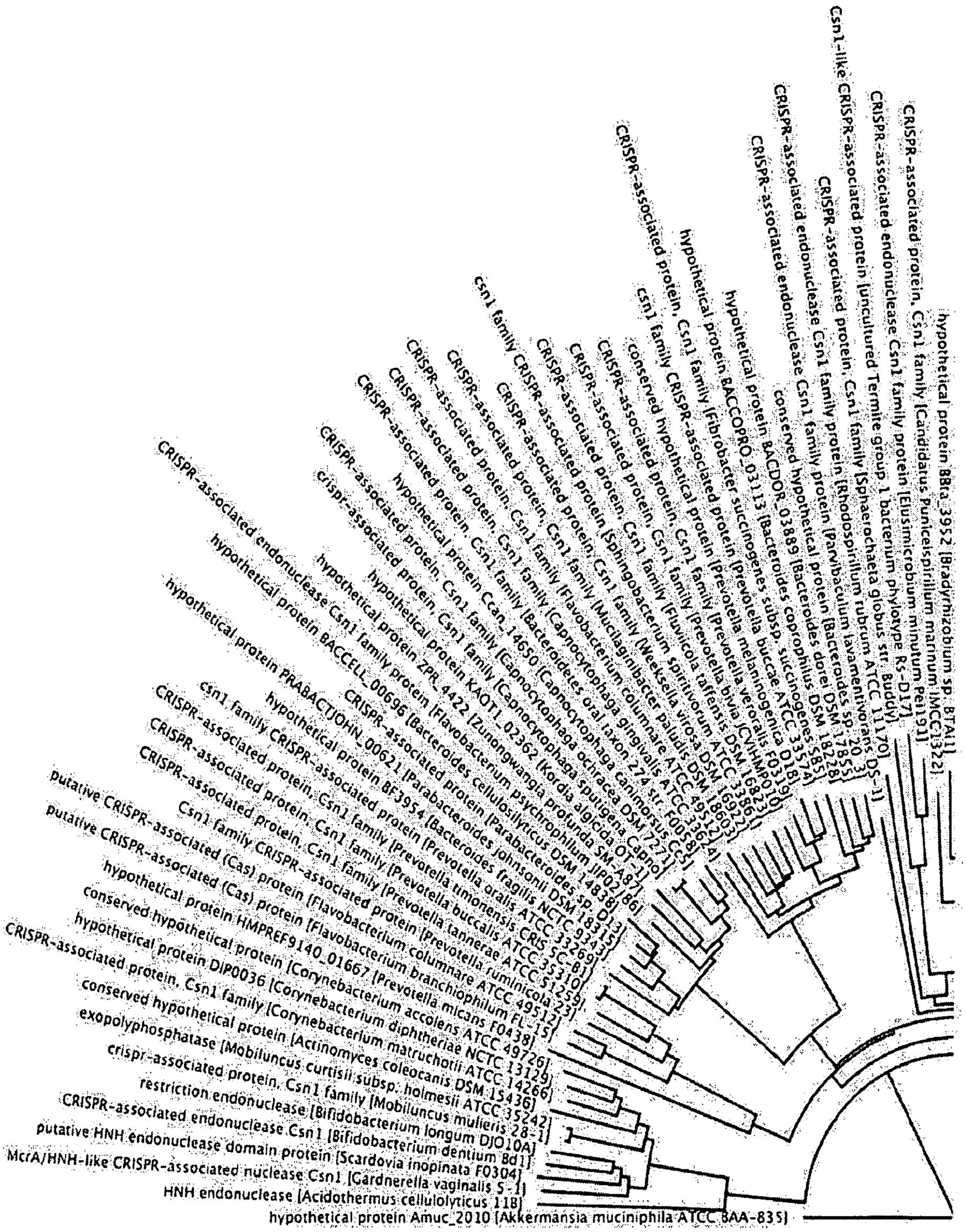

a
b
Indel (%)
0
20
40
60
N.D.
N.D.
CRNA
48*
54*
67*
85*
protospacer 1 (EMX1)
protospacer 2 (EMX1)
protospacer 3 (EMX1)
protospacer 4 (PVALB)
protospacer 5 (PVALB)

FIG. 17A-B

FIG. 18


UCSC Genome Browser on Human Feb. 2009 (GRCh37/hg19) Assembly
move <<< << < > >> >>> zoom in 1.5x 3x 10x base zoom out 1.5x 3x 10x
chr12:2,614,008-008-2,675,748 61,741 bp. enter position, gene symbol or search terms go
chr12 (p13.33)
Scale
chr12:
hg19
2,620,000
2,630,000
2,640,000
20 kb
Human SpCas9 (PAM: NGG)
UCSC Genes (RefSeq, UniProt, CCDS, Rfam, tRNAs & Comparative Genomics)
CACNA1C


hypothetical protein BBta_3952 [Bradyrhizobium sp. BTAi1]
CRISPR-associated protein, Csn1 family [Candidatus Puniceispirillum marinum IMCC1322]
CRISPR-associated endonuclease Csn1 family protein [Elusimicrobium minutum Pei191]
CRISPR-associated protein [uncultured Termite group 1 bacterium phylotype Rs-D17]
CRISPR-associated protein, Csn1 family [Sphaerochaeta globus str. Buddy]
CRISPR-associated protein [Rhodospirillum rubrum ATCC 11170]
CRISPR-associated protein, Csn1 family protein [Parvibaculum lavamentivorans DS-1]
CRISPR-associated endonuclease Csn1 family protein [Bacteroides dorei DSM 17855]
conserved hypothetical protein BACCOPRO_03889 [Bacteroides coprophilus DSM 18228]
hypothetical protein BACCOPRO_03113 [Bacteroides subsp. succinogenes ATCC 33574]
hypothetical protein [Fibrobacter succinogenes subsp. succinogenes S85]
conserved hypothetical protein [Prevotella buccae ATCC 33574]
CRISPR-associated protein, Csn1 family [Prevotella melaninogenica F0319]
CRISPR-associated protein, Csn1 family [Prevotella veroralis F0319]
hypothetical protein [Prevotella bivia JCVIHMP010]
CRISPR-associated protein, Csn1 family [Prevotella bivia DSM 16823]
CRISPR-associated protein, Csn1 family [Prevotella tannerae DSM 16922]
CRISPR-associated protein, Csn1 family [Prevotella timonensis CRIS 5C-B1]
CRISPR-associated protein, Csn1 family [Prevotella buccalis ATCC 35310]
CRISPR-associated protein, Csn1 family [Prevotella ruminicola 23]
CRISPR-associated protein, Csn1 family [Prevotella oralis ATCC 33269]
CRISPR-associated protein, Csn1 family [Prevotella oralis ATCC 33317]
CRISPR-associated protein, Csn1 family [Prevotella oris F0302]
CRISPR-associated protein, Csn1 family [Prevotella sp. oral taxon 299 str. F0039]
CRISPR-associated protein, Csn1 family [Prevotella sp. oral taxon 472 str. F0295]
CRISPR-associated protein, Csn1 family [Prevotella sp. oral taxon 782 str. F0077

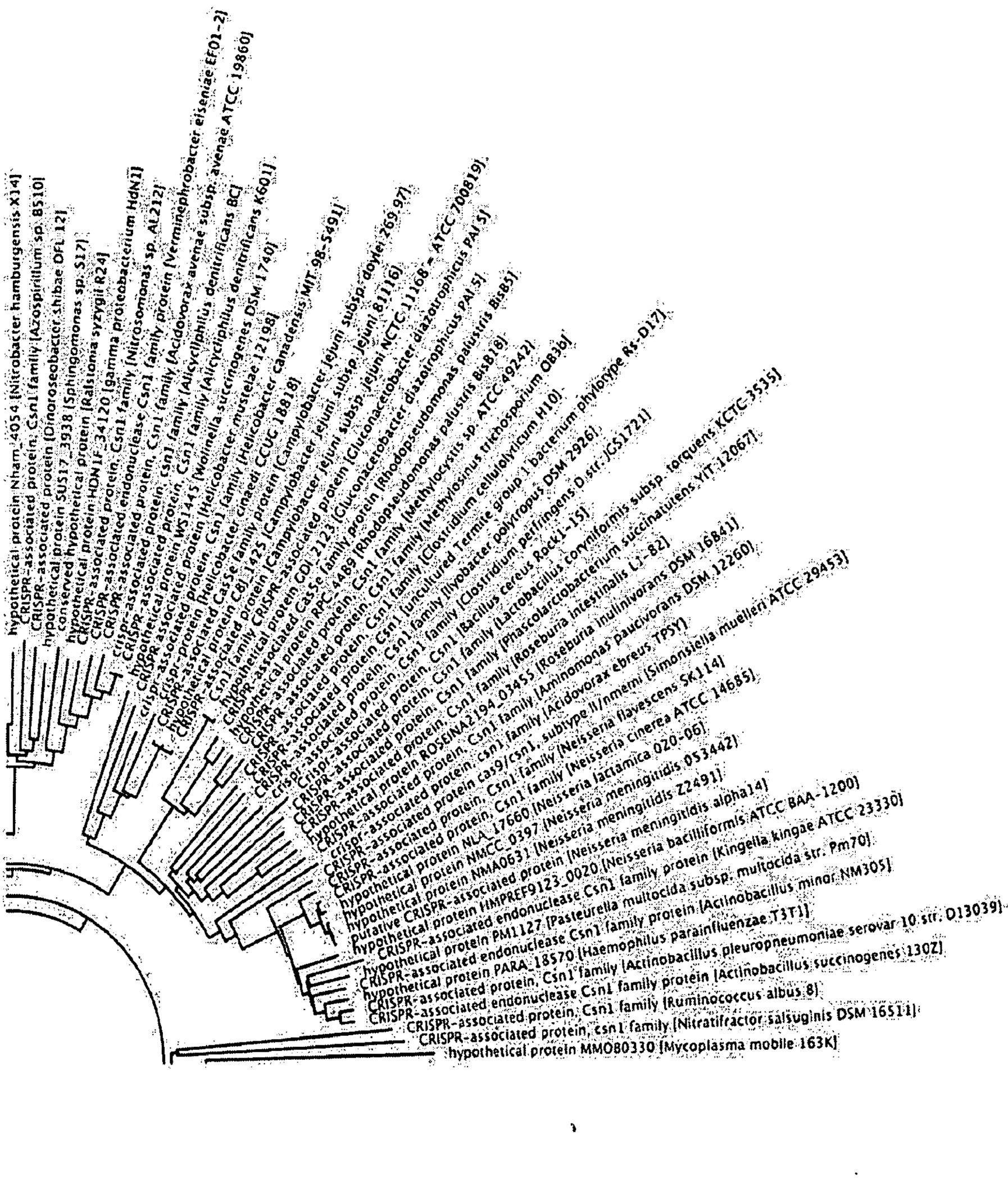

hypothetical protein Nham_4054 [Nitrobacter hamburgensis X14]
CRISPR-associated protein, Csn1 family [Azospirillum sp. B510]
hypothetical protein [Dinoroseobacter shibae DFL 12]
conserved hypothetical protein SU517_3938 [Sphingomonas sp. S17]
hypothetical protein HDN1F_34120 [gamma proteobacterium HdN1]
CRISPR-associated protein, Csn1 family [Nitrosomonas sp. AL212]
CRISPR-associated endonuclease Csn1 family [Verminephrobacter
hypothetical protein, Csn1 family [Acidovorax avenae subsp. avenae
CRISPR-associated protein WS1445 [Wolinella succinogenes DSM 1740]
CRISPR-associated protein, Csn1 family [Alicycliphilus denitrificans BC]
CRISPR-associated protein Csn1 family [Helicobacter cinaedi CCUG 18818]
hypothetical protein C8J_1425 [Campylobacter jejuni subsp. jejuni 81116]
CRISPR-associated protein [Campylobacter jejuni subsp. doylei 269.97]
hypothetical protein RPC_4489 [Rhodopseudomonas palustris BisB18]
CRISPR-associated protein, Csn1 family [Rhodopseudomonas palustris BisB5]
hypothetical protein [unicultured Termite group 1 bacterium phylotype Rs-D17]
CRISPR-associated protein, Csn1 family [Clostridium cellulolyticum H10]
hypothetical protein PM1127 [Pasteurella multocida subsp. multocida str. Pm70]
CRISPR-associated endonuclease Csn1 family protein [Kingella kingae ATCC 23330]
hypothetical protein PARA_18570 [Haemophilus parainfluenzae T3T1]
CRISPR-associated endonuclease Csn1 family protein [Actinobacillus minor NM305]
hypothetical protein HMPREF9123_0020 [Neisseria bacilliformis ATCC BAA-1200]
hypothetical protein NMA0631 [Neisseria meningitidis alpha14]
hypothetical protein NMCC_0397 [Neisseria meningitidis Z2491]
hypothetical protein NLA_17660 [Neisseria lactamica 020-06]
CRISPR-associated protein, Csn1 family [Neisseria cinerea ATCC 14685]
CRISPR-associated protein cas9/csn1, subtype II/nmeni [Simonsiella muelleri ATCC 29453]
CRISPR-associated protein ROSEINA2194_03455 [Roseburia inulinivorans DSM 16841]
CRISPR-associated protein, Csn1 family [Aminomonas paucivorans DSM 12260]
CRISPR-associated protein, Csn1 family [Acidovorax ebreus, TPSY]
CRISPR-associated protein, Csn1 family [Neisseria flavescens SK114]
hypothetical protein [Phascolarctobacterium succinatutens YIT 12067]
CRISPR-associated protein, Csn1 family [Roseburia intestinalis L1-82]
CRISPR-associated protein, Csn1 family [Lactobacillus coryniformis subsp. torquens KCTC 3535]
CRISPR-associated protein, Csn1 family [Clostridium perfringens D str. JGS1721]
CRISPR-associated protein, Csn1 family [Bacillus cereus Rock1-15]
CRISPR-associated protein, Csn1 family [Ruminococcus albus 8]
CRISPR-associated endonuclease Csn1 family protein [Actinobacillus pleuropneumoniae serovar 10 str. D13039]
CRISPR-associated protein, Csn1 family [Actinobacillus succinogenes 130Z]
CRISPR-associated protein, csn1 family [Nitratifractor salsuginis DSM 16511]
hypothetical protein MMO80330 [Mycoplasma mobile 163K]
eiseniae EF01-2]
ATCC 19860]

FIG. 19B

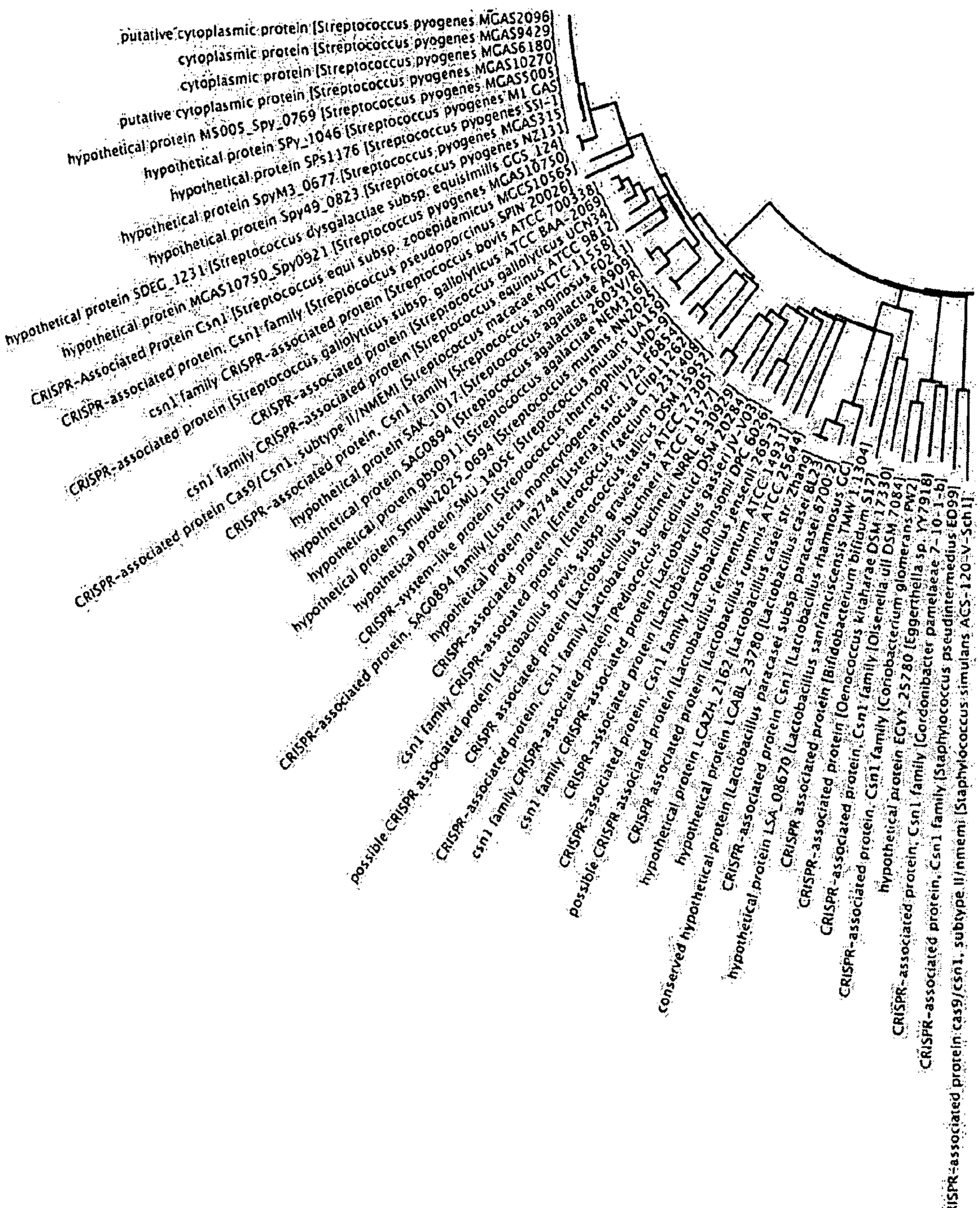
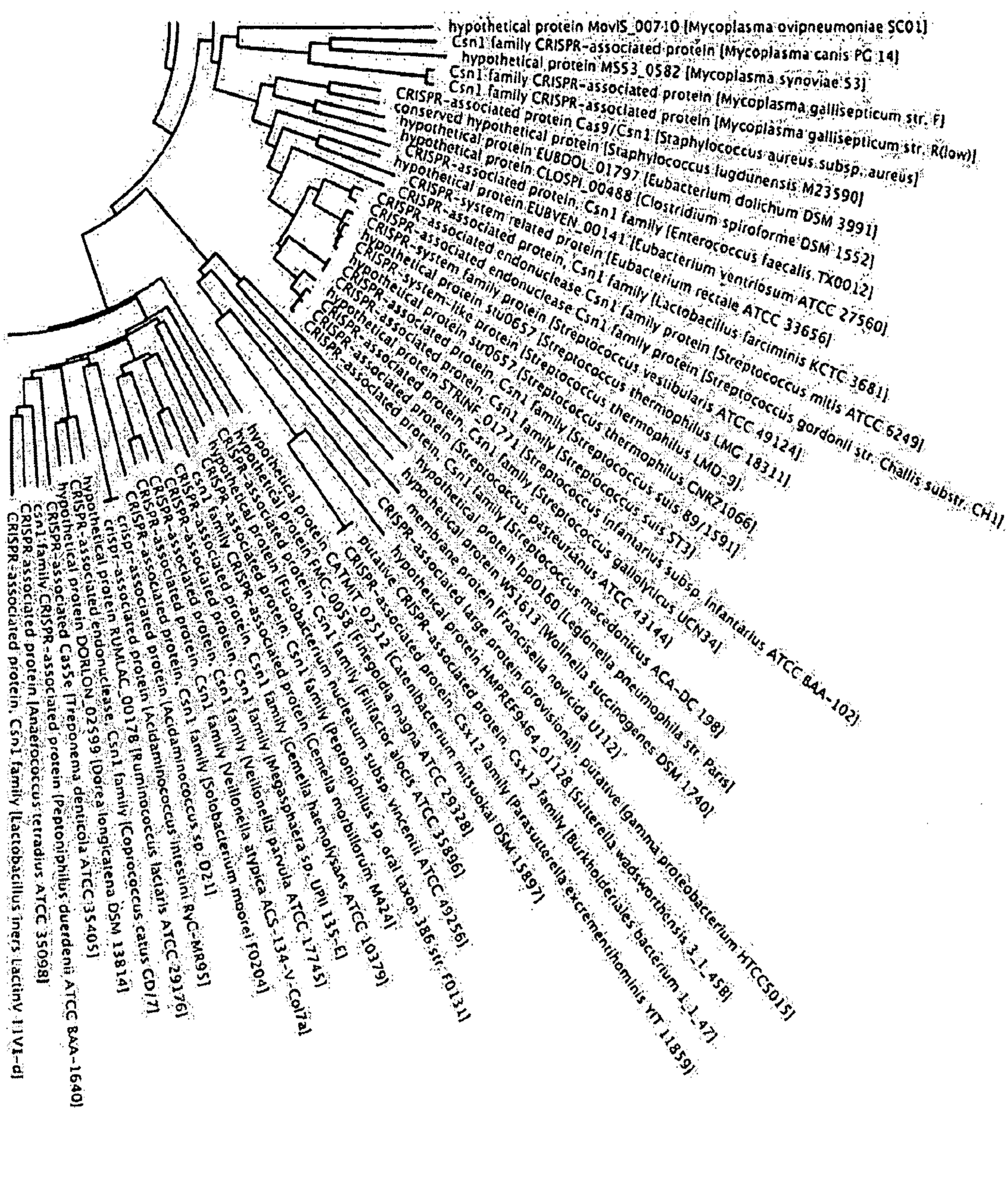

putative cytoplasmic protein [Streptococcus pyogenes MGAS2096]
cytoplasmic protein [Streptococcus pyogenes MGAS9429]
cytoplasmic protein [Streptococcus pyogenes MGAS6180]
putative cytoplasmic protein [Streptococcus pyogenes MGAS10270]
hypothetical protein MS005_Spy_0769 [Streptococcus pyogenes MGAS5005]
hypothetical protein SPy_1046 [Streptococcus pyogenes M1 GAS]
hypothetical protein SPs1176 [Streptococcus pyogenes SSI-1]
hypothetical protein SpyM3_0677 [Streptococcus pyogenes MGAS315]
hypothetical protein Spy49_0823 [Streptococcus pyogenes NZ131]
hypothetical protein SDEG_1231 [Streptococcus dysgalactiae subsp. equisimilis GGS_124]
hypothetical protein MGAS10750_Spy0921 [Streptococcus pyogenes MGAS10750]
CRISPR-Associated Protein Csn1 [Streptococcus equi subsp. zooepidemicus MGCS10565]
CRISPR-associated protein, Csn1 family [Streptococcus pseudoporcinus SPIN 20026]
csn1 family CRISPR-associated protein [Streptococcus gallolyticus subsp. gallolyticus ATCC 700338]
CRISPR-associated protein [Streptococcus bovis ATCC BAA-2069]
CRISPR-associated protein Cas9/Csn1, subtype II/NMEMI [Streptococcus gallolyticus UCN34]
csn1 family CRISPR-associated protein [Streptococcus gallolyticus ATCC 9812]
CRISPR-associated protein [Streptococcus macacae NCTC 11558]
hypothetical protein SAK_1017 [Streptococcus equinus ATCC 700338]
hypothetical protein SAG0894 [Streptococcus agalactiae 2603V/R]
hypothetical protein gbs0911 [Streptococcus agalactiae A909]
hypothetical protein SmuNN2025_0694 [Streptococcus agalactiae NEM316]
hypothetical protein SMU_1405c [Streptococcus anginosus F0211]
CRISPR-system-like protein [Streptococcus mutans NN2025]
CRISPR-associated protein, SAG0894 family [Streptococcus thermophilus LMD-9]
hypothetical protein lin2744 [Listeria monocytogenes str. 1/2a F6854]
CRISPR-associated protein [Enterococcus faecium 1,231,408]
CRISPR-associated protein [Lactobacillus brevis subsp. gravesensis ATCC 11577]
CRISPR-associated protein [Lactobacillus buchneri NRRL B-30929]
hypothetical protein [Pediococcus acidilactici DSM 20284]
CRISPR-associated protein, Csn1 family [Lactobacillus johnsonii DPC 6026]
CRISPR-associated protein [Lactobacillus gasseri JV-V03]
hypothetical protein LCABL_23780 [Lactobacillus fermentum ATCC 14931]
hypothetical protein LCAZH_2162 [Lactobacillus ruminis ATCC 25644]
CRISPR-associated protein, Csn1 [Lactobacillus casei str. Zhang]
hypothetical protein [Lactobacillus paracasei subsp. paracasei 8L23]
CRISPR-associated protein [Lactobacillus casei 6700-2]
CRISPR-associated protein [Lactobacillus sanfranciscensis TMW 1.1304]
CRISPR-associated protein [Bifidobacterium bifidum S17]
CRISPR-associated protein, Csn1 family [Oenococcus kitaharae DSM 17330]
hypothetical protein [Olsenella uli DSM 7084]
CRISPR-associated protein, Csn1 family [Coriobacterium glomerans PW2]
hypothetical protein EGYY_25780 [Eggerthella sp. YY7918]
CRISPR-associated protein, Csn1 family [Gordonibacter pamelaeae 7-10-1-b]
CRISPR-associated protein, Csn1 family [Staphylococcus pseudintermedius ED99]
CRISPR-associated protein Cas9/Csn1, subtype II/nmemi [Staphylococcus simulans ACS-120-V-Sch1]

FIG. 19C


hypothetical protein MovIS_007 10 [Mycoplasma ovipneumoniae SC01]
Csn1 family CRISPR-associated protein [Mycoplasma canis PG 14]
hypothetical protein MS53_0582 [Mycoplasma synoviae 53]
Csn1 family CRISPR-associated protein [Mycoplasma gallisepticum str. F]
Csn1 family CRISPR-associated protein [Mycoplasma gallisepticum str. R(low)]
CRISPR-associated protein Cas9/Csn1 [Staphylococcus aureus subsp. aureus]
conserved hypothetical protein [Staphylococcus lugdunensis M23590]
hypothetical protein EUBDOL_01797 [Eubacterium dolichum DSM 3991]
hypothetical protein CLOSPI_00488 [Clostridium spiroforme DSM 1552]
CRISPR-associated protein, Csn1 family [Enterococcus faecalis TX0012]
hypothetical protein EUBVEN_00141 [Eubacterium ventriosum ATCC 27560]
CRISPR-system related protein [Eubacterium rectale ATCC 33656]
CRISPR-associated endonuclease Csn1 family [Lactobacillus farciminis KCTC 3681]
CRISPR-system family protein [Streptococcus vestibularis ATCC 49124]
hypothetical protein stu0657 [Streptococcus thermophilus LMG 18311]
CRISPR-system-like protein str0657 [Streptococcus thermophilus CNRZ1066]
hypothetical protein, Csn1 family [Streptococcus thermophilus LMD-9]
CRISPR-associated protein, Csn1 family [Streptococcus suis 89/1591]
hypothetical protein STRINF_01771 [Streptococcus infantarius subsp. infantarius ATCC BAA-102]
CRISPR-associated protein, Csn1 family [Streptococcus pasteurianus ATCC 43144]
hypothetical protein [Streptococcus macedonicus ACA-DC 198]
membrane protein WS1613 [Wolinella succinogenes DSM 1740]
hypothetical protein lpp0160 [Legionella pneumophila str. Paris]
CRISPR-associated large protein HMPREF9464_01128 [Sutterella wadsworthensis 3_1_45B]
hypothetical protein (provisional) , putative (gamma proteobacterium HTCC5015]
putative CRISPR-associated protein, Csx12 family [Burkholderiales bacterium 1_1_47]
CRISPR-associated protein, Csx12 family [Parasutterella excrementihominis YIT 11859]
hypothetical protein, Csx12 family [Parasutterella secunda DSM 15897]
hypothetical protein FMG_0058 [Finegoldia magna ATCC 29328]
hypothetical protein CATMIT_02512 [Catenibacterium mitsuokai DSM 15897]
CRISPR-associated protein, Csn1 family [Filifactor alocis ATCC 35896]
hypothetical protein [Peptoniphilus sp. oral taxon 386 str. F0131]
CRISPR-associated protein, Csn1 family [Peptoniphilus sp. oral taxon 386 str. F0131]
hypothetical protein [Gemella haemolysans ATCC 10379]
CRISPR-associated protein (Gemella haemolysans ATCC 10379)
hypothetical protein [Megasphaera sp. UPII 135-E]
CRISPR-associated protein, Csn1 family [Megasphaera sp. UPII 135-E]
hypothetical protein [Veillonella parvula ATCC 17745]
CRISPR-associated protein, Csn1 family [Veillonella atypica ACS-134-V-Col7a]
CRISPR-associated protein, Csn1 family [Veillonella atypica ACS-134-V-Col7a]
csn1 family CRISPR-associated protein, Csn1 family [Veillonella parvula ATCC 17745]
CRISPR-associated protein, Csn1 family [Veillonella atypica ACS-134-V-Col7a

CA 02894681 2015-06-10

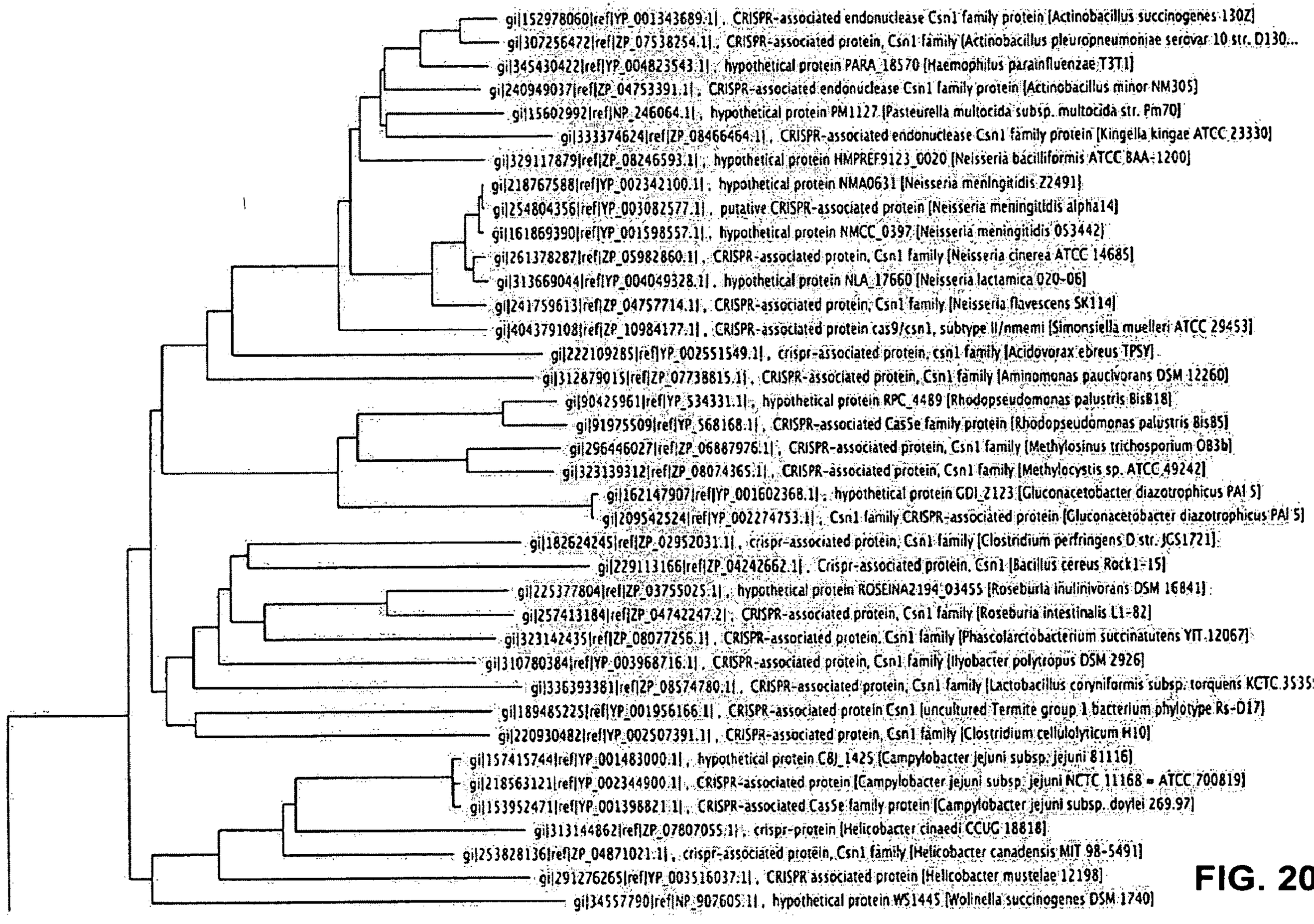


FIG. 20A

CA 02894681 2015-06-10

SUBSTITUTE SHEET (RULE 26)

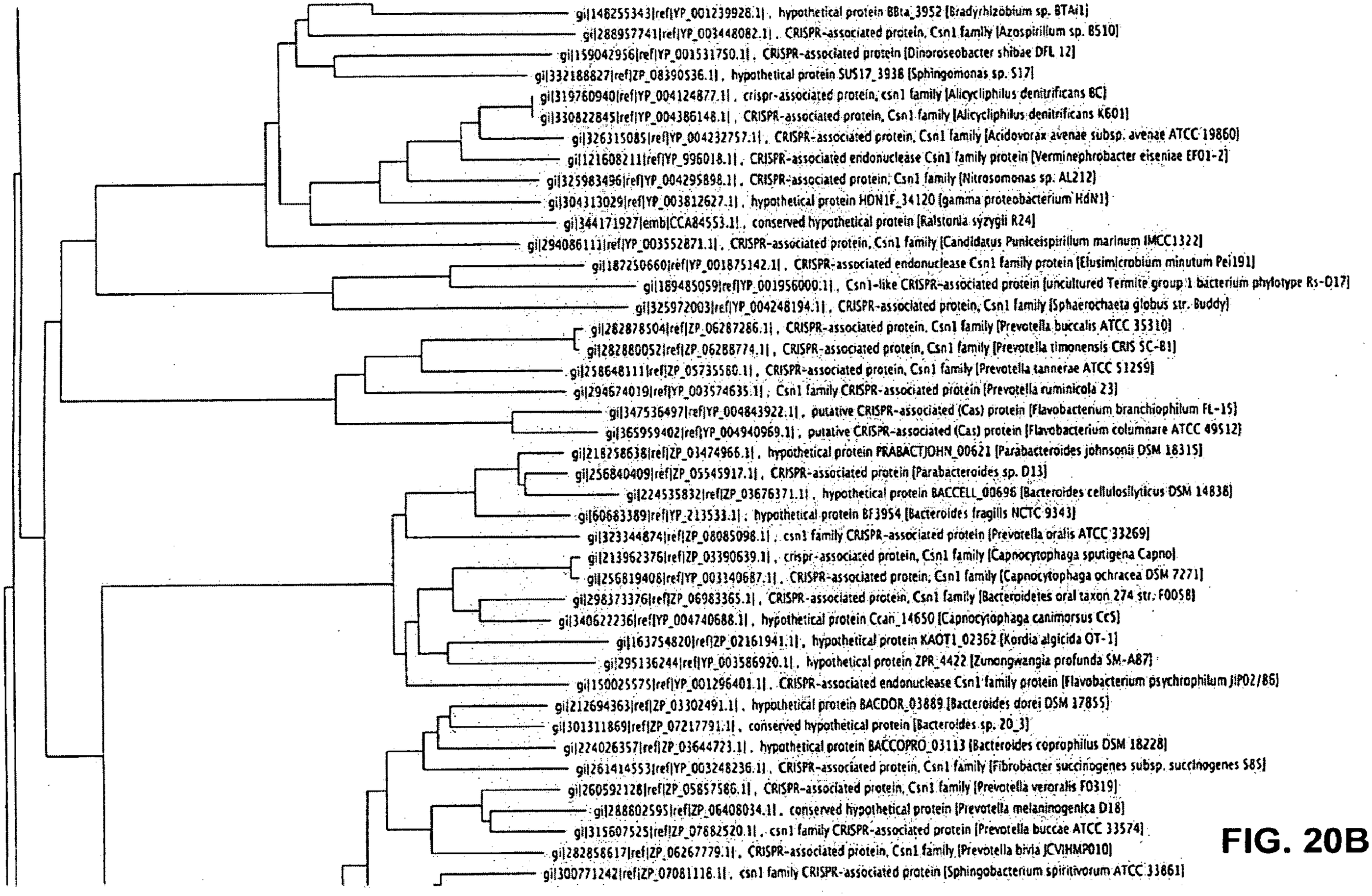


FIG. 20B

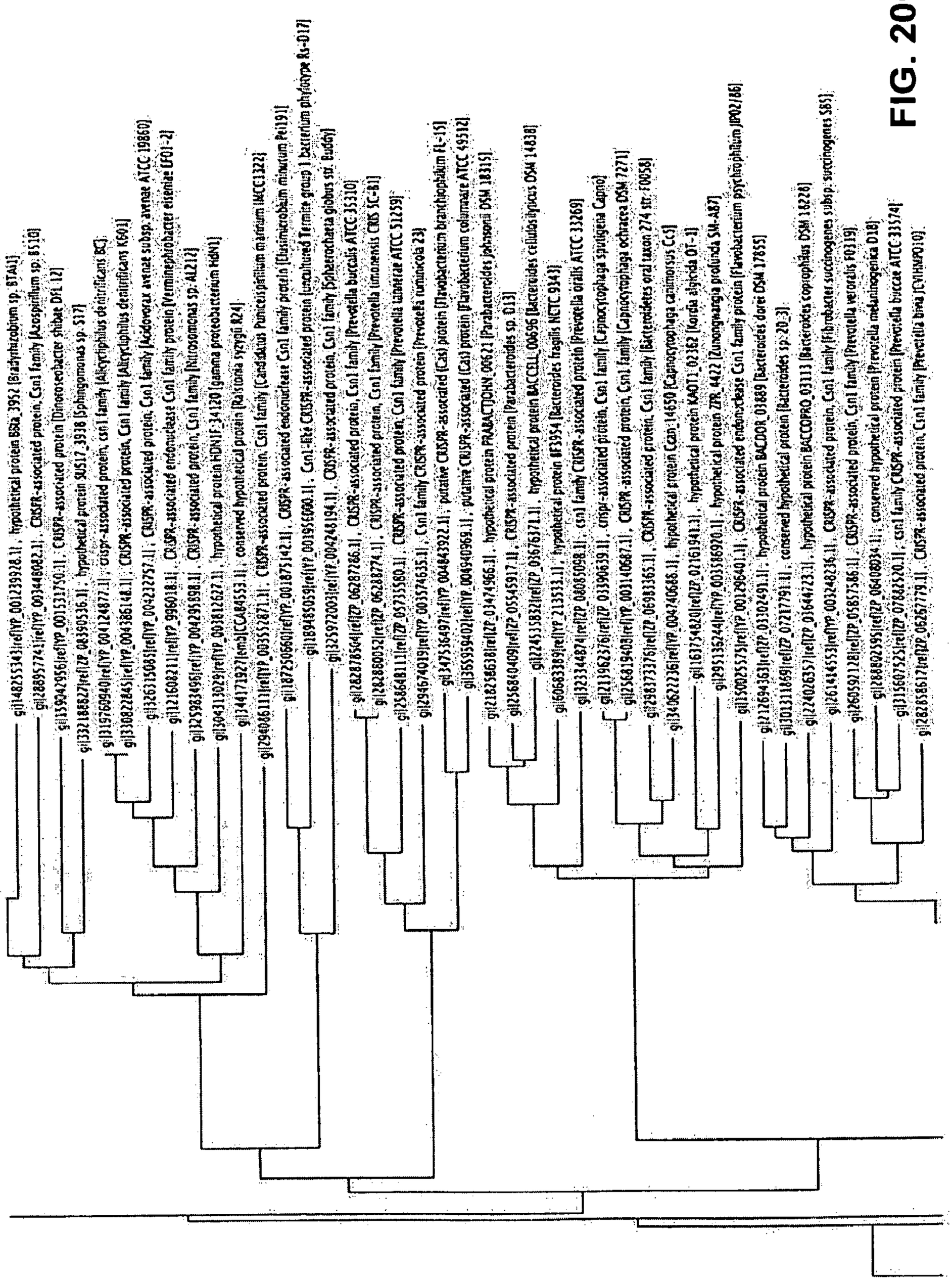

gi|148255343|ref|YP_001239928.1| hypothetical protein BBta_3952 [Bradyrhizobium sp. BTAi1]
gi|288957741|ref|YP_003446082.1| CRISPR-associated protein, Csn1 family [Azospirillum sp. B510]
gi|159042956|ref|YP_001531750.1| CRISPR-associated protein [Dinoroseobacter shibae DFL 12]
gi|332188827|ref|ZP_08390536.1| hypothetical protein SUS17_3938 [Sphingomonas sp. S17]
gi|315769540|ref|YP_004124877.1| cas9-associated protein, csn1 family [Alicycliphilus denitrificans BC]
gi|330822845|ref|YP_004386148.1| CRISPR-associated protein, Csn1 family [Alicycliphilus denitrificans K601]
gi|326315085|ref|YP_004232757.1| CRISPR-associated protein, Csn1 family [Acidovorax avenae subsp. avenae ATCC 19860]
gi|121608211|ref|YP_996018.1| CRISPR-associated endonuclease Csn1 family protein [Verminephrobacter eiseniae EF01-2]
gi|325983496|ref|YP_004295898.1| CRISPR-associated protein, Csn1 family [Nitrosomonas sp. AL212]
gi|304311029|ref|YP_003812627.1| hypothetical protein HDN1F_34120 [gamma proteobacterium HdN1]
gi|344171927|emb|CCA84553.1| conserved hypothetical protein [Ralstonia syzygii R24]
gi|296086111|ref|YP_003552871.1| CRISPR-associated protein, Csn1 family [Candidatus Puniceispirillum marinum IMCC1322]
gi|187750142.1| CRISPR-associated endonuclease Csn1 family protein [Dissimicrobium minutum Pei19]
gi|189485059|ref|YP_001875142.1| Csn1-like CRISPR-associated protein, Csn1 family [Sphaerochaeta globus str. Buddy]
gi|325972003|ref|YP_004244194.1| CRISPR-associated protein, Csn1 family [Sphaerochaeta globus str. Buddy]
gi|282878564|ref|ZP_06287286.1| CRISPR-associated protein, Csn1 family [Prevotella buccalis ATCC 35310]
gi|282860052|ref|ZP_06288774.1| CRISPR-associated protein, Csn1 family [Prevotella timonensis CRIS 5C-B1]
gi|258648111|ref|ZP_05735580.1| CRISPR-associated protein, Csn1 family [Prevotella tannerae ATCC 51259]
gi|294674019|ref|YP_003574635.1| CRISPR-associated protein, Csn1 family [Prevotella ruminicola 23]
gi|347536497|ref|YP_004843922.1| putative CRISPR-associated (Cas) protein [Flavobacterium branchiophilum FL-15]
gi|355959402|ref|YP_004940969.1| putative CRISPR-associated (Cas) protein [Flavobacterium columnare ATCC 49512]
gi|323344874|ref|ZP_08085098.1| hypothetical protein PRABACTJOHN_06621 [Parabacteroides johnsonii DSM 18315]
gi|218256638|ref|ZP_03474966.1| hypothetical protein BF3954 [Bacteroides fragilis NCTC 9343]
gi|325685917.1| CRISPR-associated protein [Parabacteroides sp. D13]
gi|325685098.1| csn1 family CRISPR-associated protein [Prevotella oralis ATCC 33269]
gi|ZP_03676371.1| hypothetical protein BACCELL_00696 [Bacteroides cellulosilyticus DSM 14838]
gi|255619403|ref|YP_003114068.1| crispr-associated protein, Csn1 family [Capnocytophaga sputigena Capno]
gi|325683365.1| CRISPR-associated protein, Csn1 family [Capnocytophaga ochracea DSM 7271]
gi|325983365.1| CRISPR-associated protein, Csn1 family [Bacteroidetes oral taxon 274 str. F0058]
gi|298373376|ref|ZP_06983365.1| hypothetical protein Csan_14650 [Capnocytophaga canimorsus Cc5]
gi|340622236|ref|YP_004740648.1| hypothetical protein KAOT1_02382 [Kordia algicida OT-1]
gi|161754820|ref|ZP_02161941.1| hypothetical protein ZPR_4422 [Zunongwangia profunda SM-A87]
gi|295135244|ref|YP_003586920.1| hypothetical protein BACCOPRO_03113 [Bacteroides coprophilus DSM 18228]
gi|150025575|ref|YP_001296401.1| CRISPR-associated endonuclease Csn1 family protein [Flavobacterium psychrophilum JIP02/86]
gi|212694363|ref|ZP_03302491.1| hypothetical protein BACDOR_01889 [Bacteroides dorei DSM 17855]
gi|301311869|ref|ZP_07217791.1| conserved hypothetical protein [Bacteroides sp. 20_3]
gi|224026357|ref|ZP_03644723.1| hypothetical protein BACCOPRO_03113 [Bacteroides coprophilus DSM 18228]
gi|261414553|ref|YP_003248236.1| CRISPR-associated protein, Csn1 family [Fibrobacter succinogenes subsp. succinogenes S85]
gi|260592128|ref|ZP_05857586.1| CRISPR-associated protein, Csn1 family [Prevotella veroralis F0319]
gi|288802595|ref|ZP_06408034.1| conserved hypothetical protein [Prevotella melaninogenica D18]
gi|315607525|ref|ZP_07882520.1| csn1 family CRISPR-associated protein [Prevotella buccae ATCC 33574]
gi|282858617|ref|ZP_06267779.1| CRISPR-associated protein, Csn1 family [Prevotella bivia JCVIHMP010]

FIG. 20C

CA 02894681 2015-06-10

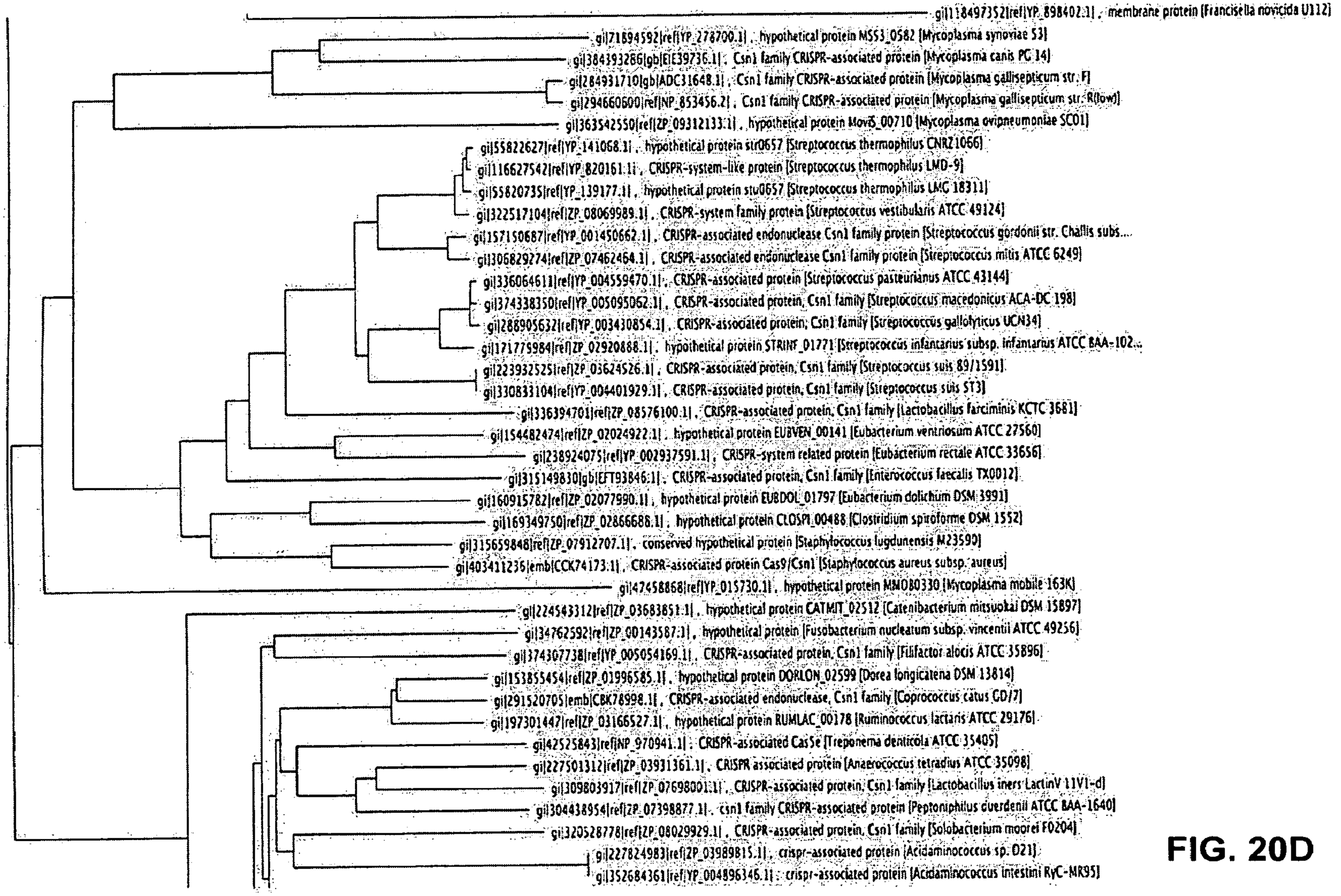


FIG. 20D

SUBSTITUTE SHEET (RULE 26)

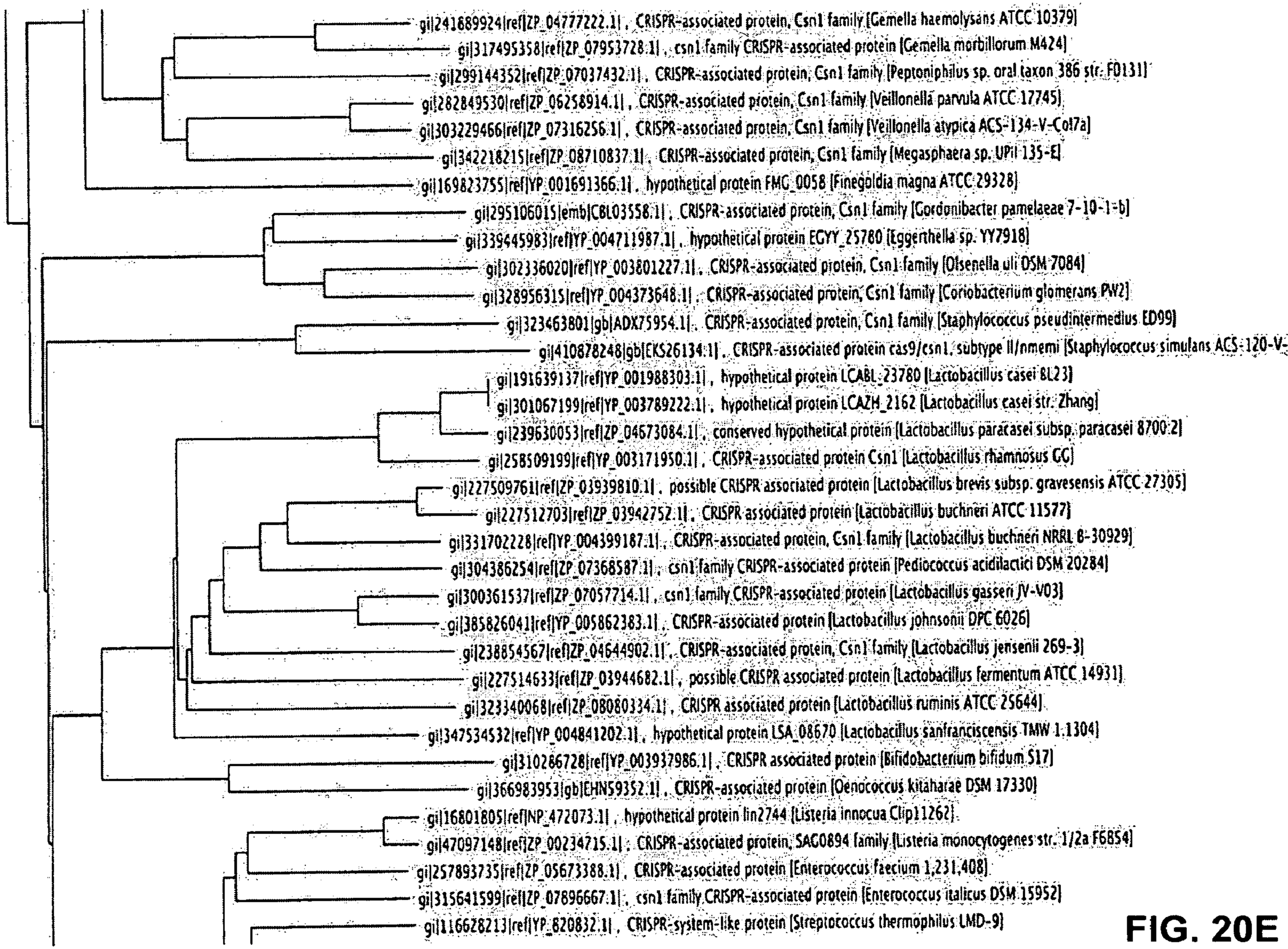


FIG. 20E

SUBSTITUTE SHEET (RULE 26)

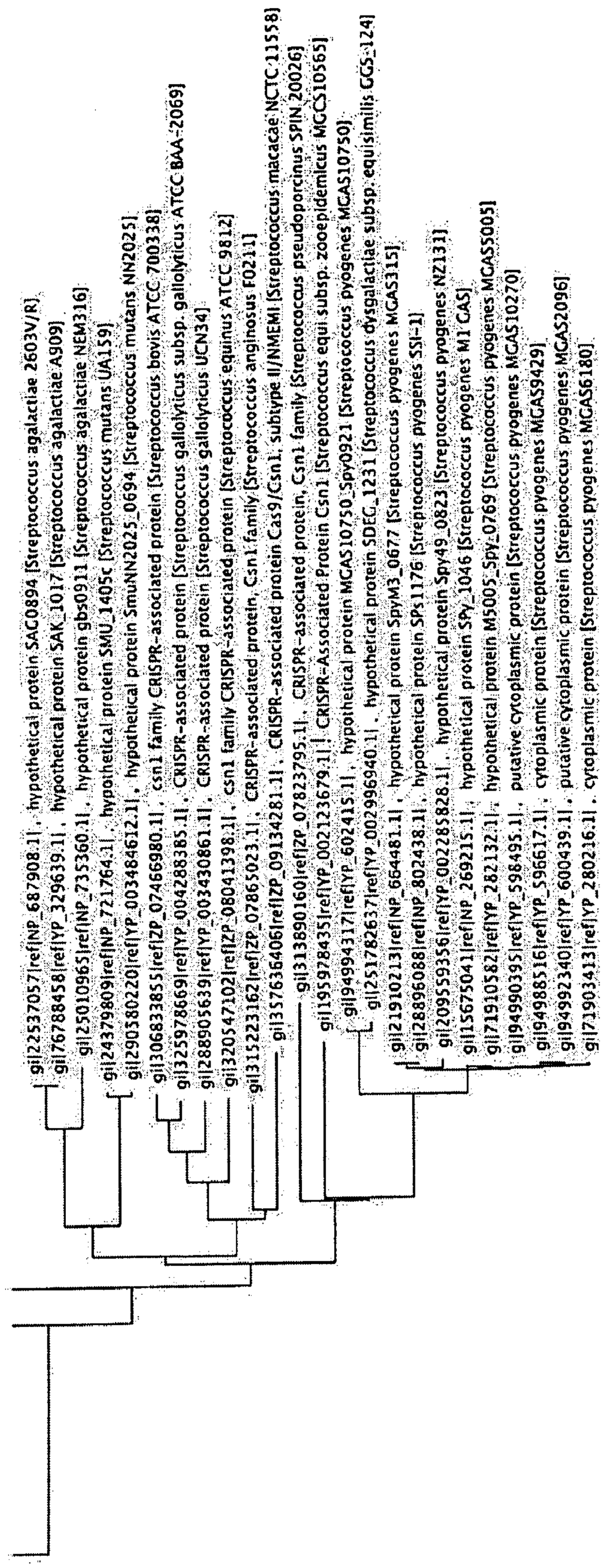

gi|22537057|ref|NP_687908.1|, hypothetical protein SAG0894 [Streptococcus agalactiae 2603V/R]
gi|76788458|ref|YP_329639.1|, hypothetical protein SAK_1017 [Streptococcus agalactiae A909]
gi|25010965|ref|NP_735360.1|, hypothetical protein gbs0911 [Streptococcus agalactiae NEM316]
gi|24379809|ref|NP_721764.1|, hypothetical protein SMU_1405c [Streptococcus mutans UA159]
gi|29058022O|ref|YP_003484612.1|, hypothetical protein SmuNN2025_0694 [Streptococcus mutans NN2025]
gi|30683385S|ref|ZP_07466980.1|, csn1 family CRISPR-associated protein [Streptococcus bovis ATCC 700338]
gi|32597866|ref|YP_004288385.1|, CRISPR-associated protein [Streptococcus gallolyticus subsp. gallolyticus ATCC BAA-2069]
gi|28890563|ref|YP_003430861.1|, CRISPR-associated protein [Streptococcus gallolyticus UCN34]
gi|32054710|ref|ZP_08041398.1|, CRISPR-associated protein, Csn1 family [Streptococcus anginosus F0211]
gi|31522316|ref|ZP_07865023.1|, CRISPR-associated protein Cas9/Csn1, subtype II/NMEMI [Streptococcus pseudoporcinus SPIN 20026]
gi|357636406|ref|ZP_09134281.1|, CRISPR-associated protein, Csn1 family [Streptococcus anginosus MGCS10565]
gi|313890160|ref|ZP_07823795.1|, CRISPR-associated protein, Csn1 family [Streptococcus pseudoporcinus SPIN 20026]
gi|195978435|ref|YP_002123679.1|, CRISPR-Associated Protein Csn1 [Streptococcus equi subsp. zooepidemicus MGCS10565]
gi|94994317|ref|YP_602415.1|, hypothetical protein MGAS10750_Spy0921 [Streptococcus pyogenes MGAS10750]
gi|251782637|ref|YP_002996940.1|, hypothetical protein SDEG_1231 [Streptococcus dysgalactiae subsp. equisimilis GGS_124]
gi|21910213|ref|NP_664481.1|, hypothetical protein SpyM3_0677 [Streptococcus pyogenes MGAS315]
gi|28896088|ref|NP_802438.1|, hypothetical protein SPs1176 [Streptococcus pyogenes SSI-1]
gi|20955935S|ref|YP_002285828.1|, hypothetical protein Spy49_0823 [Streptococcus pyogenes NZ131]
gi|15675041|ref|NP_269215.1|, hypothetical protein SPY_1046 [Streptococcus pyogenes M1 GAS]
gi|71910582|ref|YP_282132.1|, hypothetical protein M5005_Spy_0769 [Streptococcus pyogenes MGAS5005]
gi|94990395|ref|YP_598495.1|, putative cytoplasmic protein [Streptococcus pyogenes MGAS10270]
gi|94988516|ref|YP_596617.1|, cytoplasmic protein [Streptococcus pyogenes MGAS9429]
gi|94992340|ref|YP_600439.1|, putative cytoplasmic protein [Streptococcus pyogenes MGAS2096]
gi|71903413|ref|YP_280216.1|, cytoplasmic protein [Streptococcus pyogenes MGAS6180]

FIG. 20F

a

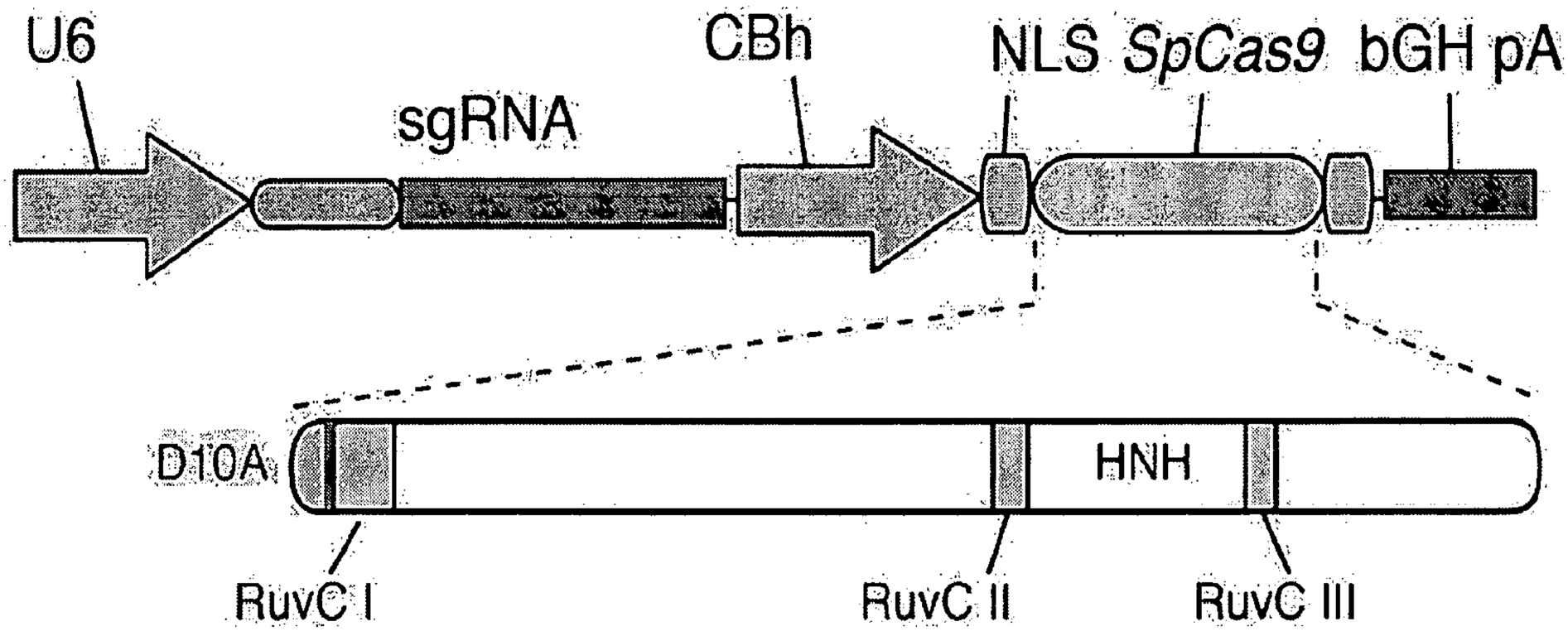


FIG. 21A

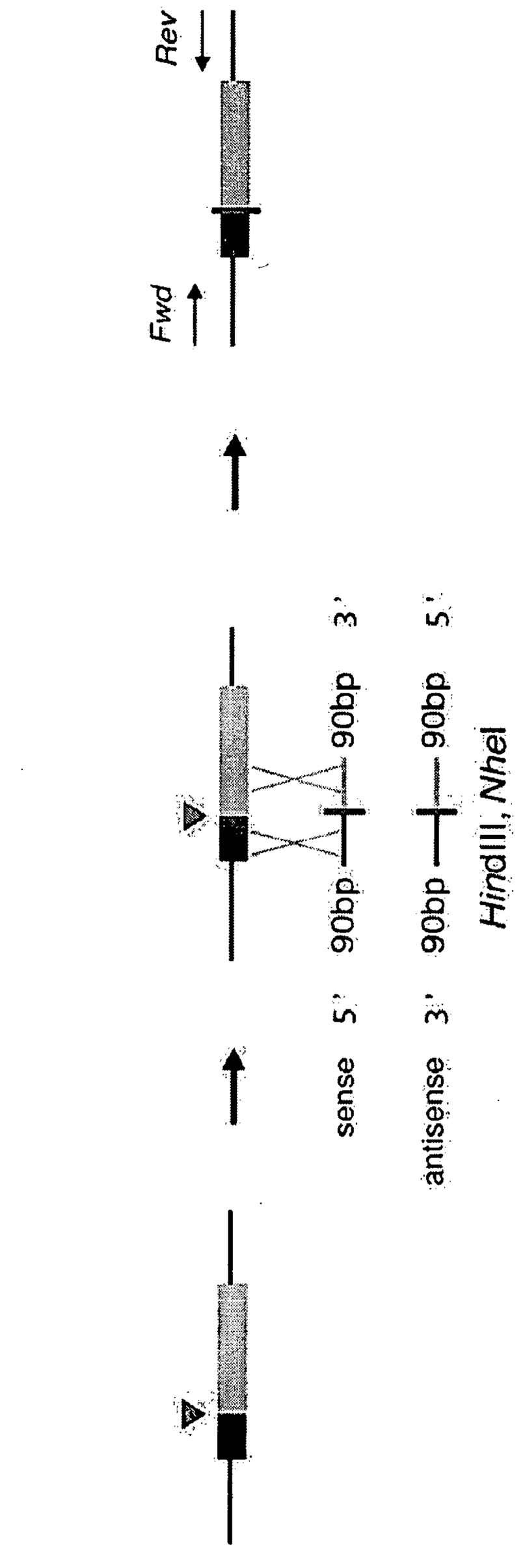

b
hEMX1 locus
ssODN
repair template
Rev
Fwd
90bp
90bp
3'
5'
HindIII, NheI
sense 5'
antisense 3'

FIG. 21B

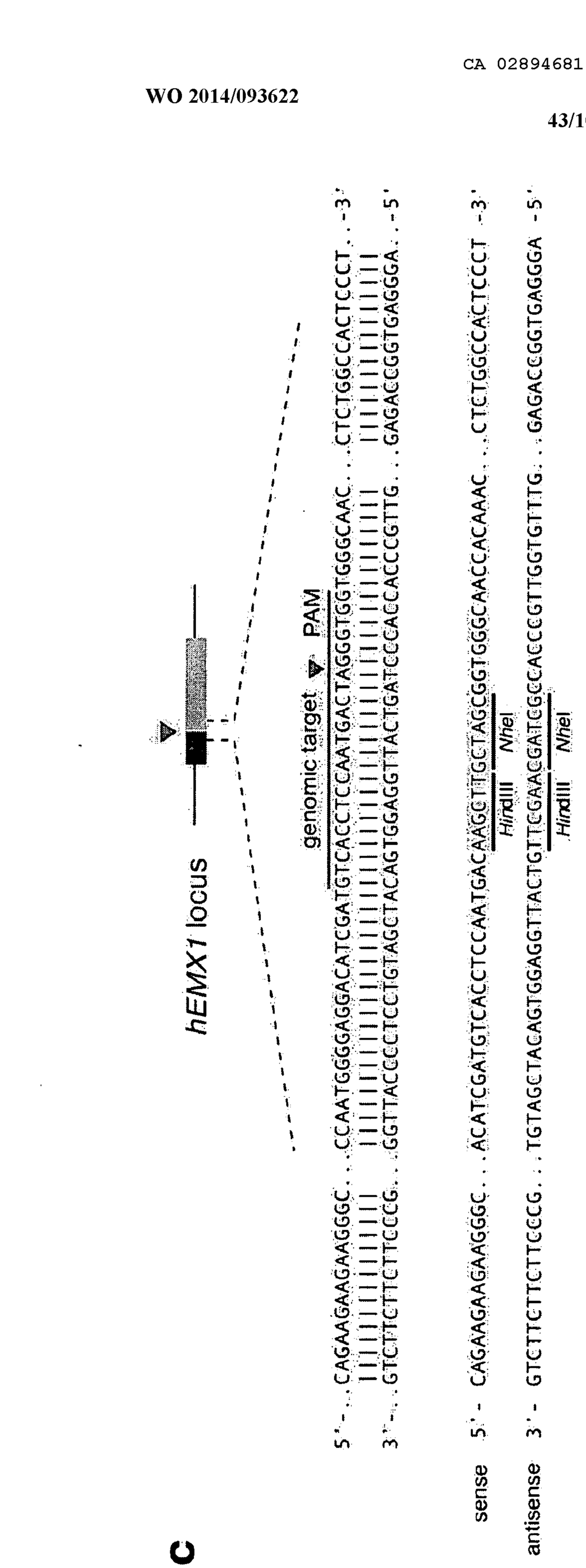

hEMX1 locus
genomic target
PAM
HindIII
NheI
HindIII
NheI
sense
antisense
5'-
3'-
5'-
3'-

FIG. 21C

d

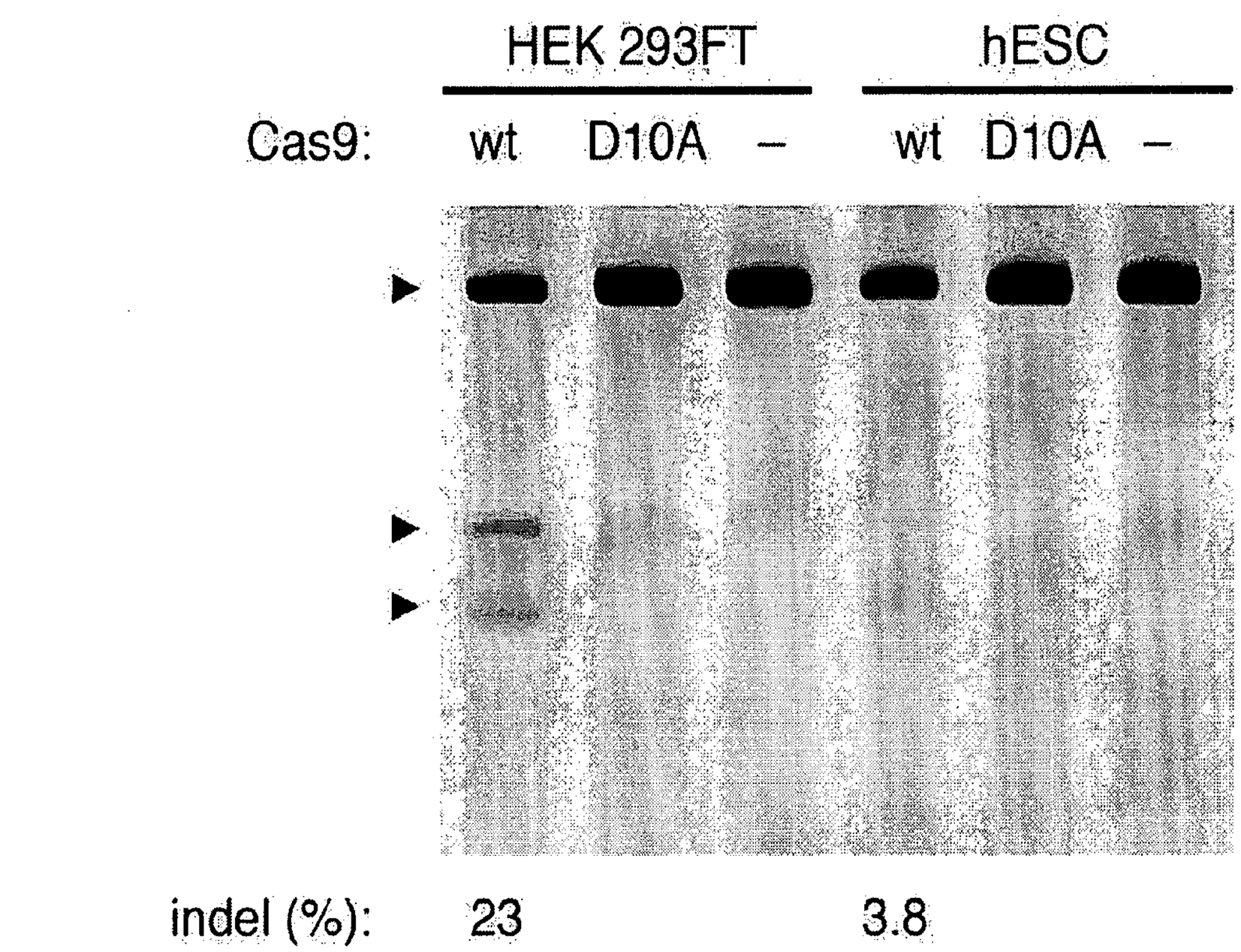


FIG. 21D

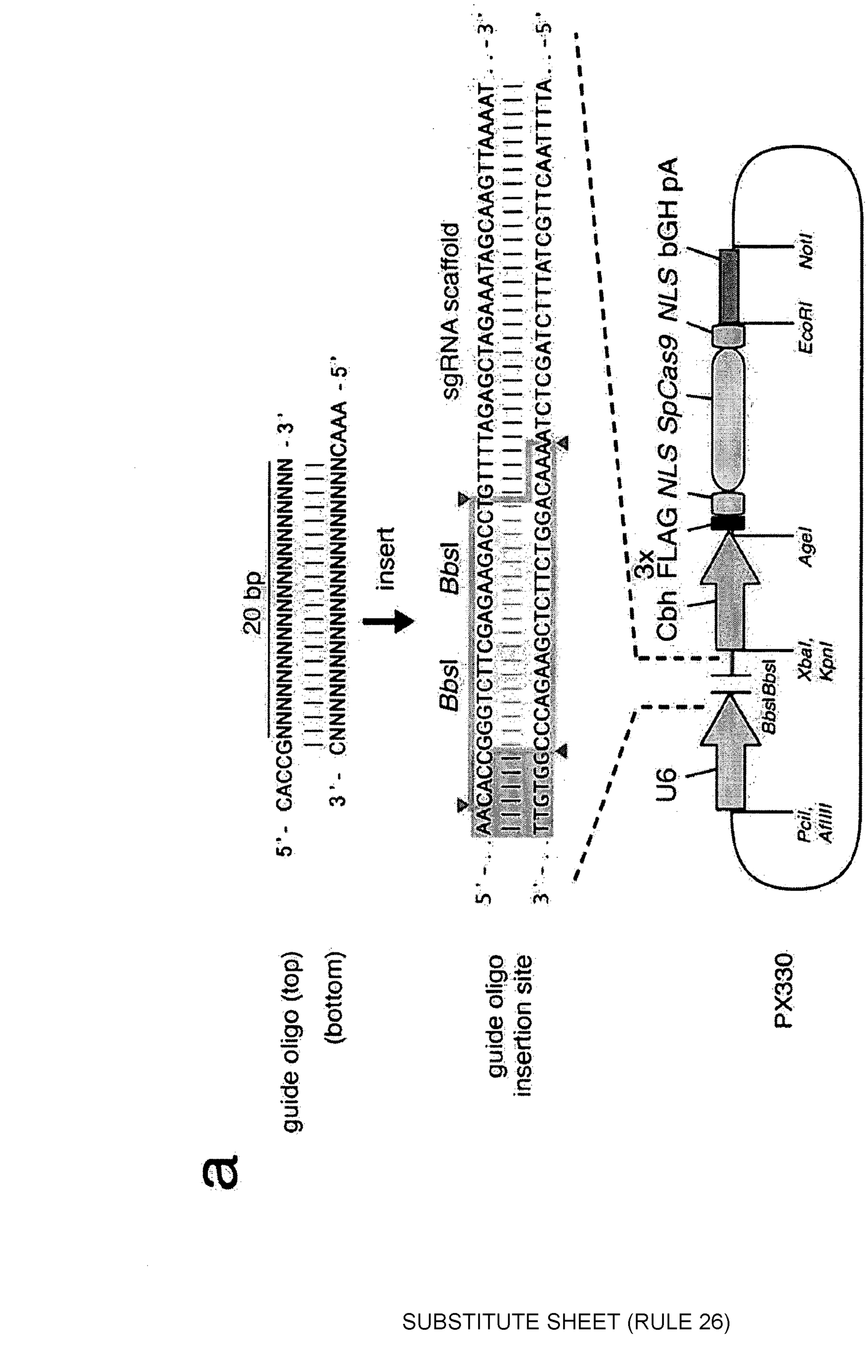

a
guide oligo (top)
(bottom)
guide oligo
insertion site
PX330
20 bp
5'- CACCGNNNNNNNNNNNNNNNNNNN - 3'
3'- CNNNNNNNNNNNNNNNNNNNCAAA - 5'
insert
BbsI
sgRNA scaffold
5'- ...AACACCGGGTCTTCAGAGACCTGTTTTAGAGCTAGAAATAGCAAG...
3'- ...TTGTGGCCCAGAAGTCTCTGGACAAAATCTCGATCTTTATCGTTC...
3x
Cbh
FLAG NLS SpCas9 NLS bGH pA
U6
BbsI BbsI
AgeI
EcoRI
NotI
PciI, AflIII
XbaI, KpnI

FIG. 22A

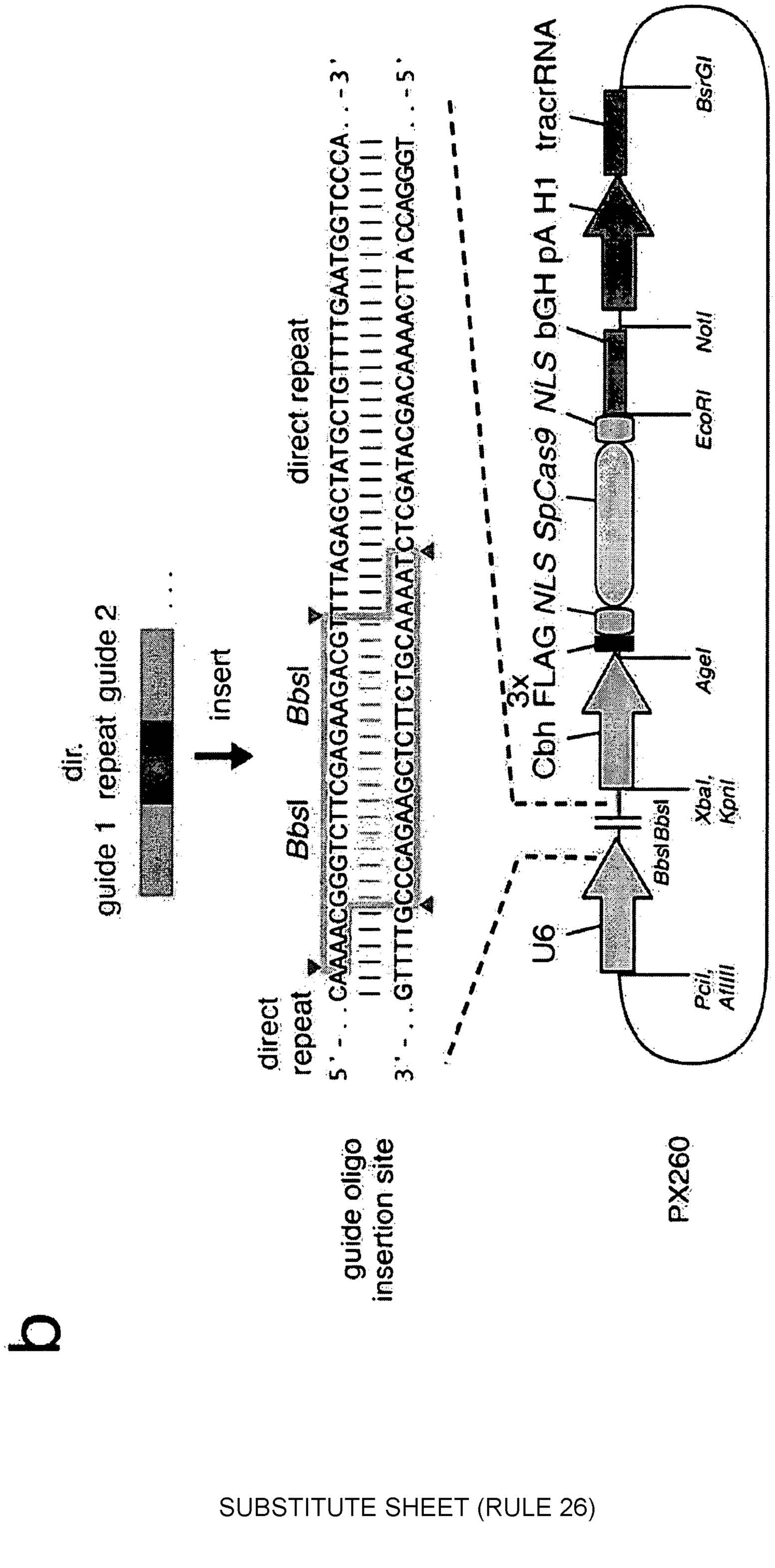

b
guide oligo insertion site
PX260
dir. repeat guide 2
guide 1
insert
direct repeat
BbsI
BbsI
5' - . . . CAAACGGGTCTTCGAGAAGACGTTTTAGAGCTATGCTGTTTTGAATGGTCCCA . . . - 3'
3' - . . . GTTTTGCCCAGAAGCTCTTCTGCAAAATCTCGATACGACAAAACTTACCAGGGT . . . - 5'
direct repeat
tracrRNA
BsrGI
U6
3x Cbh FLAG NLS SpCas9 NLS bGH pA H1
BbsI BbsI
XbaI, KpnI
AgeI
EcoRI
NotI
PciI, AflIII

FIG. 22B

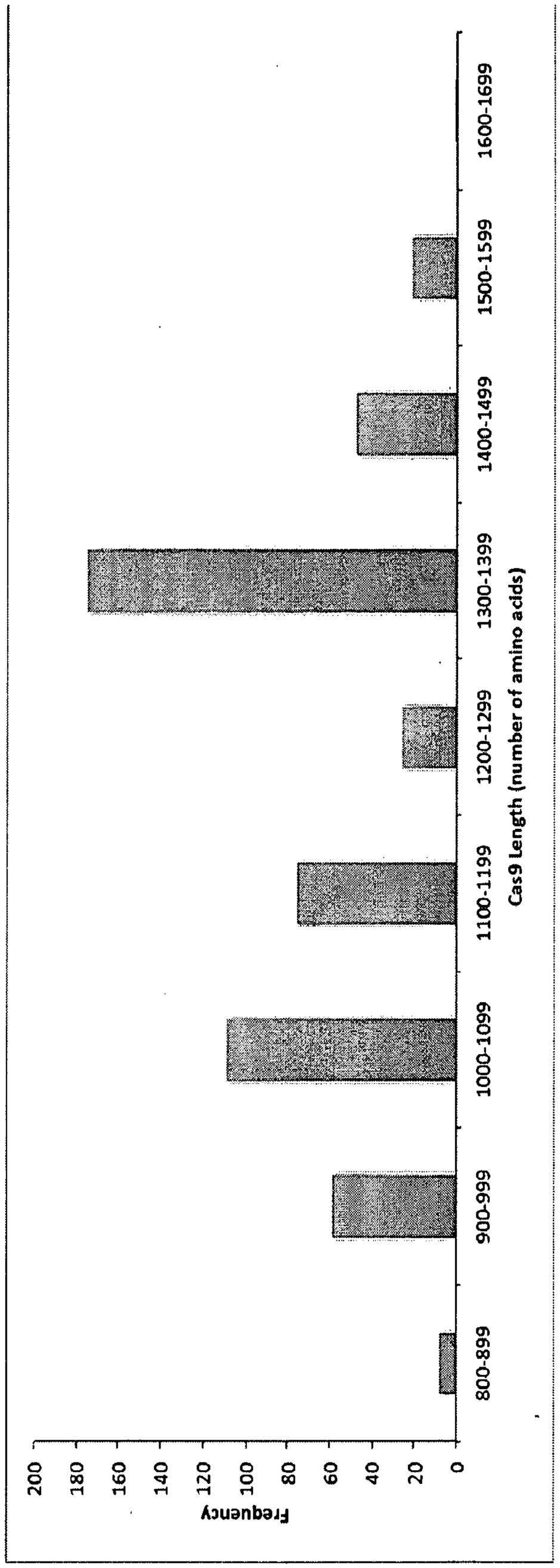

Cas9 Length (number of amino acids)
Frequency
800-899
900-999
1000-1099
1100-1199
1200-1299
1300-1399
1400-1499
1500-1599
1600-1699
0
20
40
60
80
100
120
140
160
180
200

FIG. 23

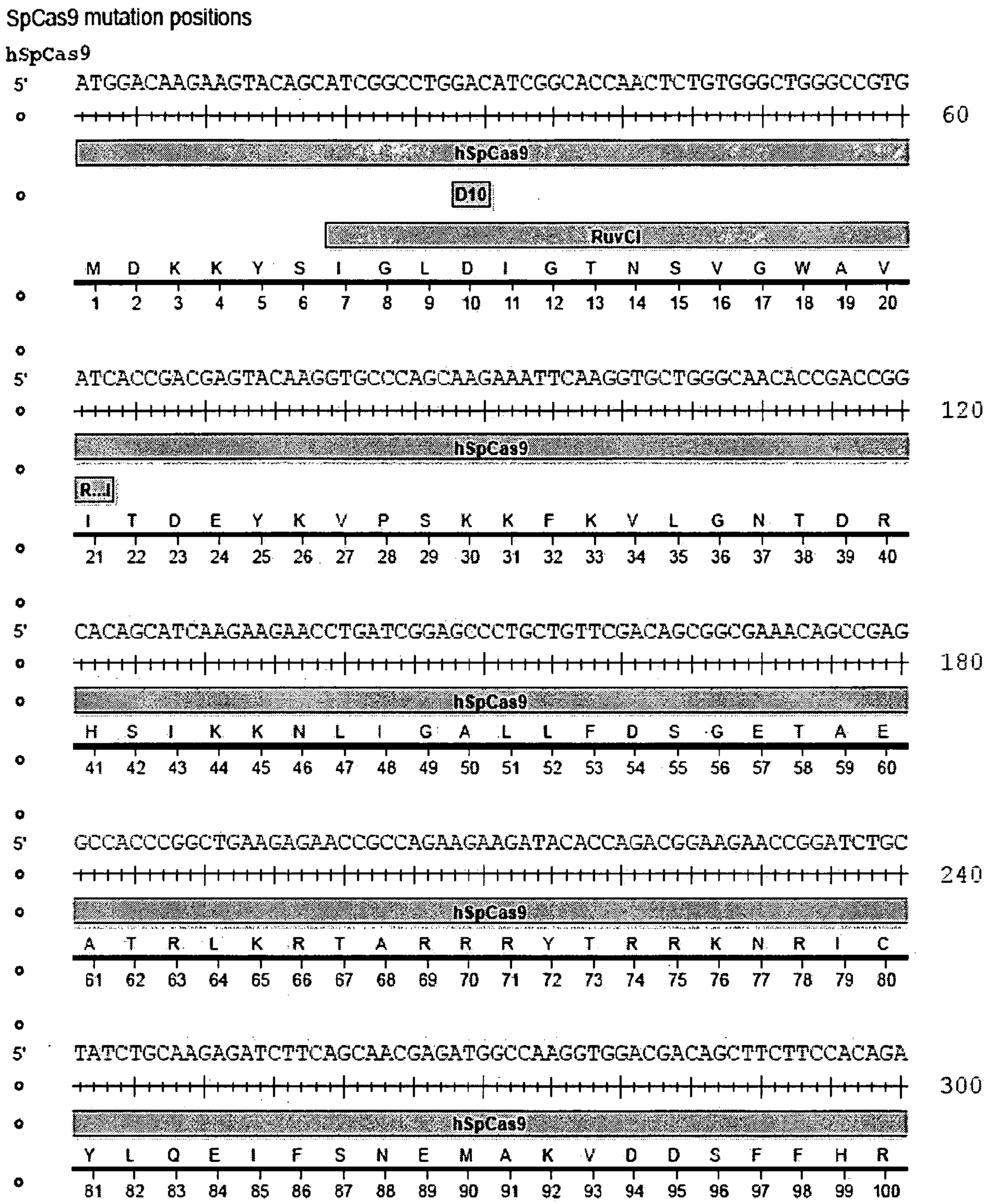

SpCas9 mutation positions
hSpCas9
5'
ATGGACAAGAAGTACAGCATCGGCCTGGACATCGGCACCAACTCTGTGGGCTGGGCCGTG
60
hSpCas9
D10
RuvCI
M D K K Y S I G L D I G T N S V G W A V
1 2 3 4 5 6 7 8 9 10 11 12 13 14 15 16 17 18 19 20
ATCACCGACGAGTACAAGGTGCCCAGCAAGAAATTCAAGGTGCTGGGCAACACCGACCGG
120
hSpCas9
R...I
I T D E Y K V P S K K F K V L G N T D R
21 22 23 24 25 26 27 28 29 30 31 32 33 34 35 36 37 38 39 40
CACAGCATCAAGAAGAACCTGATCGGAGCCCTGCTGTTCGACAGCGGCGAAACAGCCGAG
180
hSpCas9
H S I K K N L I G A L L F D S G E T A E
41 42 43 44 45 46 47 48 49 50 51 52 53 54 55 56 57 58 59 60
GCCACCCGGCTGAAGAGAACCGCCAGAAGAAGATACACCAGACGGAAGAACCGGATCTGC
240
hSpCas9
A T R L K R T A R R R Y T R R K N R I C
61 62 63 64 65 66 67 68 69 70 71 72 73 74 75 76 77 78 79 80
TATCTGCAAGAGATCTTCAGCAACGAGATGGCCAAGGTGGACGACAGCTTCTTCCACAGA
300
hSpCas9
Y L Q E I F S N E M A K V D D S F F H R
81 82 83 84 85 86 87 88 89 90 91 92 93 94 95 96 97 98 99 100

FIG. 24A

hSpCas9
5' CTGGAAGAGTCCTTCCTGGTGGAAGAGGATAAGAAGCACGAGCGGCACCCCATCTTCGGC
360
hSpCas9
L E E S F L V E E D K K H E R H P I F G
101 102 103 104 105 106 107 108 109 110 111 112 113 114 115 116 117 118 119 120
5' AACATCGTGGACGAGGTGGCCTACCACGAGAAGTACCCCACCATCTACCACCTGAGAAAG
420
hSpCas9
N I V D E V A Y H E K Y P T I Y H L R K
121 122 123 124 125 126 127 128 129 130 131 132 133 134 135 136 137 138 139 140
5' AAACTGGTGGACAGCACCGACAAGGCCGACCTGCGGCTGATCTATCTGGCCCTGGCCCAC
480
hSpCas9
K L V D S T D K A D L R L I Y L A L A H
141 142 143 144 145 146 147 148 149 150 151 152 153 154 155 156 157 158 159 160
5' ATGATCAAGTTCCGGGGCCACTTCCTGATCGAGGGCGACCTGAACCCCGACAACAGCGAC
540
hSpCas9
M I K F R G H F L I E G D L N P D N S D
161 162 163 164 165 166 167 168 169 170 171 172 173 174 175 176 177 178 179 180
5' GTGGACAAGCTGTTCATCCAGCTGGTGCAGACCTACAACCAGCTGTTCGAGGAAAACCCC
600
hSpCas9
V D K L F I Q L V Q T Y N Q L F E E N P
181 182 183 184 185 186 187 188 189 190 191 192 193 194 195 196 197 198 199 200
5' ATCAACGCCAGCGGCGTGGACGCCAAGGCCATCCTGTCTGCCAGACTGAGCAAGAGCAGA
660
hSpCas9
I N A S G V D A K A I L S A R L S K S R
201 202 203 204 205 206 207 208 209 210 211 212 213 214 215 216 217 218 219 220

FIG. 24B

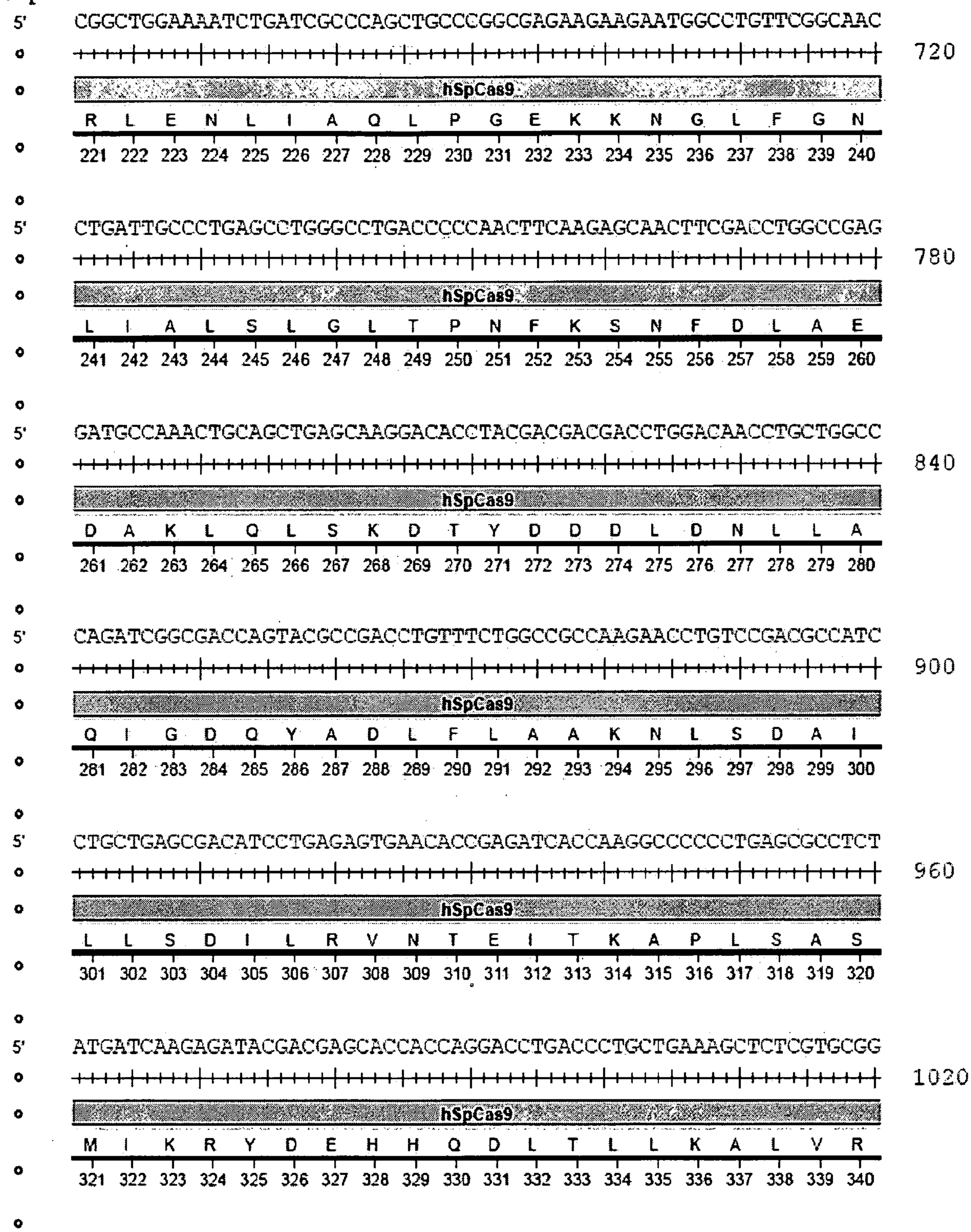

hSpCas9
5' CGGCTGGAAAATCTGATCGCCCAGCTGCCCGGCGAGAAGAAGAATGGCCTGTTCGGCAAC
720
hSpCas9
R L E N L I A Q L P G E K K N G L F G N
221 222 223 224 225 226 227 228 229 230 231 232 233 234 235 236 237 238 239 240
5' CTGATTGCCCTGAGCCTGGGCCTGACCCCCAACTTCAAGAGCAACTTCGACCTGGCCGAG
780
hSpCas9
L I A L S L G L T P N F K S N F D L A E
241 242 243 244 245 246 247 248 249 250 251 252 253 254 255 256 257 258 259 260
5' GATGCCAAACTGCAGCTGAGCAAGGACACCTACGACGACGACCTGGACAACCTGCTGGCC
840
hSpCas9
D A K L Q L S K D T Y D D D L D N L L A
261 262 263 264 265 266 267 268 269 270 271 272 273 274 275 276 277 278 279 280
5' CAGATCGGCGACCAGTACGCCGACCTGTTTCTGGCCGCCAAGAACCTGTCCGACGCCATC
900
hSpCas9
Q I G D Q Y A D L F L A A K N L S D A I
281 282 283 284 285 286 287 288 289 290 291 292 293 294 295 296 297 298 299 300
5' CTGCTGAGCGACATCCTGAGAGTGAACACCGAGATCACCAAGGCCCCCCTGAGCGCCTCT
960
hSpCas9
L L S D I L R V N T E I T K A P L S A S
301 302 303 304 305 306 307 308 309 310 311 312 313 314 315 316 317 318 319 320
5' ATGATCAAGAGATACGACGAGCACCACCAGGACCTGACCCTGCTGAAAGCTCTCGTGCGG
1020
hSpCas9
M I K R Y D E H H Q D L T L L K A L V R
321 322 323 324 325 326 327 328 329 330 331 332 333 334 335 336 337 338 339 340

FIG. 24C

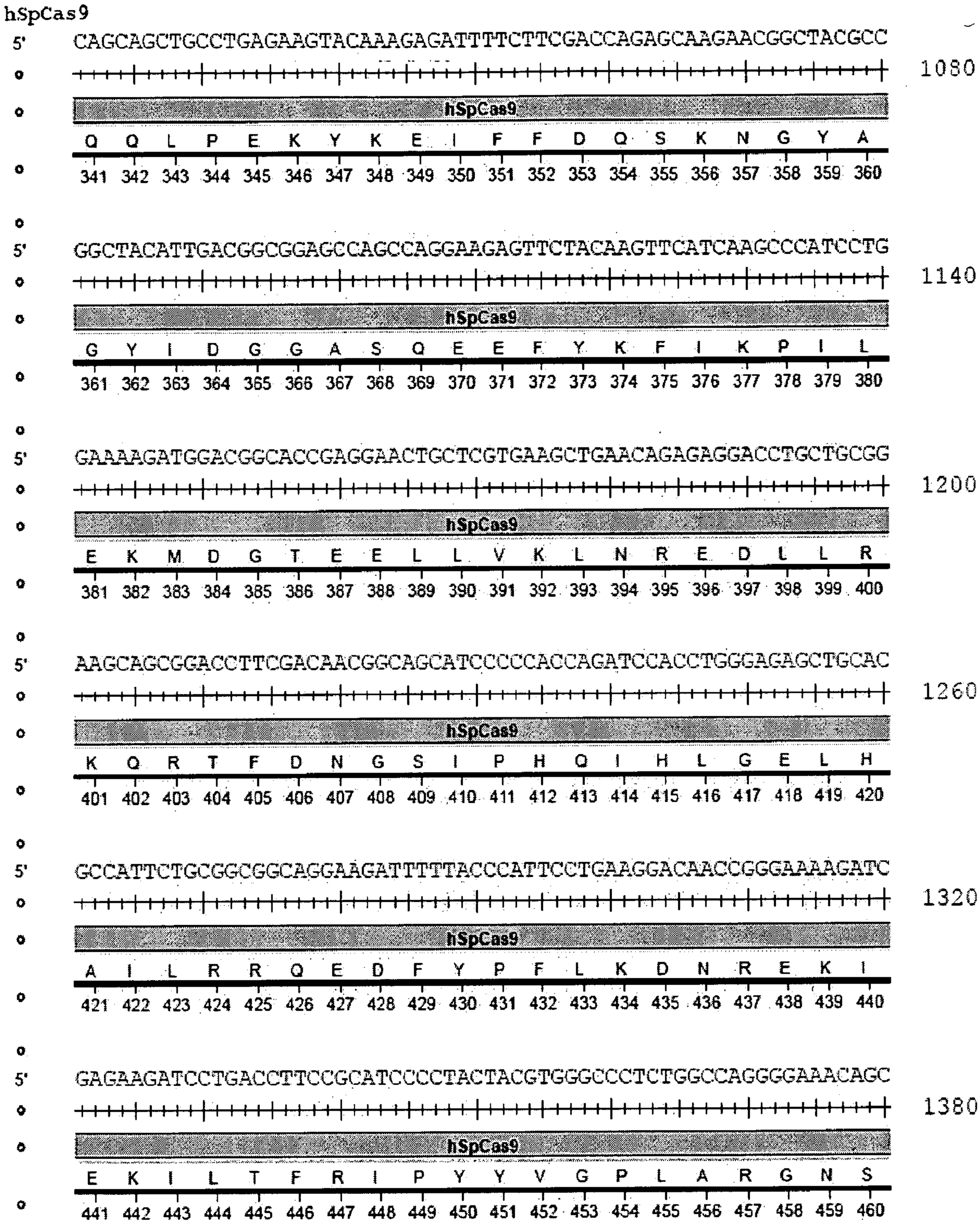

hSpCas9
5' CAGCAGCTGCCTGAGAAGTACAAAGAGATTTTCTTCGACCAGAGCAAGAACGGCTACGCC
1080
hSpCas9
Q Q L P E K Y K E I F F D Q S K N G Y A
341 342 343 344 345 346 347 348 349 350 351 352 353 354 355 356 357 358 359 360
5' GGCTACATTGACGGCGGAGCCAGCCAGGAAGAGTTCTACAAGTTCATCAAGCCCATCCTG
1140
hSpCas9
G Y I D G G A S Q E E F Y K F I K P I L
361 362 363 364 365 366 367 368 369 370 371 372 373 374 375 376 377 378 379 380
5' GAAAAGATGGACGGCACCGAGGAACTGCTCGTGAAGCTGAACAGAGAGGACCTGCTGCGG
1200
hSpCas9
E K M D G T E E L L V K L N R E D L L R
381 382 383 384 385 386 387 388 389 390 391 392 393 394 395 396 397 398 399 400
5' AAGCAGCGGACCTTCGACAACGGCAGCATCCCCCACCAGATCCACCTGGGAGAGCTGCAC
1260
hSpCas9
K Q R T F D N G S I P H Q I H L G E L H
401 402 403 404 405 406 407 408 409 410 411 412 413 414 415 416 417 418 419 420
5' GCCATTCTGCGGCGGCAGGAAGATTTTTACCCATTCCTGAAGGACAACCGGGAAAAGATC
1320
hSpCas9
A I L R R Q E D F Y P F L K D N R E K I
421 422 423 424 425 426 427 428 429 430 431 432 433 434 435 436 437 438 439 440
5' GAGAAGATCCTGACCTTCCGCATCCCCTACTACGTGGGCCCTCTGGCCAGGGGAAACAGC
1380
hSpCas9
E K I L T F R I P Y Y V G P L A R G N S
441 442 443 444 445 446 447 448 449 450 451 452 453 454 455 456 457 458 459 460

FIG. 24D

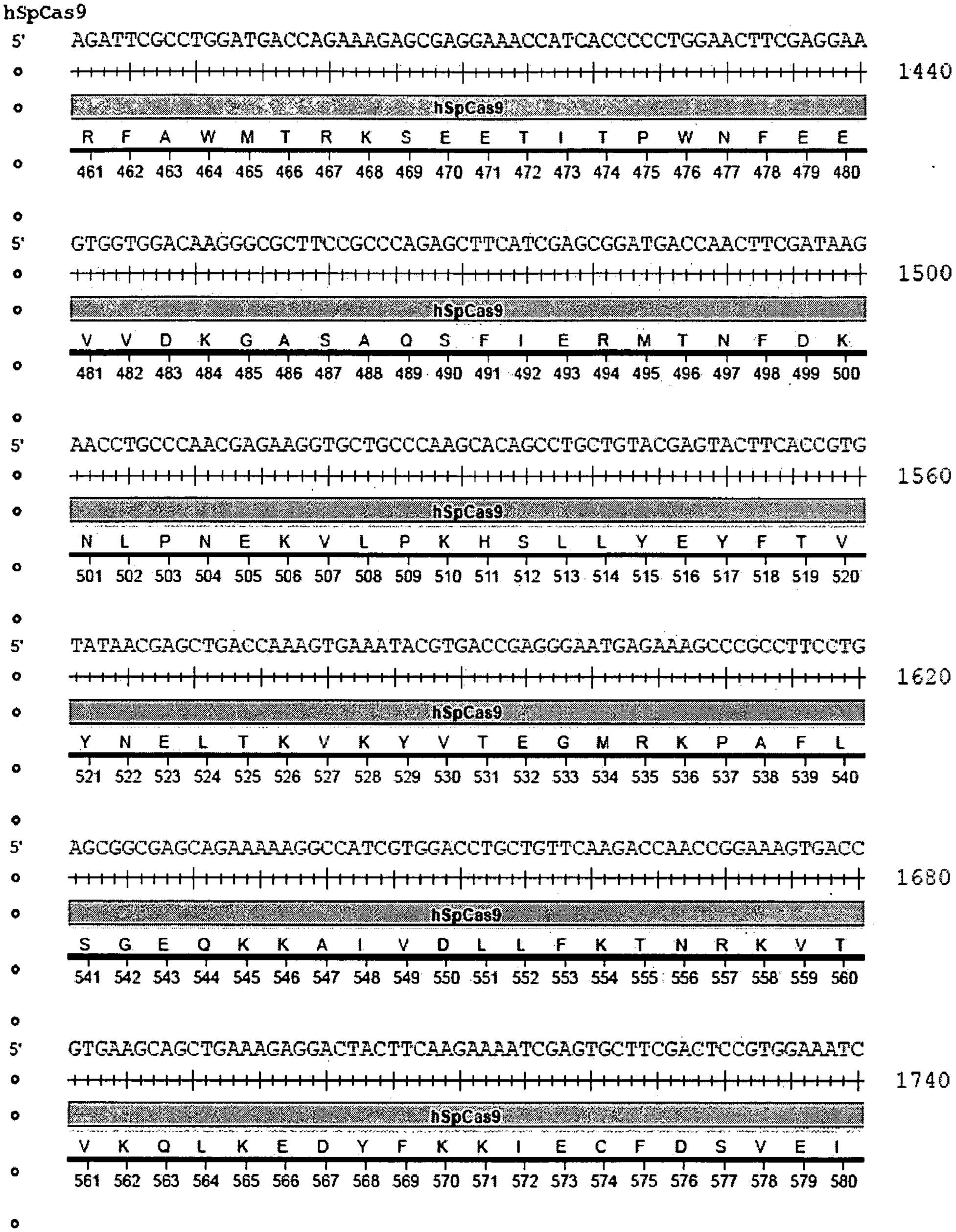

hSpCas9
5' AGATTCGCCTGGATGACCAGAAAGAGCGAGGAAACCATCACCCCCTGGAACTTCGAGGAA
1440
hSpCas9
R F A W M T R K S E E T I T P W N F E E
461 462 463 464 465 466 467 468 469 470 471 472 473 474 475 476 477 478 479 480
5' GTGGTGGACAAGGGCGCTTCCGCCCAGAGCTTCATCGAGCGGATGACCAACTTCGATAAG
1500
hSpCas9
V V D K G A S A Q S F I E R M T N F D K
481 482 483 484 485 486 487 488 489 490 491 492 493 494 495 496 497 498 499 500
5' AACCTGCCCAACGAGAAGGTGCTGCCCAAGCACAGCCTGCTGTACGAGTACTTCACCGTG
1560
hSpCas9
N L P N E K V L P K H S L L Y E Y F T V
501 502 503 504 505 506 507 508 509 510 511 512 513 514 515 516 517 518 519 520
5' TATAACGAGCTGACCAAAGTGAAATACGTGACCGAGGGAATGAGAAAGCCCGCCTTCCTG
1620
hSpCas9
Y N E L T K V K Y V T E G M R K P A F L
521 522 523 524 525 526 527 528 529 530 531 532 533 534 535 536 537 538 539 540
5' AGCGGCGAGCAGAAAAAGGCCATCGTGGACCTGCTGTTCAAGACCAACCGGAAAGTGACC
1680
hSpCas9
S G E Q K K A I V D L L F K T N R K V T
541 542 543 544 545 546 547 548 549 550 551 552 553 554 555 556 557 558 559 560
5' GTGAAGCAGCTGAAAGAGGACTACTTCAAGAAAATCGAGTGCTTCGACTCCGTGGAAATC
1740
hSpCas9
V K Q L K E D Y F K K I E C F D S V E I
561 562 563 564 565 566 567 568 569 570 571 572 573 574 575 576 577 578 579 580

FIG. 24E

hSpCas9
5' TCCGGCGTGGAAGATCGGTTCAACGCCTCCCTGGGCACATACCACGATCTGCTGAAAATT
1800
hSpCas9
S G V E D R F N A S L G T Y H D L L K I
581 582 583 584 585 586 587 588 589 590 591 592 593 594 595 596 597 598 599 600
5' ATCAAGGACAAGGACTTCCTGGACAATGAGGAAAACGAGGACATTCTGGAAGATATCGTG
1860
hSpCas9
I K D K D F L D N E E N E D I L E D I V
601 602 603 604 605 606 607 608 609 610 611 612 613 614 615 616 617 618 619 620
5' CTGACCCTGACACTGTTTGAGGACAGAGAGATGATCGAGGAACGGCTGAAAACCTATGCC
1920
hSpCas9
L T L T L F E D R E M I E E R L K T Y A
621 622 623 624 625 626 627 628 629 630 631 632 633 634 635 636 637 638 639 640
5' CACCTGTTCGACGACAAAGTGATGAAGCAGCTGAAGCGGCGGAGATACACCGGCTGGGGC
1980
hSpCas9
H L F D D K V M K Q L K R R R Y T G W G
641 642 643 644 645 646 647 648 649 650 651 652 653 654 655 656 657 658 659 660
5' AGGCTGAGCCGGAAGCTGATCAACGGCATCCGGGACAAGCAGTCCGGCAAGACAATCCTG
2040
hSpCas9
R L S R K L I N G I R D K Q S G K T I L
661 662 663 664 665 666 667 668 669 670 671 672 673 674 675 676 677 678 679 680
5' GATTTCCTGAAGTCCGACGGCTTCGCCAACAGAAACTTCATGCAGCTGATCCACGACGAC
2100
hSpCas9
D F L K S D G F A N R N F M Q L I H D D
681 682 683 684 685 686 687 688 689 690 691 692 693 694 695 696 697 698 699 700

FIG. 24F

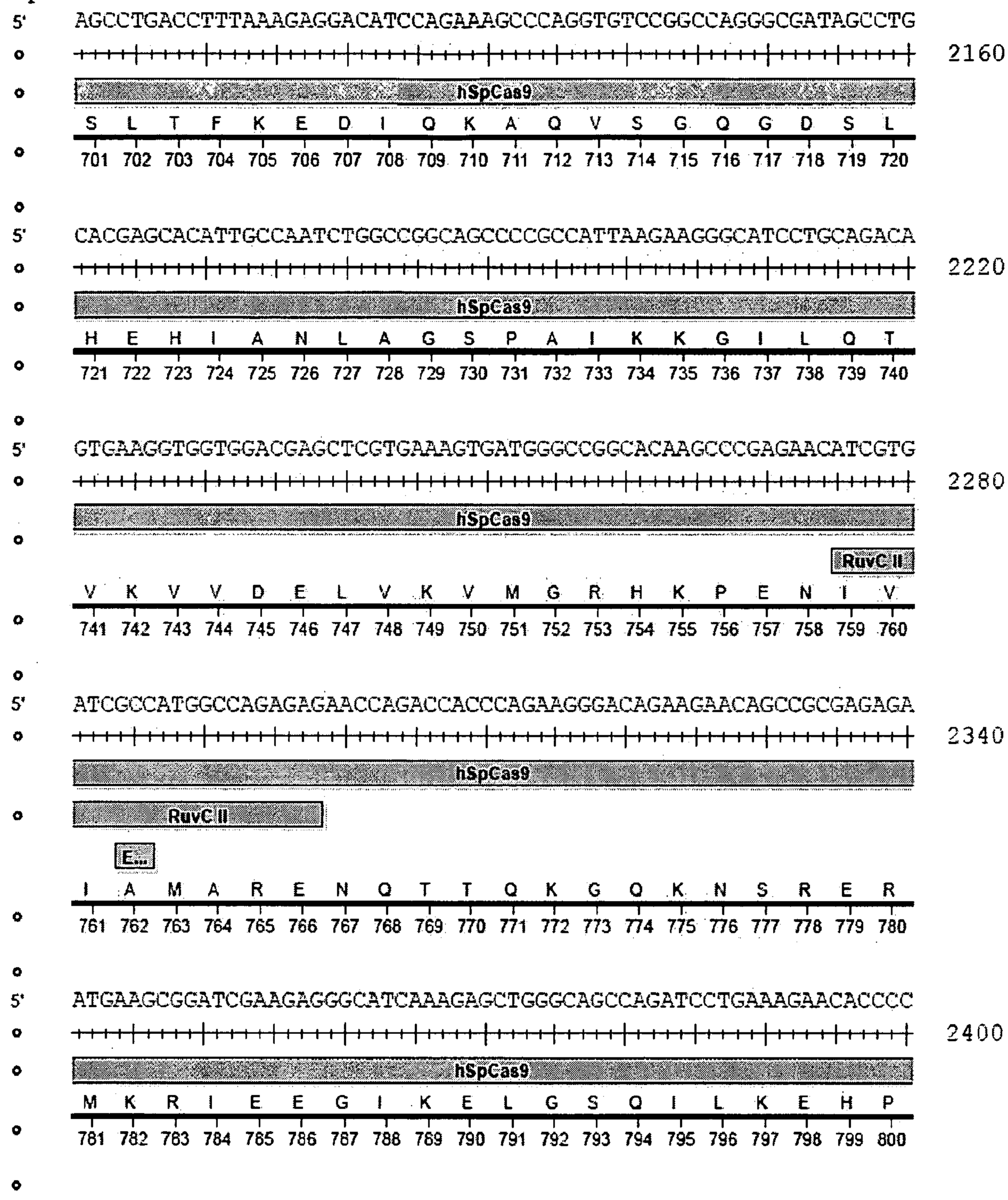

hSpCas9
5' AGCCTGACCTTTAAAGAGGACATCCAGAAAGCCCAGGTGTCCGGCCAGGGCGATAGCCTG
2160
hSpCas9
S L T F K E D I Q K A Q V S G Q G D S L
701 702 703 704 705 706 707 708 709 710 711 712 713 714 715 716 717 718 719 720
5' CACGAGCACATTGCCAATCTGGCCGGCAGCCCCGCCATTAAGAAGGGCATCCTGCAGACA
2220
hSpCas9
H E H I A N L A G S P A I K K G I L Q T
721 722 723 724 725 726 727 728 729 730 731 732 733 734 735 736 737 738 739 740
5' GTGAAGGTGGTGGACGAGCTCGTGAAAGTGATGGGCCGGCACAAGCCCGAGAACATCGTG
2280
hSpCas9
RuvC II
V K V V D E L V K V M G R H K P E N I V
741 742 743 744 745 746 747 748 749 750 751 752 753 754 755 756 757 758 759 760
5' ATCGCCATGGCCAGAGAGAACCAGACCACCCAGAAGGGACAGAAGAACAGCCGCGAGAGA
2340
hSpCas9
RuvC II
E...
I A M A R E N Q T T Q K G Q K N S R E R
761 762 763 764 765 766 767 768 769 770 771 772 773 774 775 776 777 778 779 780
5' ATGAAGCGGATCGAAGAGGGCATCAAAGAGCTGGGCAGCCAGATCCTGAAAGAACACCCC
2400
hSpCas9
M K R I E E G I K E L G S Q I L K E H P
781 782 783 784 785 786 787 788 789 790 791 792 793 794 795 796 797 798 799 800

FIG. 24G

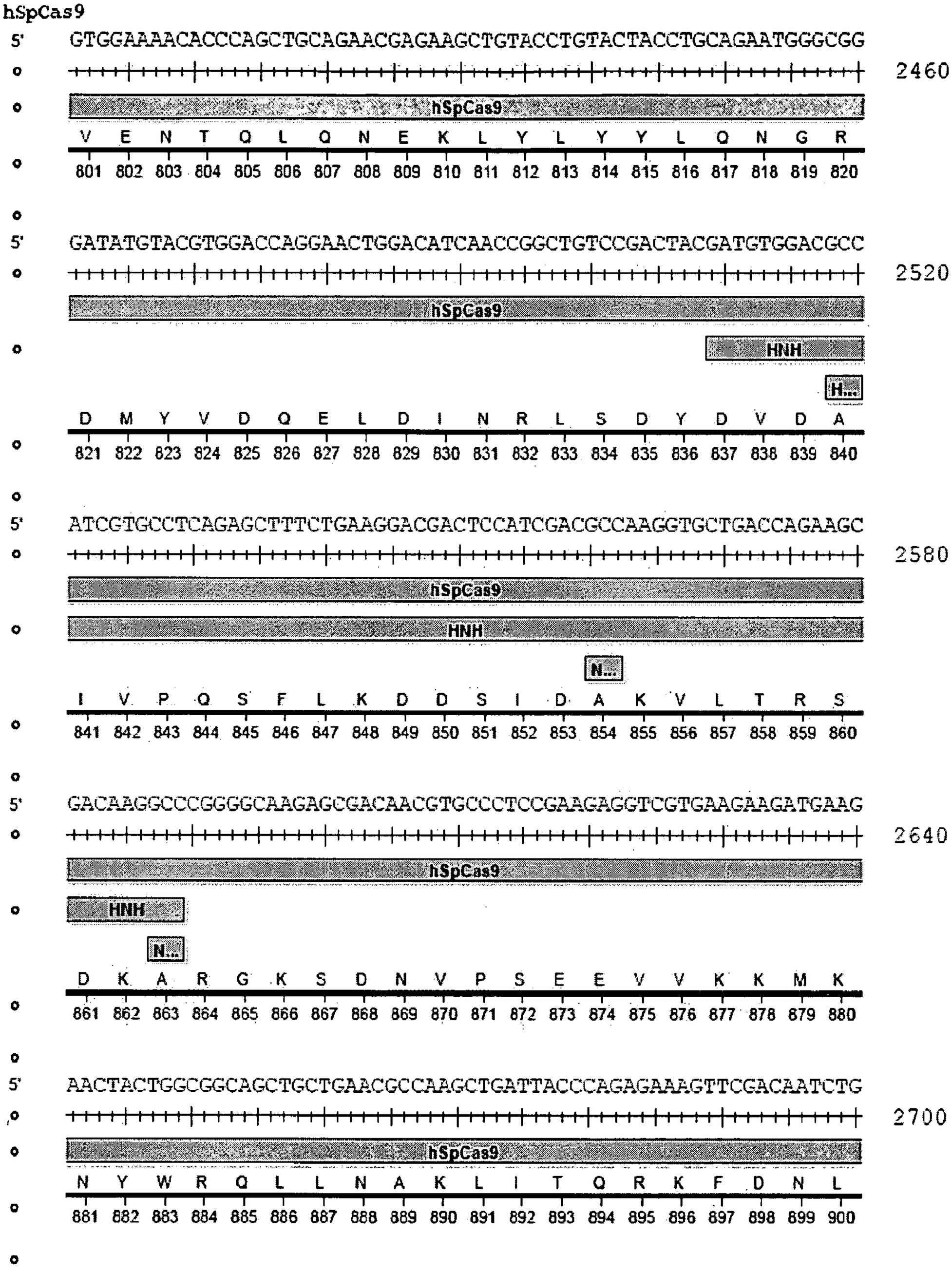

hSpCas9
5' GTGGAAAACACCCAGCTGCAGAACGAGAAGCTGTACCTGTACTACCTGCAGAATGGGCGG
2460
hSpCas9
V E N T Q L Q N E K L Y L Y Y L Q N G R
801 802 803 804 805 806 807 808 809 810 811 812 813 814 815 816 817 818 819 820
5' GATATGTACGTGGACCAGGAACTGGACATCAACCGGCTGTCCGACTACGATGTGGACGCC
2520
hSpCas9
HNH
H...
D M Y V D Q E L D I N R L S D Y D V D A
821 822 823 824 825 826 827 828 829 830 831 832 833 834 835 836 837 838 839 840
5' ATCGTGCCTCAGAGCTTTCTGAAGGACGACTCCATCGACGCCAAGGTGCTGACCAGAAGC
2580
hSpCas9
HNH
N...
I V P Q S F L K D D S I D A K V L T R S
841 842 843 844 845 846 847 848 849 850 851 852 853 854 855 856 857 858 859 860
5' GACAAGGCCCGGGGCAAGAGCGACAACGTGCCCTCCGAAGAGGTCGTGAAGAAGATGAAG
2640
hSpCas9
HNH
N...
D K A R G K S D N V P S E E V V K K M K
861 862 863 864 865 866 867 868 869 870 871 872 873 874 875 876 877 878 879 880
5' AACTACTGGCGGCAGCTGCTGAACGCCAAGCTGATTACCCAGAGAAAGTTCGACAATCTG
2700
hSpCas9
N Y W R Q L L N A K L I T Q R K F D N L
881 882 883 884 885 886 887 888 889 890 891 892 893 894 895 896 897 898 899 900

FIG. 24H

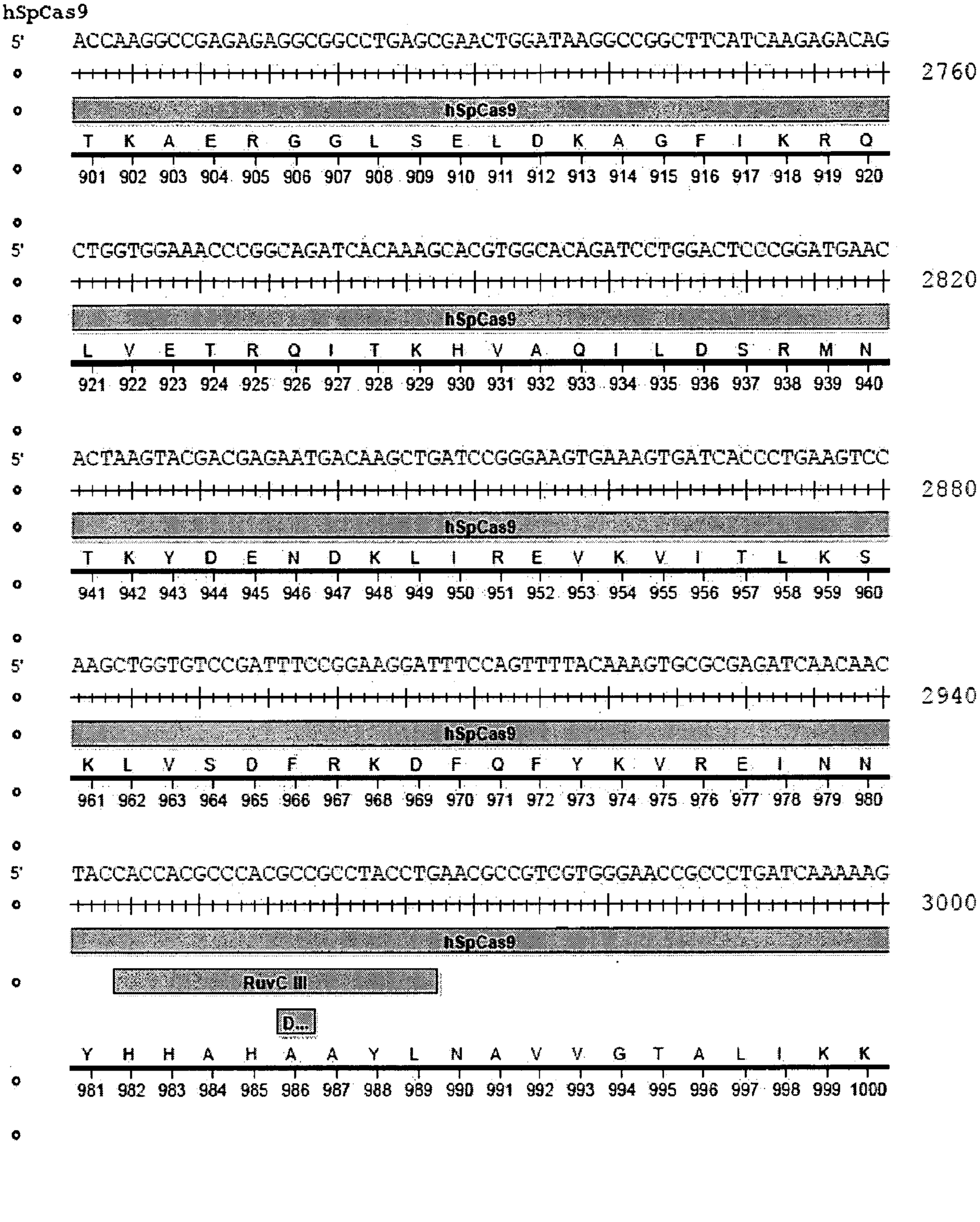

hSpCas9
5' ACCAAGGCCGAGAGAGGCGGCCTGAGCGAACTGGATAAGGCCGGCTTCATCAAGAGACAG
2760
hSpCas9
T K A E R G G L S E L D K A G F I K R Q
901 902 903 904 905 906 907 908 909 910 911 912 913 914 915 916 917 918 919 920
5' CTGGTGGAAACCCGGCAGATCACAAAGCACGTGGCACAGATCCTGGACTCCCGGATGAAC
2820
hSpCas9
L V E T R Q I T K H V A Q I L D S R M N
921 922 923 924 925 926 927 928 929 930 931 932 933 934 935 936 937 938 939 940
5' ACTAAGTACGACGAGAATGACAAGCTGATCCGGGAAGTGAAAGTGATCACCCTGAAGTCC
2880
hSpCas9
T K Y D E N D K L I R E V K V I T L K S
941 942 943 944 945 946 947 948 949 950 951 952 953 954 955 956 957 958 959 960
5' AAGCTGGTGTCCGATTTCCGGAAGGATTTCCAGTTTTACAAAGTGCGCGAGATCAACAAC
2940
hSpCas9
K L V S D F R K D F Q F Y K V R E I N N
961 962 963 964 965 966 967 968 969 970 971 972 973 974 975 976 977 978 979 980
5' TACCACCACGCCCACGCCGCCTACCTGAACGCCGTCGTGGGAACCGCCCTGATCAAAAAG
3000
hSpCas9
RuvC III
D...
Y H H A H A A Y L N A V V G T A L I K K
981 982 983 984 985 986 987 988 989 990 991 992 993 994 995 996 997 998 999 1000

FIG. 24I

hSpCas9
5' TACCCTAAGCTGGAAAGCGAGTTCGTGTACGGCGACTACAAGGTGTACGACGTGCGGAAG
3060
hSpCas9
Y P K L E S E F V Y G D Y K V Y D V R K
1001 1002 1003 1004 1005 1006 1007 1008 1009 1010 1011 1012 1013 1014 1015 1016 1017 1018 1019 1020
5' ATGATCGCCAAGAGCGAGCAGGAAATCGGCAAGGCTACCGCCAAGTACTTCTTCTACAGC
3120
hSpCas9
M I A K S E Q E I G K A T A K Y F F Y S
1021 1022 1023 1024 1025 1026 1027 1028 1029 1030 1031 1032 1033 1034 1035 1036 1037 1038 1039 1040
5' AACATCATGAACTTTTTCAAGACCGAGATTACCCTGGCCAACGGCGAGATCCGGAAGCGG
3180
hSpCas9
N I M N F F K T E I T L A N G E I R K R
1041 1042 1043 1044 1045 1046 1047 1048 1049 1050 1051 1052 1053 1054 1055 1056 1057 1058 1059 1060
5' CCTCTGATCGAGACAAACGGCGAAACCGGGGAGATCGTGTGGGATAAGGGCCGGGATTTT
3240
hSpCas9
P L I E T N G E T G E I V W D K G R D F
1061 1062 1063 1064 1065 1066 1067 1068 1069 1070 1071 1072 1073 1074 1075 1076 1077 1078 1079 1080
5' GCCACCGTGCGGAAAGTGCTGAGCATGCCCCAAGTGAATATCGTGAAAAAGACCGAGGTG
3300
hSpCas9
A T V R K V L S M P Q V N I V K K T E V
1081 1082 1083 1084 1085 1086 1087 1088 1089 1090 1091 1092 1093 1094 1095 1096 1097 1098 1099 1100
5' CAGACAGGCGGCTTCAGCAAAGAGTCTATCCTGCCCAAGAGGAACAGCGATAAGCTGATC
3360
hSpCas9
Q T G G F S K E S I L P K R N S D K L I
1101 1102 1103 1104 1105 1106 1107 1108 1109 1110 1111 1112 1113 1114 1115 1116 1117 1118 1119 1120

FIG. 24J

hSpCas9

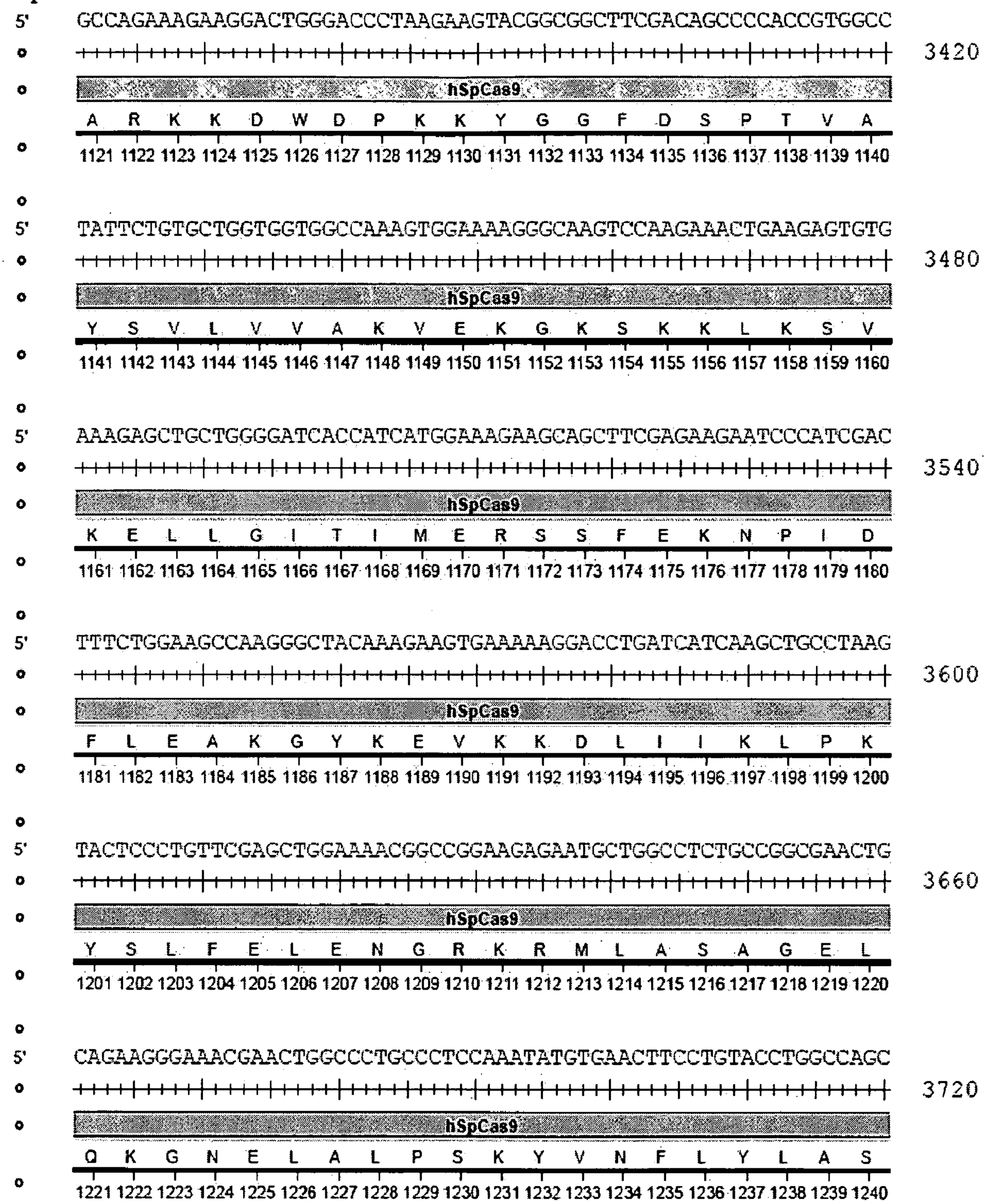
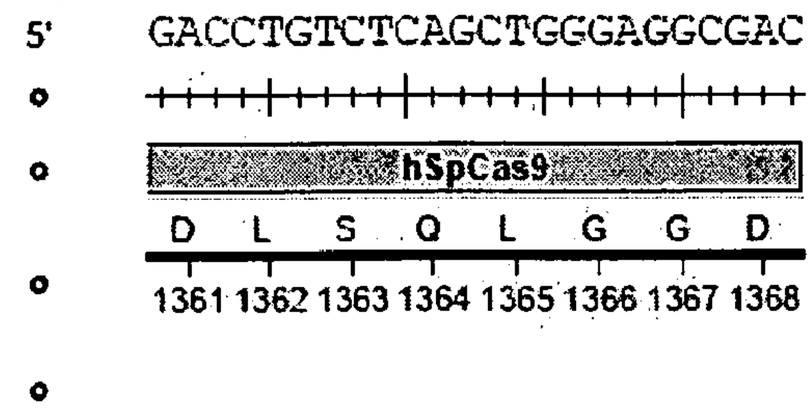


FIG. 24K

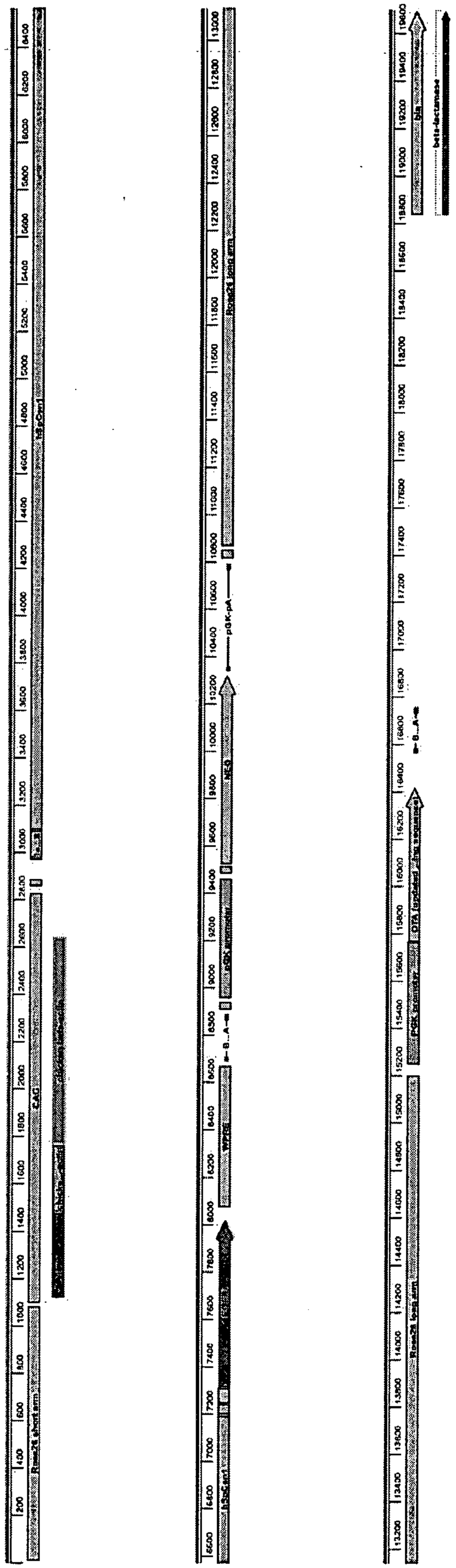
hSpCas9
5' CACTATGAGAAGCTGAAGGGCTCCCCCGAGGATAATGAGCAGAAACAGCTGTTTGTGGAA
3780
hSpCas9
H Y E K L K G S P E D N E Q K Q L F V E
1241 1242 1243 1244 1245 1246 1247 1248 1249 1250 1251 1252 1253 1254 1255 1256 1257 1258 1259 1260
5' CAGCACAAGCACTACCTGGACGAGATCATCGAGCAGATCAGCGAGTTCTCCAAGAGAGTG
3840
hSpCas9
Q H K H Y L D E I I E Q I S E F S K R V
1261 1262 1263 1264 1265 1266 1267 1268 1269 1270 1271 1272 1273 1274 1275 1276 1277 1278 1279 1280
5' ATCCTGGCCGACGCTAATCTGGACAAAGTGCTGTCCGCCTACAACAAGCACCGGGATAAG
3900
hSpCas9
I L A D A N L D K V L S A Y N K H R D K
1281 1282 1283 1284 1285 1286 1287 1288 1289 1290 1291 1292 1293 1294 1295 1296 1297 1298 1299 1300
5' CCCATCAGAGAGCAGGCCGAGAATATCATCCACCTGTTTACCCTGACCAATCTGGGAGCC
3960
hSpCas9
P I R E Q A E N I I H L F T L T N L G A
1301 1302 1303 1304 1305 1306 1307 1308 1309 1310 1311 1312 1313 1314 1315 1316 1317 1318 1319 1320
5' CCTGCCGCCTTCAAGTACTTTGACACCACCATCGACCGGAAGAGGTACACCAGCACCAAA
4020
hSpCas9
P A A F K Y F D T T I D R K R Y T S T K
1321 1322 1323 1324 1325 1326 1327 1328 1329 1330 1331 1332 1333 1334 1335 1336 1337 1338 1339 1340
5' GAGGTGCTGGACGCCACCCTGATCCACCAGAGCATCACCGGCCTGTACGAGACACGGATC
4080
hSpCas9
E V L D A T L I H Q S I T G L Y E T R I
1341 1342 1343 1344 1345 1346 1347 1348 1349 1350 1351 1352 1353 1354 1355 1356 1357 1358 1359 1360

FIG. 24L


hSpCas9
5'
GACCTGTCTCAGCTGGGAGGCGAC
3'
hSpCas9
D L S Q L G G D
1361 1362 1363 1364 1365 1366 1367 1368

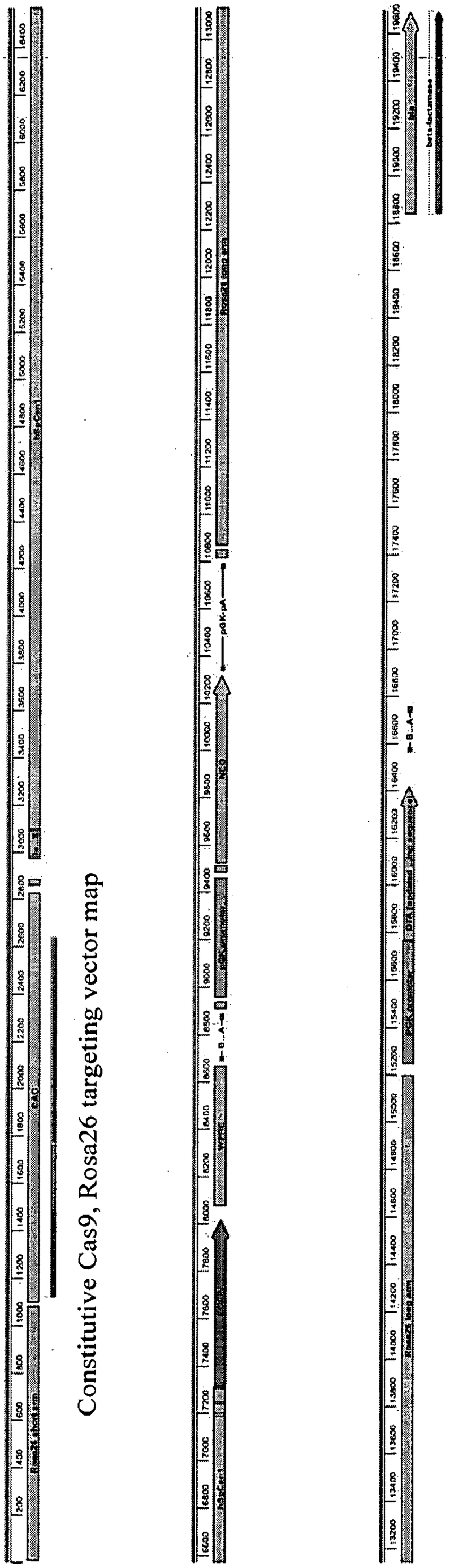
4104

FIG. 24M



Conditional Cas9, Rosa26 targeting vector map

FIG. 25A



Constitutive Cas9, Rosa26 targeting vector map

FIG. 25B

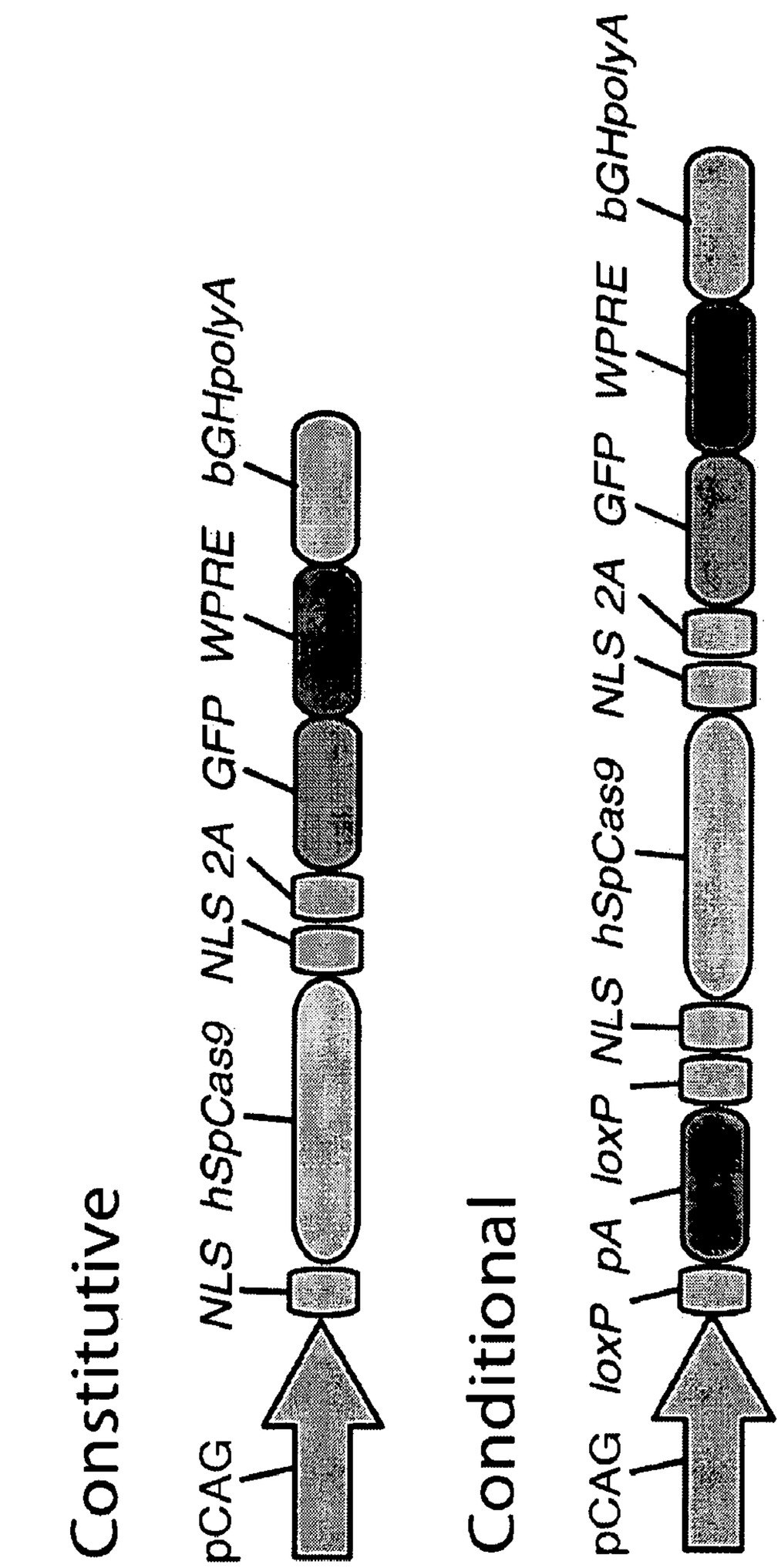
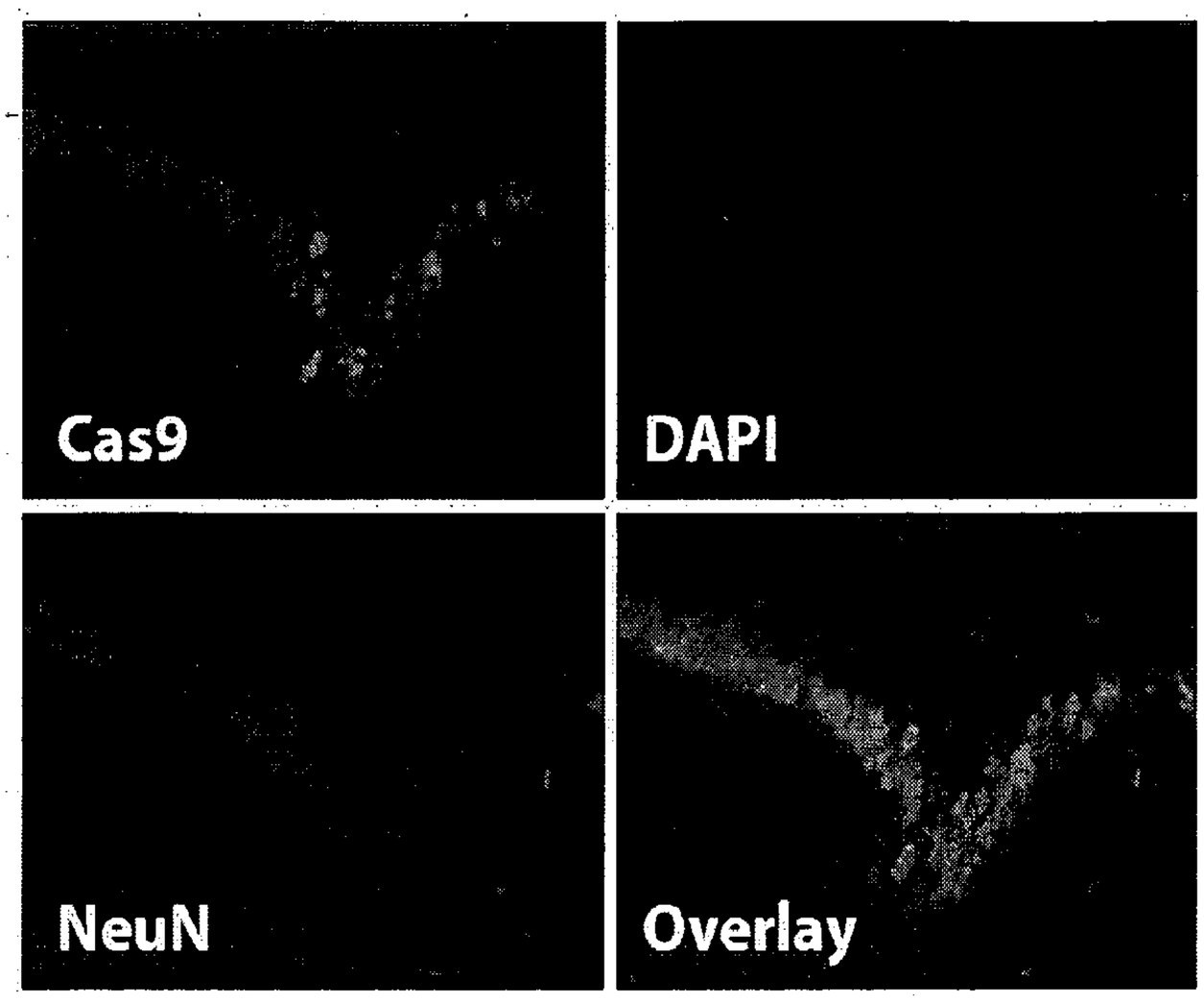

Constitutive
pCAG
NLS hSpCas9 NLS 2A GFP WPRE bGHpolyA
Conditional
pCAG loxP pA loxP NLS hSpCas9 NLS 2A GFP WPRE bGHp

FIG. 26


Cas9 Expression in Mouse Hippocampus (AAV)
Cas9
DAPI
NeuN
Overlay

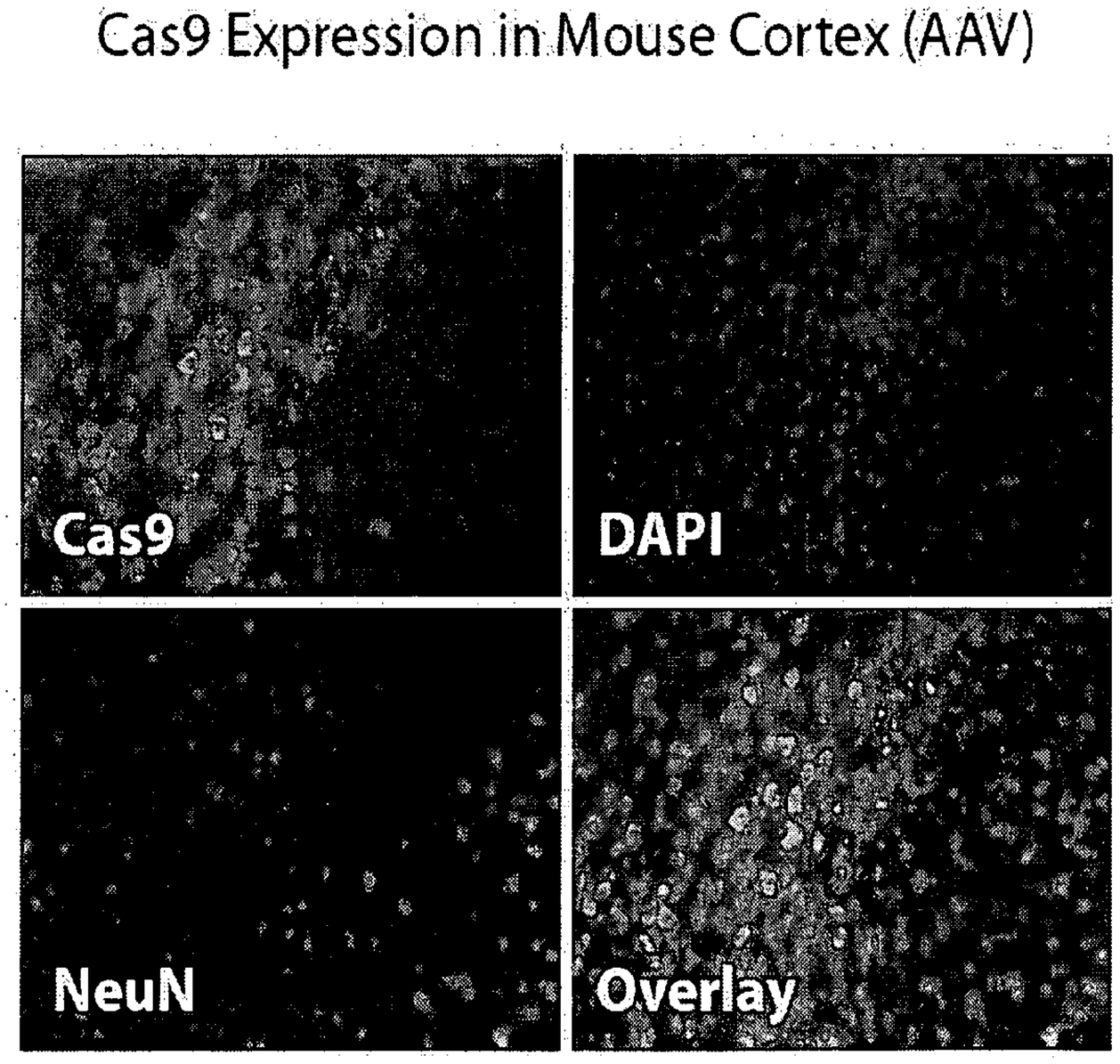

Cas9 Expression in Mouse Cortex (AAV)
Cas9
DAPI
NeuN
Overlay

FIG. 27

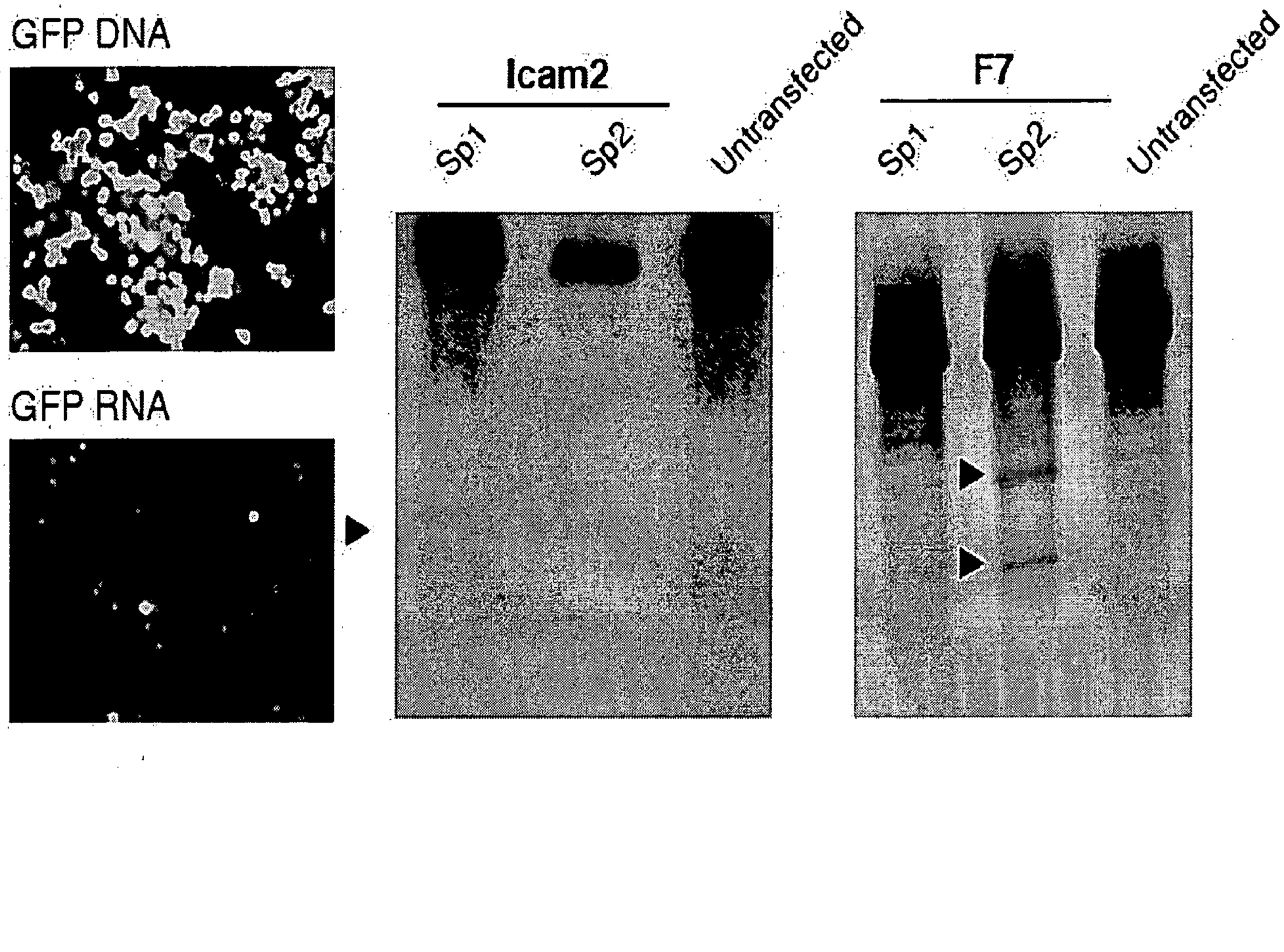

A
B
C
GFP DNA
GFP RNA
Icam2
Sp1
Sp2
Untransfected
F7
Sp1
Sp2
Untransfected

FIG. 28

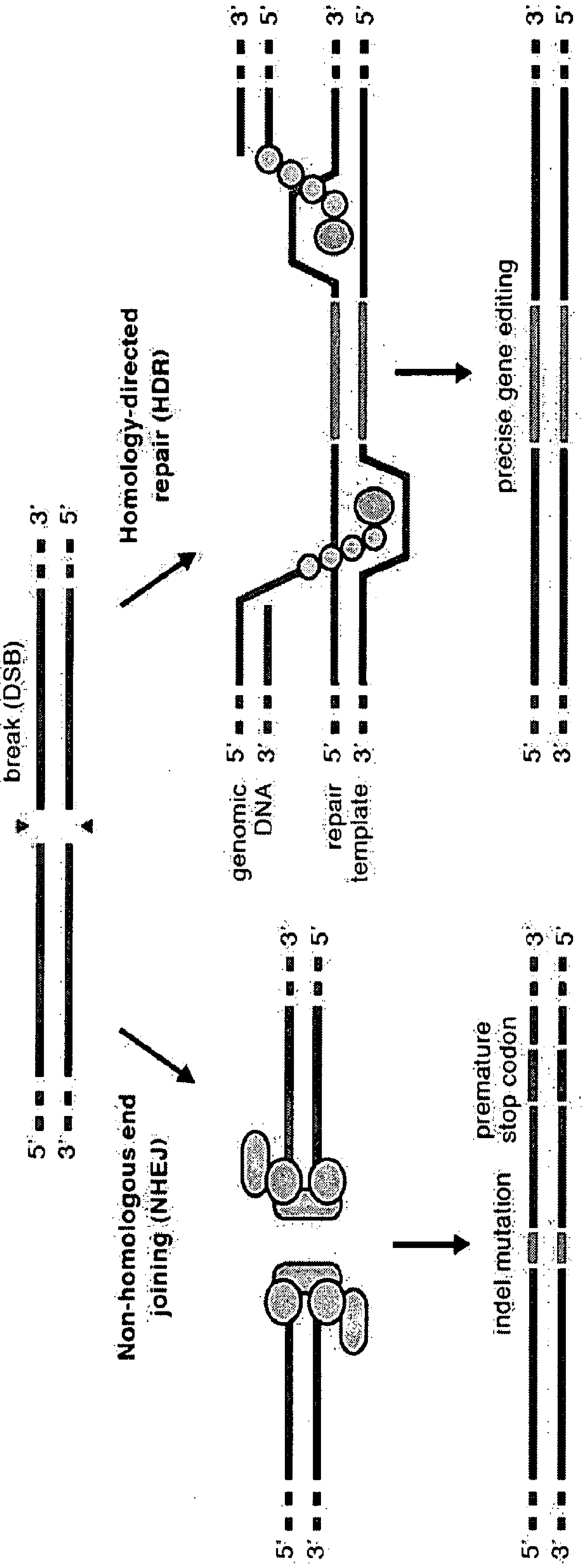

3'
5'
3'
5'
double stranded break (DSB)
Homology-directed repair (HDR)
genomic DNA
repair template
precise gene editing
Non-homologous end joining (NHEJ)
premature stop codon
indel mutation

FIG. 29

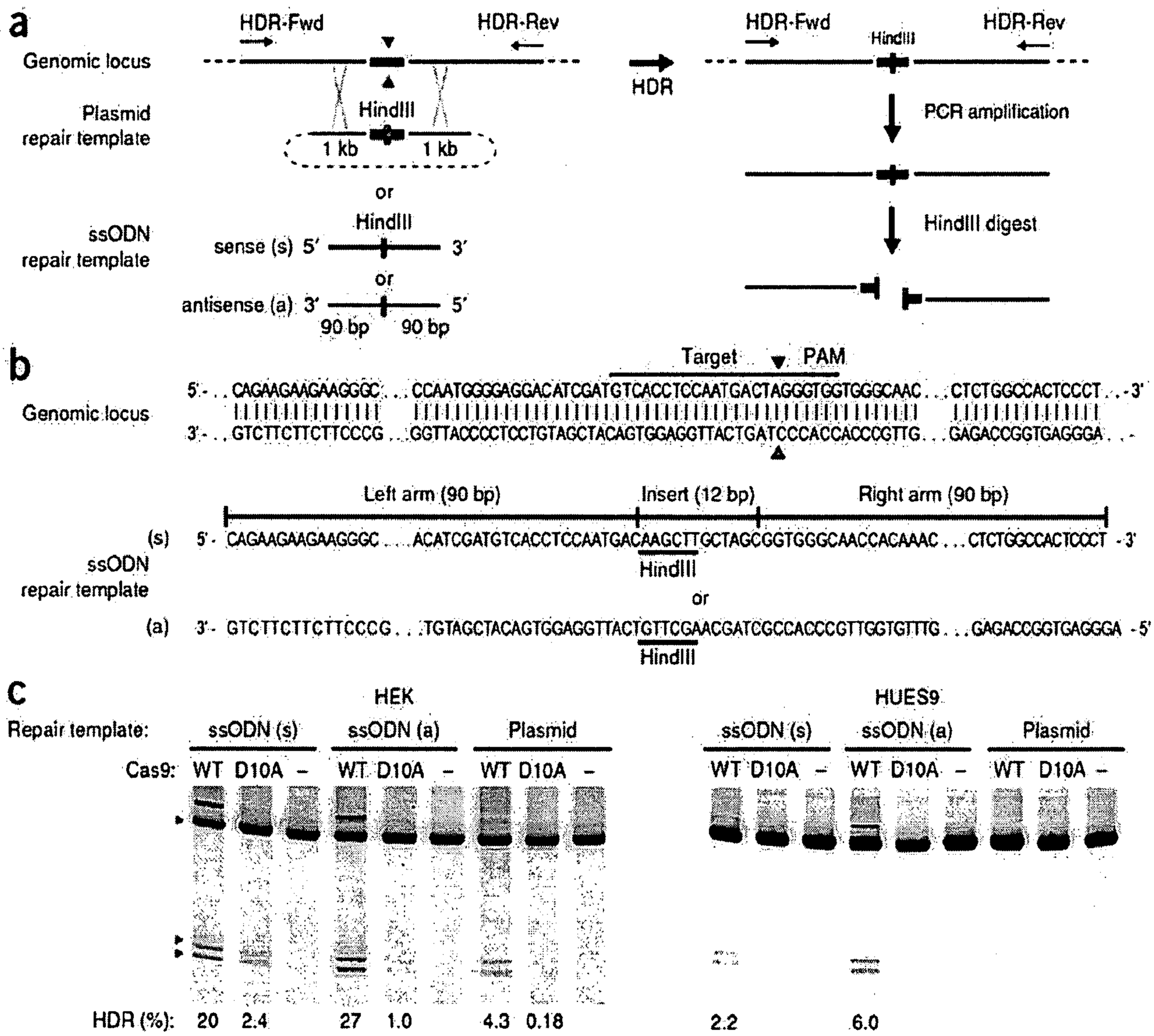

a
Genomic locus
Plasmid repair template
ssODN repair template
HDR-Fwd
HDR-Rev
HindIII
1 kb
1 kb
or
sense (s) 5′
3′
antisense (a) 3′
5′
90 bp
90 bp
HDR
PCR amplification
HindIII digest
b
Genomic locus
Target
PAM
5′- . . CAGAAGAAGAAGGGC . . . CCAATGGGGAGGACATCGATGTCACCTCCAATGACTAGGGTGGTGGGCAAC . . . CTCTGGCCACTCCCT . . -3′
3′- . . GTCTTCTTCTTCCCG . . . GGTTACCCCTCCTGTAGCTACAGTGGAGGTTACTGATCCCACCACCCGTTG . . . GAGACCGGTGAGGGA . . -
Left arm (90 bp)
Insert (12 bp)
Right arm (90 bp)
(s) 5′- CAGAAGAAGAAGGGC . . . ACATCGATGTCACCTCCAATGACAAGCTTGCTAGCGGTGGGCAACCACAAAC . . . CTCTGGCCACTCCCT -3′
ssODN repair template
HindIII
or
(a) 3′- GTCTTCTTCTTCCCG . . . TGTAGCTACAGTGGAGGTTACTGTTCGAACGATCGCCACCCGTTGGTGTTTG . . . GAGACCGGTGAGGGA -5′
HindIII
C
HEK
HUES9
Repair template:
ssODN (s)
ssODN (a)
Plasmid
Cas9: WT D10A –
HDR (%): 20 2.4 27 1.0 4.3 0.18 2.2 6.0

FIG. 30A-C

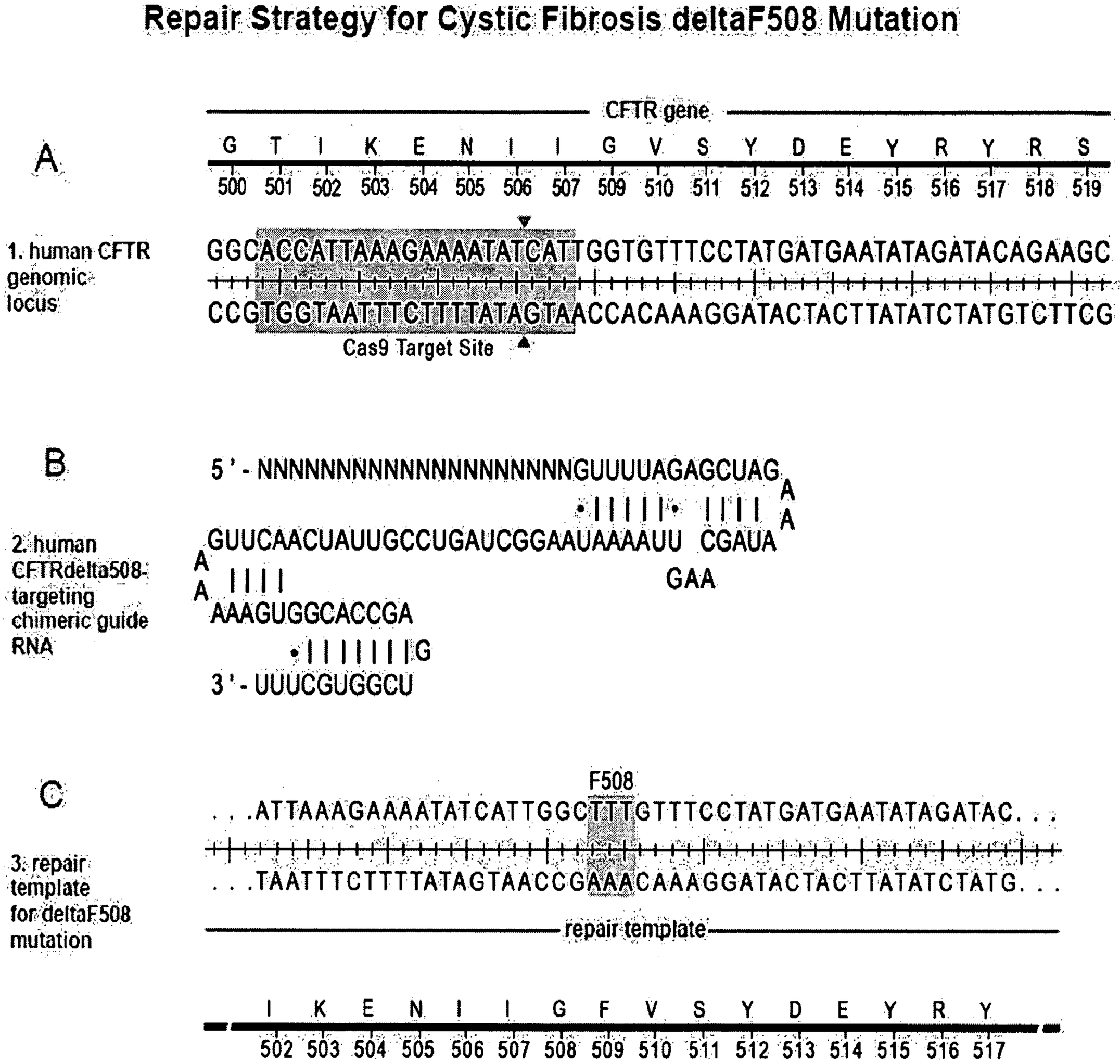
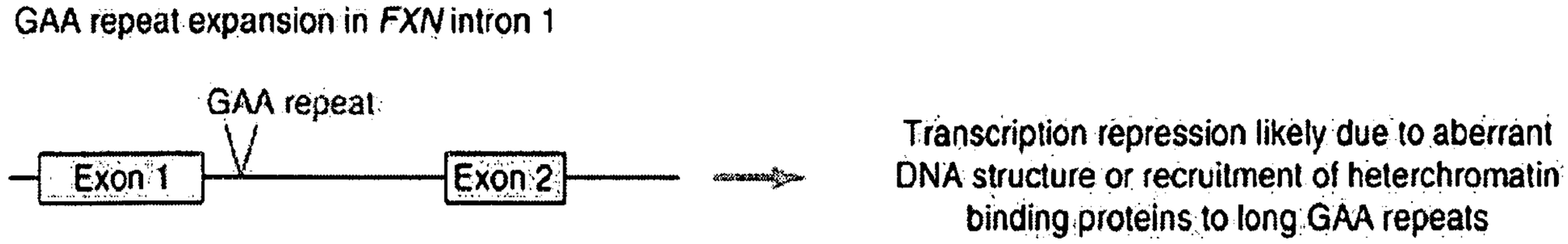

Repair Strategy for Cystic Fibrosis deltaF508 Mutation
A
CFTR gene
G T I K E N I I G V S Y D E Y R Y R S
500 501 502 503 504 505 506 507 509 510 511 512 513 514 515 516 517 518 519
1. human CFTR genomic locus
GGCACCATTAAAGAAAATATCATTGGTGTTTCCTATGATGAATATAGATACAGAAGC
CCGTGGTAATTTCTTTTATAGTAACCACAAAGGATACTACTTATATCTATGTCTTCG
Cas9 Target Site
B
5 ' - NNNNNNNNNNNNNNNNNNNNGUUUUAGAGCUAG
GUUCAACUAUUGCCUGAUCGGAAUAAAAUU CGAUA
GAA
AAAGUGGCACCGA
3 ' - UUUCGUGGCU
2. human CFTRdelta508-targeting chimeric guide RNA
C
F508
. . .ATTAAAGAAAATATCATTGGCTTTGTTTCCTATGATGAATATAGATAC. . .
. . .TAATTTCTTTTATAGTAACCGAAACAAAGGATACTACTTATATCTATG. . .
3. repair template for deltaF508 mutation
repair template
I K E N I I G F V S Y D E Y R Y
502 503 504 505 506 507 508 509 510 511 512 513 514 515 516 517

FIG. 31

A


GAA repeat expansion in *FXN* intron 1
GAA repeat
Exon 1
Exon 2
Transcription repression likely due to aberrant DNA structure or recruitment of heterchromatin binding proteins to long GAA repeats

B

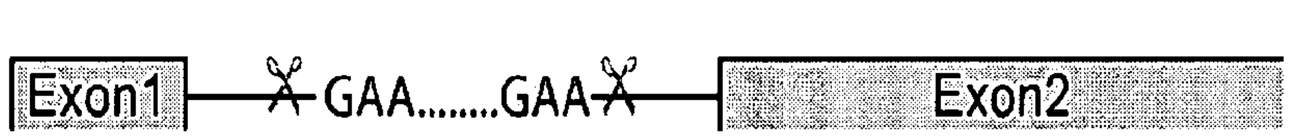

Exon1
GAA.....GAA
Exon2

FIG. 32

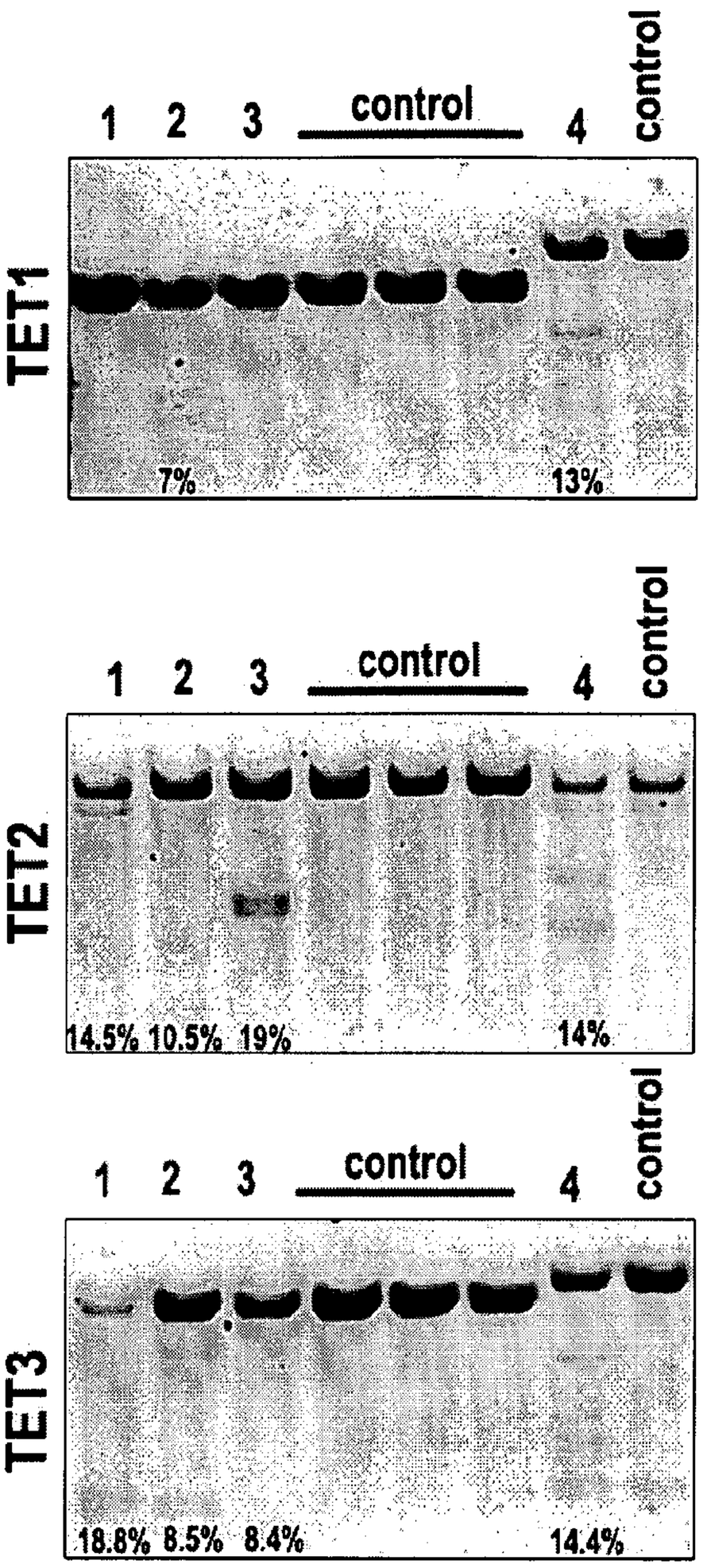

1 2 3 control 4 control
TET1
7%
13%
1 2 3 control 4 control
TET2
14.5% 10.5% 19%
14%
1 2 3 control 4 con
TET3
18.8% 8.5% 8.4%
14.4%
1 control 2 control
DNMT1
1 control 2 3 control
DNMT3a
18.5%
20.4% 11.5%
1 2 control 3 control
DNMT3b
16.3% 21%
14%

FIG. 33

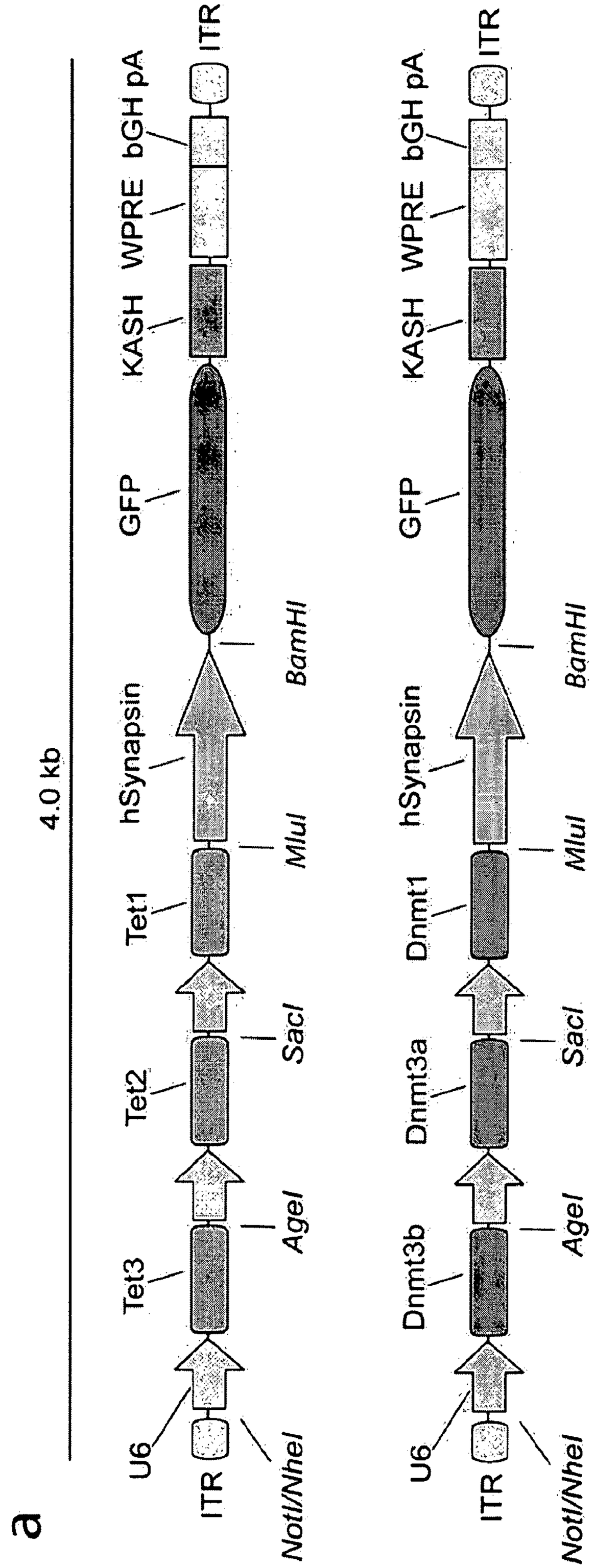

a
4.0 kb
U6
ITR
NotI/NheI
Tet3
AgeI
Tet2
SacI
Tet1
MluI
hSynapsin
GFP
KASH
WPRE
bGH pA
ITR
BamHI
Dnmt3b
Dnmt3a
Dnmt1
SpCas9
NLS
HA
NLS
ITR

FIG. 34

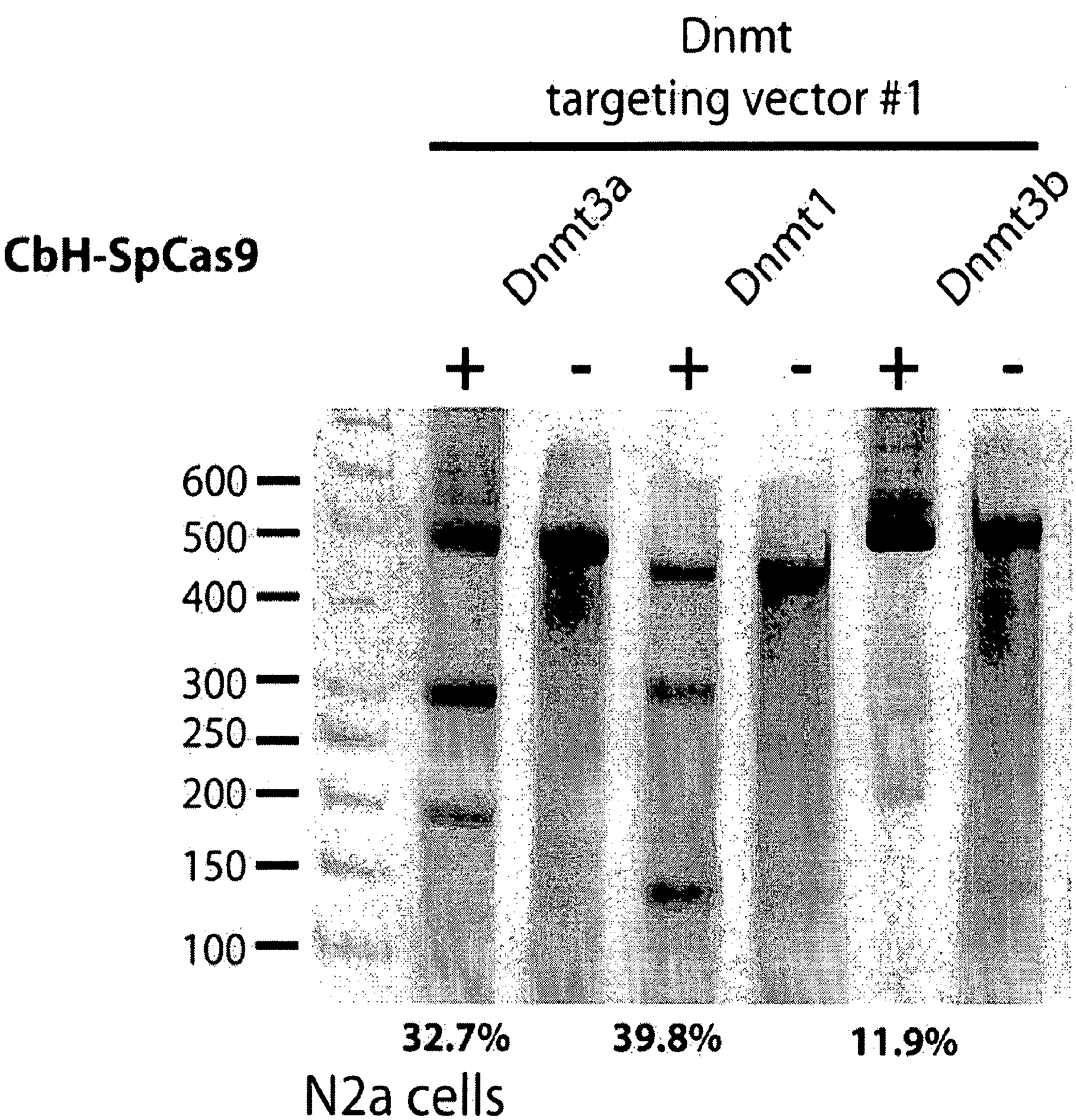

Dnmt
targeting vector #1
CbH-SpCas9
Dnmt3a
Dnmt1
Dnmt3b
+
-
+
-
+
-
600
500
400
300
250
200
150
100
32.7%
39.8%
11.9%
N2a cells

FIG. 35

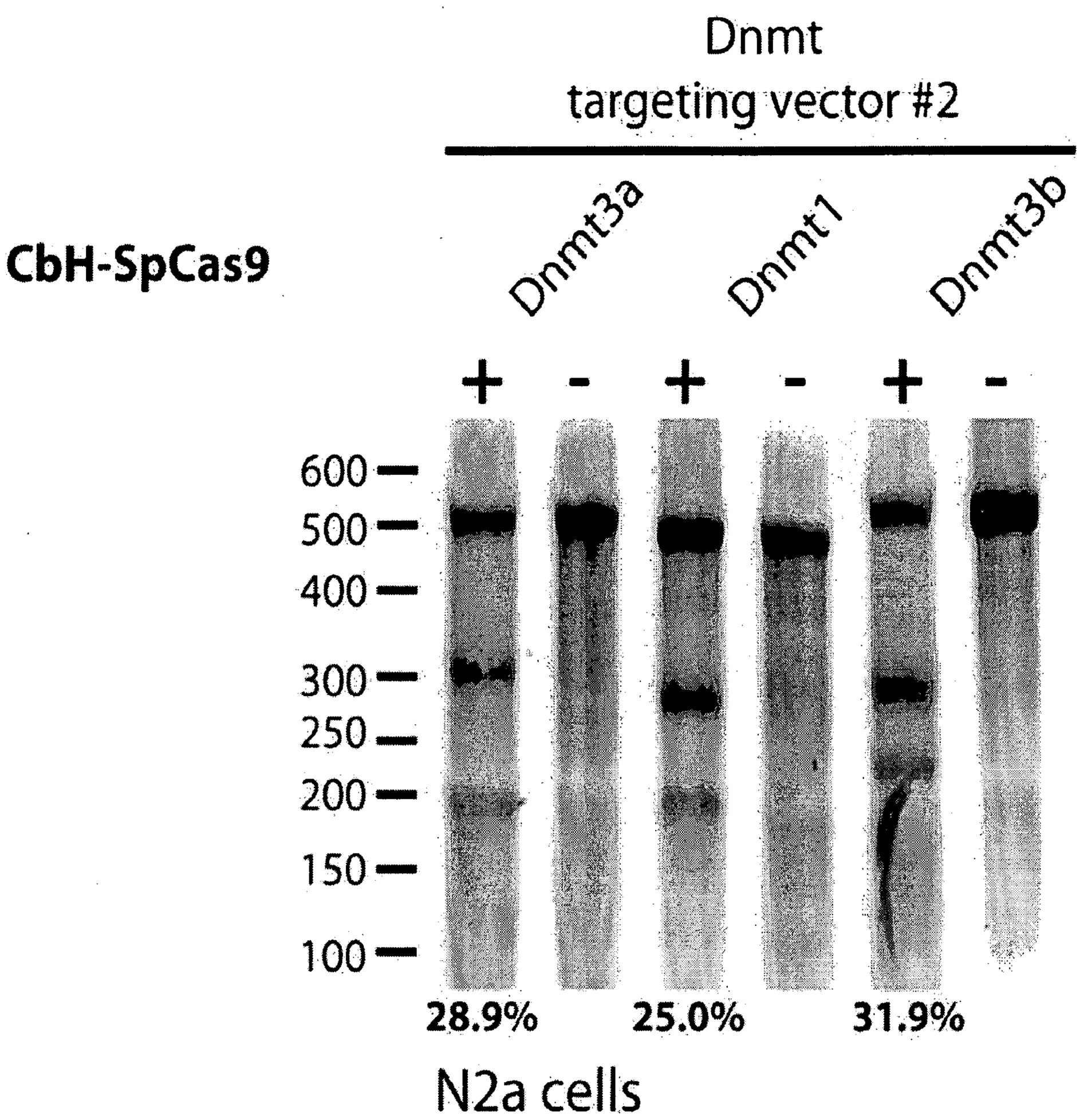

Dnmt
targeting vector #2
CbH-SpCas9
Dnmt3a
Dnmt1
Dnmt3b
+
-
+
-
+
-
600
500
400
300
250
200
150
100
28.9%
25.0%
31.9%
N2a cells

FIG. 36

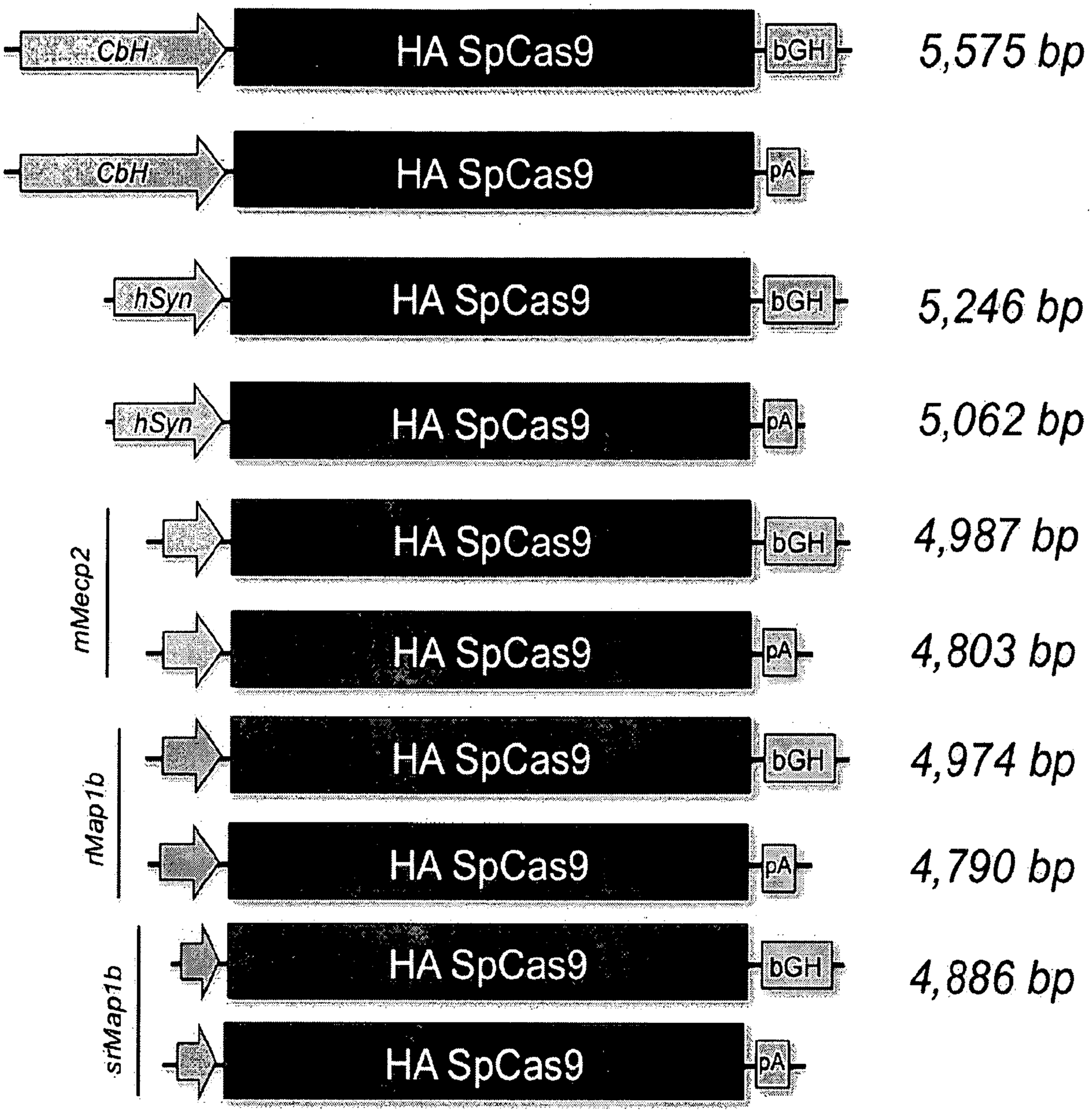

CbH
HA SpCas9
bGH
5,575 bp
CbH
HA SpCas9
pA
hSyn
HA SpCas9
bGH
5,246 bp
hSyn
HA SpCas9
pA
5,062 bp
mMecp2
HA SpCas9
bGH
4,987 bp
HA SpCas9
pA
4,803 bp
rMap1b
HA SpCas9
bGH
4,974 bp
HA SpCas9
pA
4,790 bp
srMap1b
HA SpCas9
bGH
4,886 bp
HA SpCas9
pA

FIG. 37

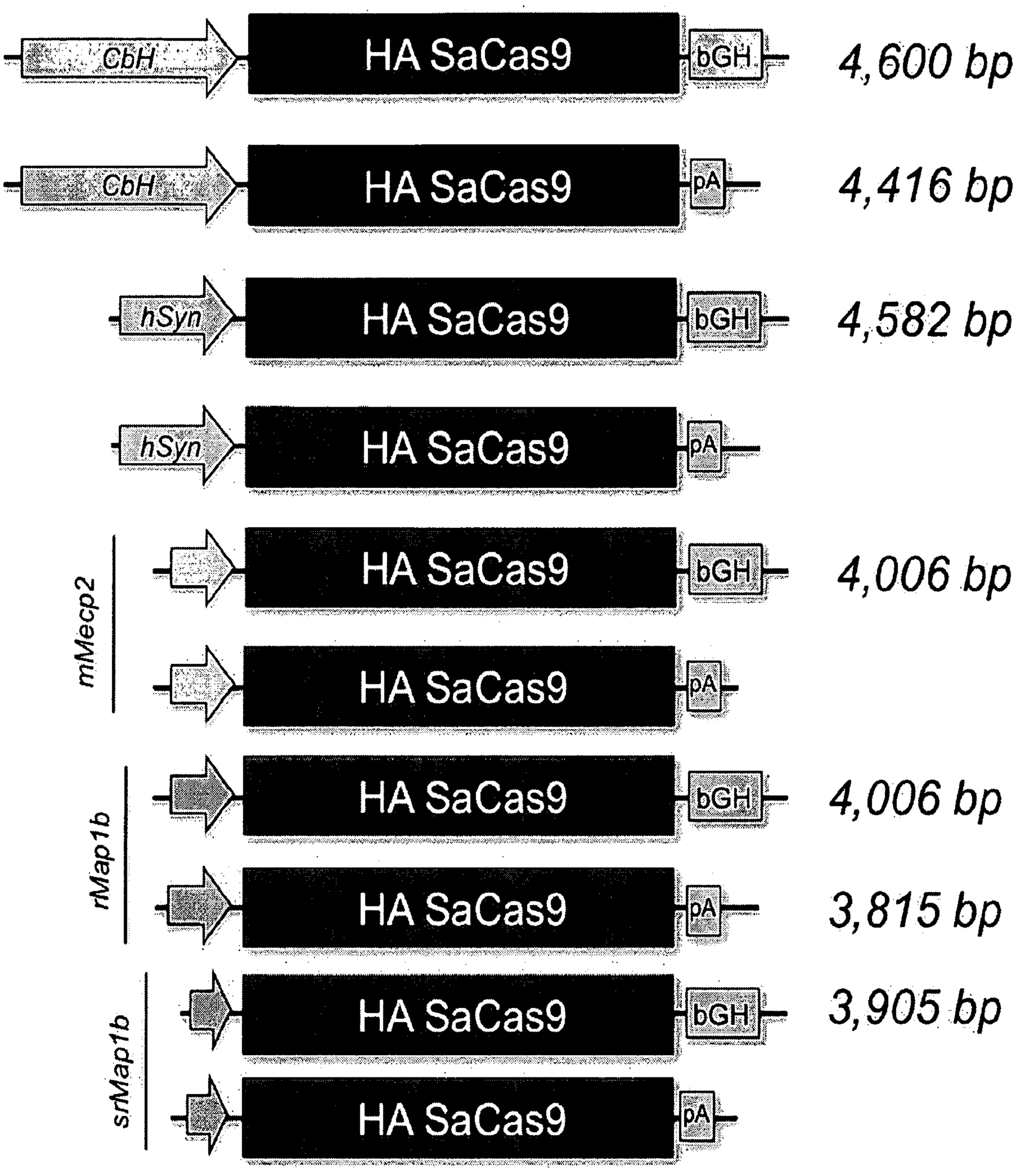

CbH
HA SaCas9
bGH
4,600 bp
CbH
HA SaCas9
pA
4,416 bp
hSyn
HA SaCas9
bGH
4,582 bp
hSyn
HA SaCas9
pA
mMecp2
HA SaCas9
bGH
4,006 bp
HA SaCas9
pA
rMap1b
HA SaCas9
bGH
4,006 bp
HA SaCas9
pA
3,815 bp
srMap1b
HA SaCas9
bGH
3,905 bp
HA SaCas9
pA

FIG. 38

Expression of SpCas9 & SaCas9 in N2a cells

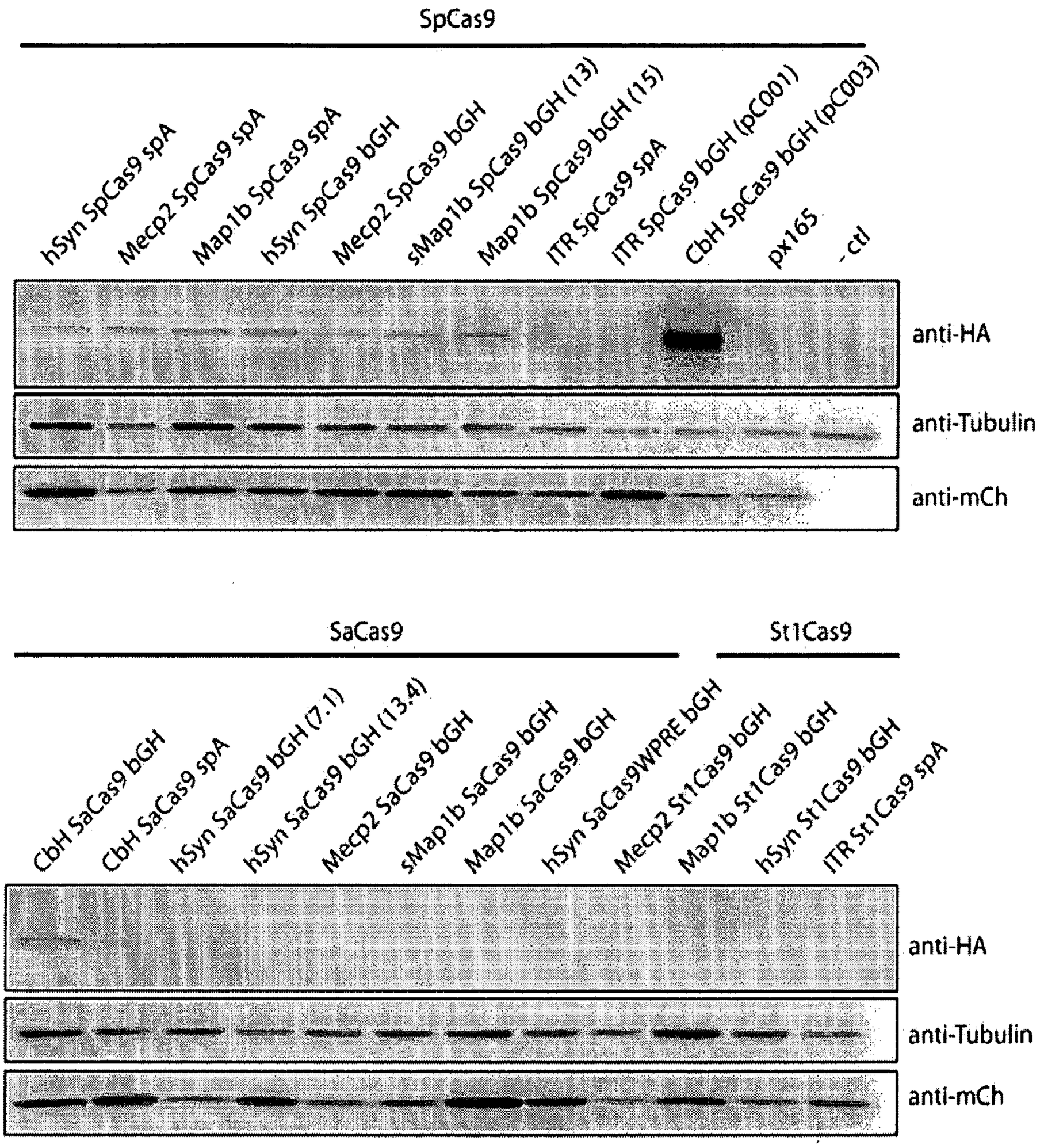


FIG. 39

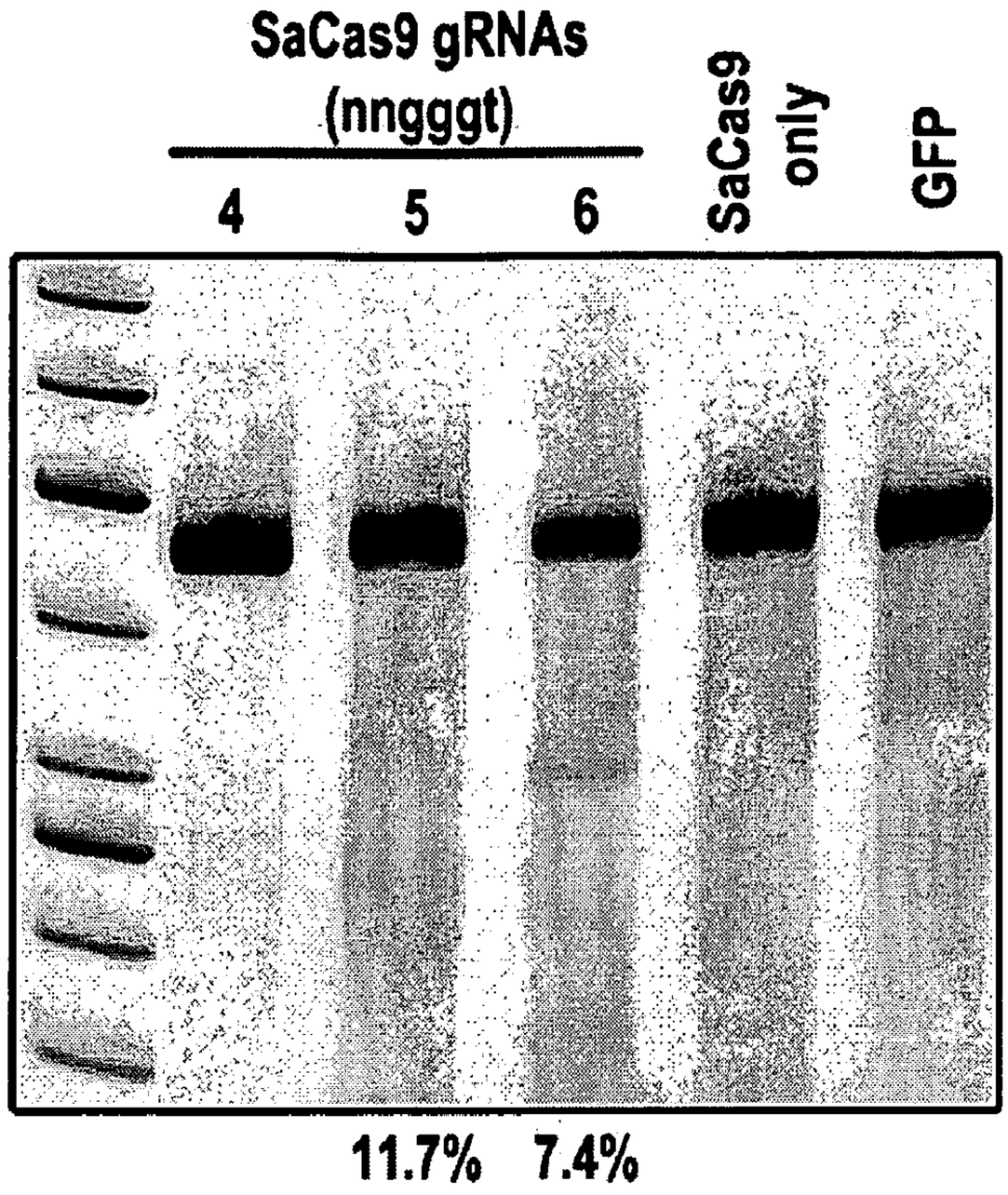


FIG. 40

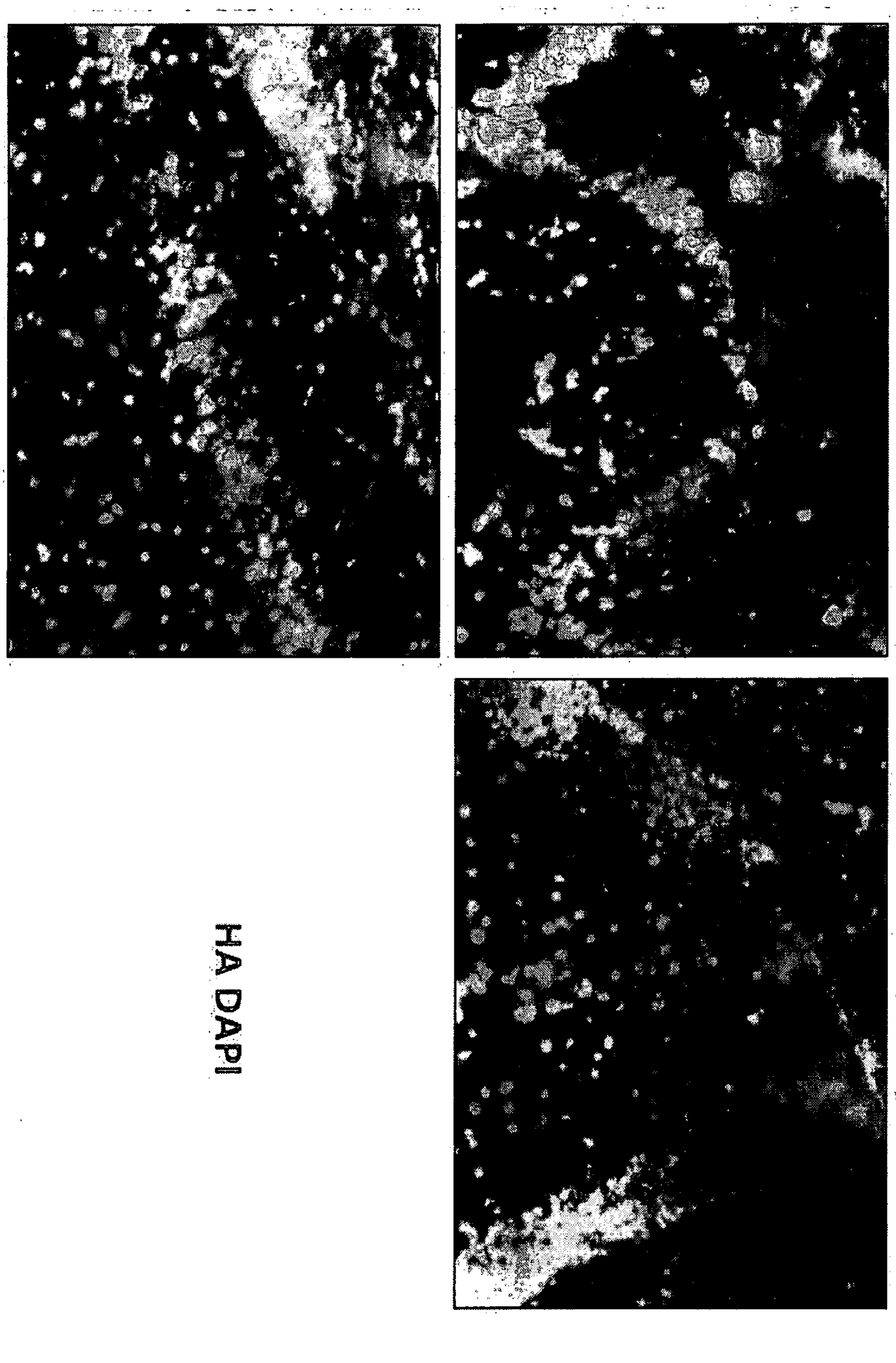


FIG. 41

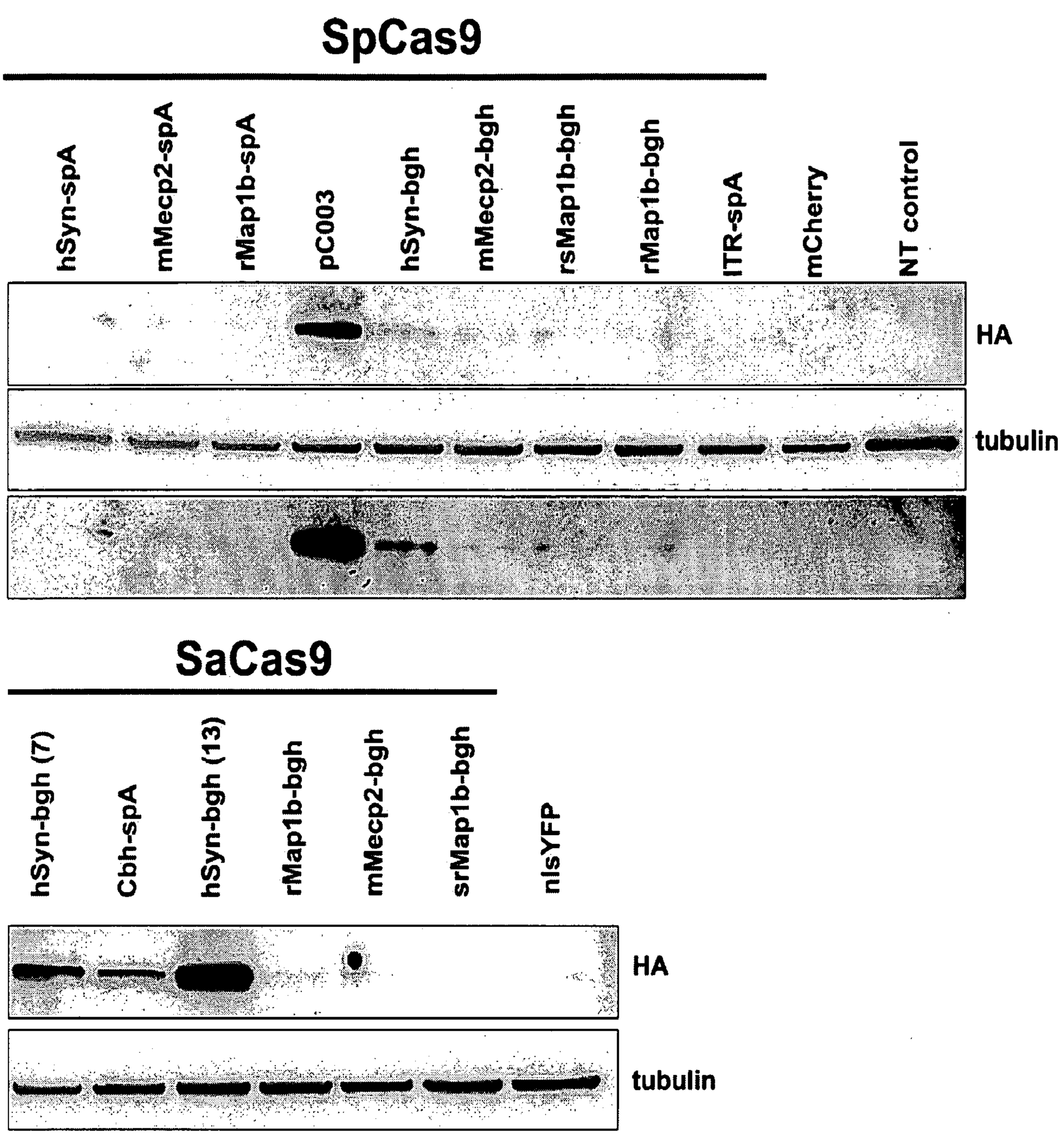

SpCas9
hSyn-spA
mMecp2-spA
rMap1b-spA
pC003
hSyn-bgh
mMecp2-bgh
srMap1b-bgh
rMap1b-bgh
ITR-spA
mCherry
NT control
HA
tubulin
SaCas9
hSyn-bgh (7)
Cbh-spA
hSyn-bgh (13
rMap1b-bgh
mMecp2-bgh
srMap1b-bgh
nlsYFP
HA
tubulin

FIG. 42

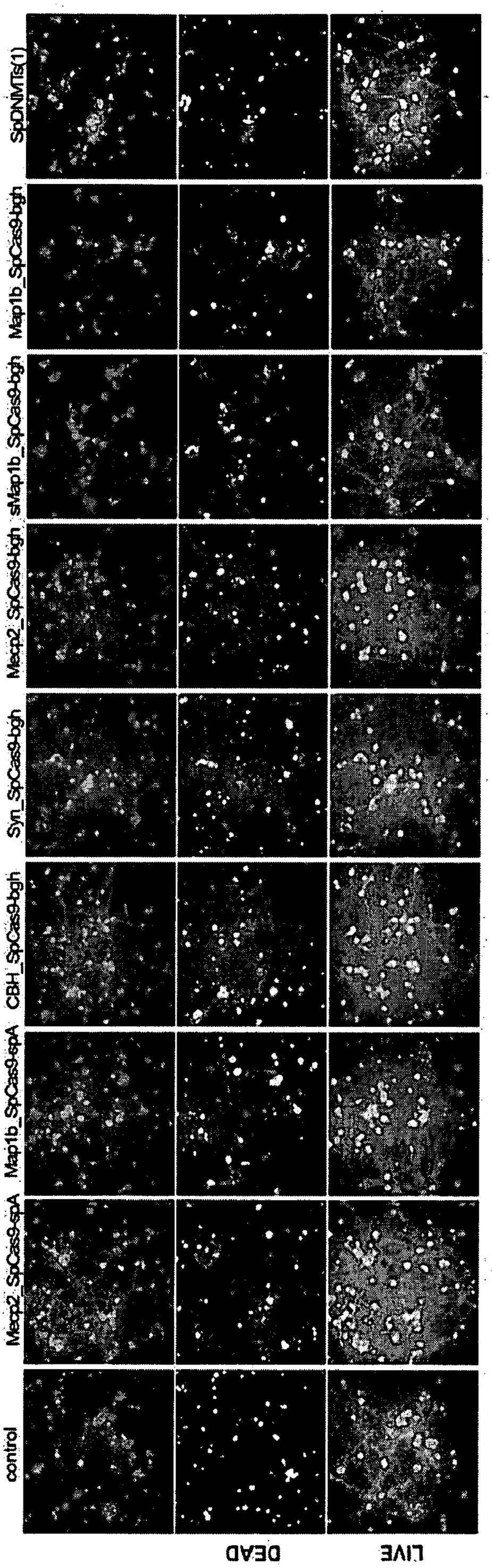

SpDNMTis(1)
Map1b_SpCas9-bgh
Mecp2_SpCas9-bgh
sMap1b_SpCas9-bgh
Syn_SpCas9-bgh
CBH_SpCas9-bgh
Map1b_SpCas9-spA
Mecp2_SpCas9-spA
control
DEAD
LIVE

FIG. 43

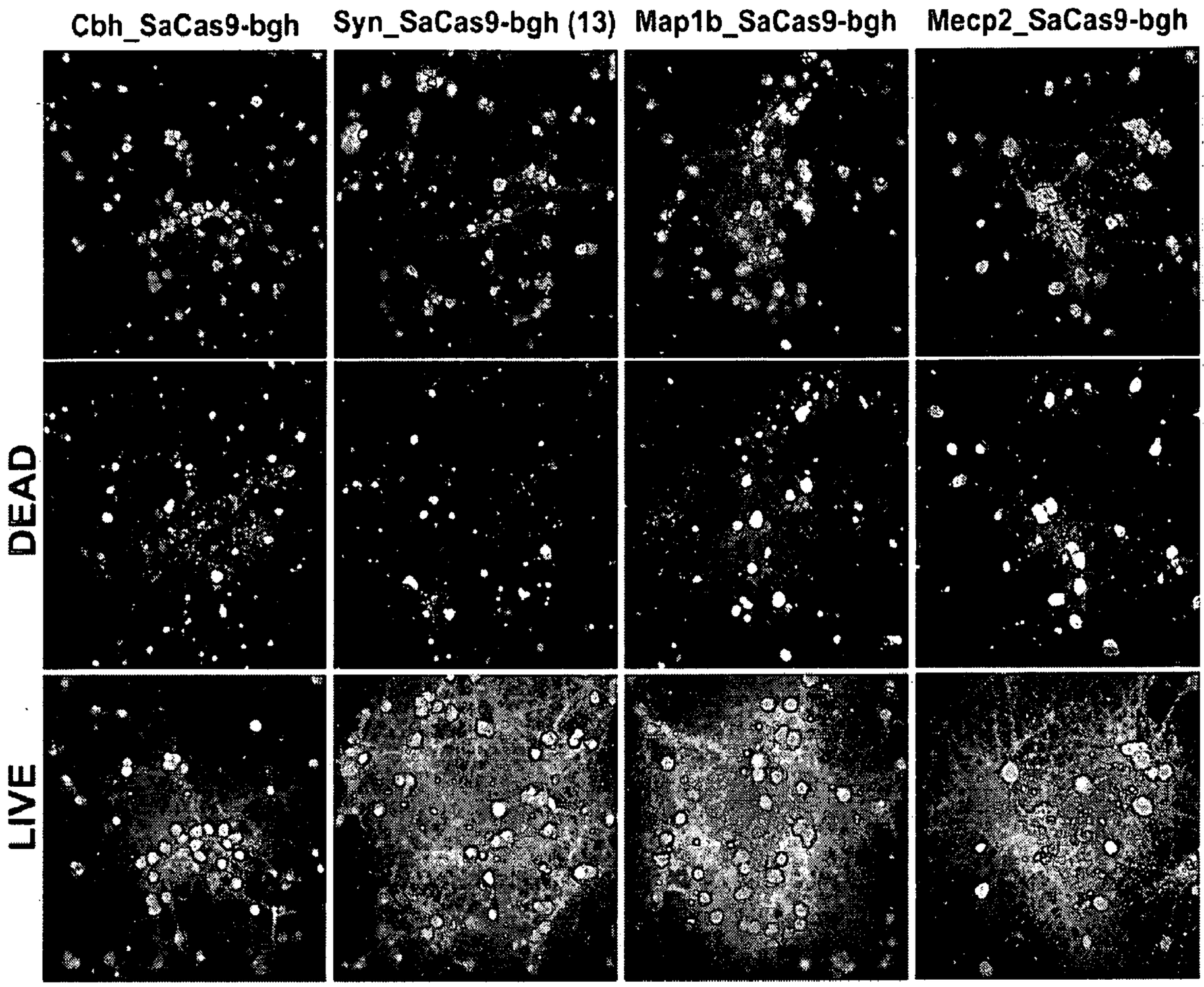

Cbh_SaCas9-bgh
Syn_SaCas9-bgh (13)
Map1b_SaCas9-bgh
Mecp2_SaCas9-bgh
DEAD
LIVE

FIG. 44

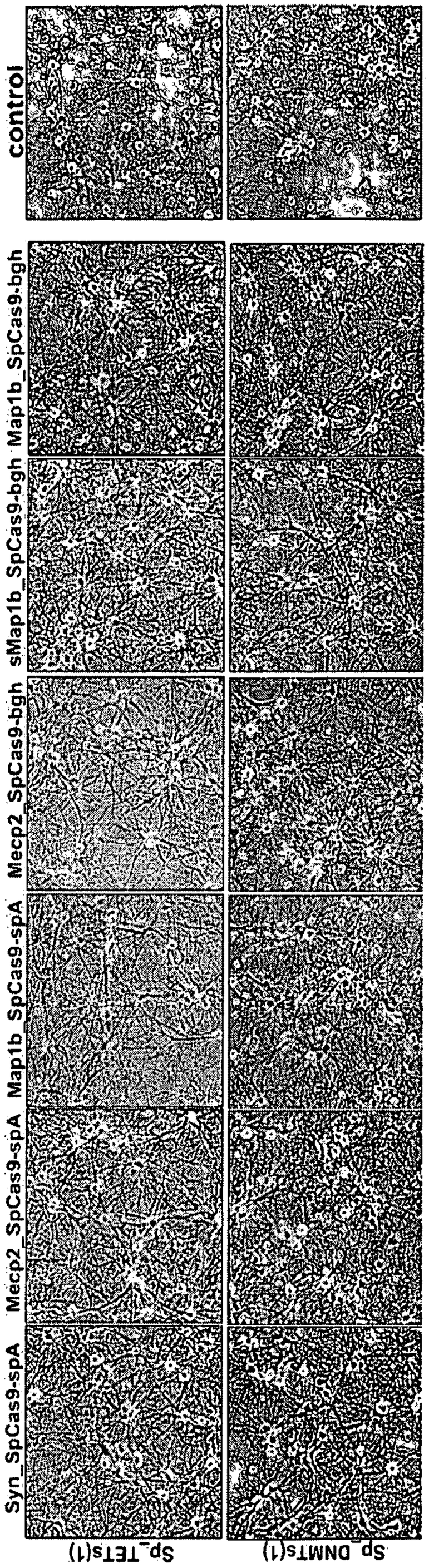

control
sMap1b_SpCas9-bgh
Map1b_SpCas9-bgh
Mecp2_SpCas9-bgh
Mecp2_SpCas9-spA
Map1b_SpCas9-spA
Syn_SpCas9-spA
Sp_TETs(1)
Sp_DNMTs(1)

FIG. 45

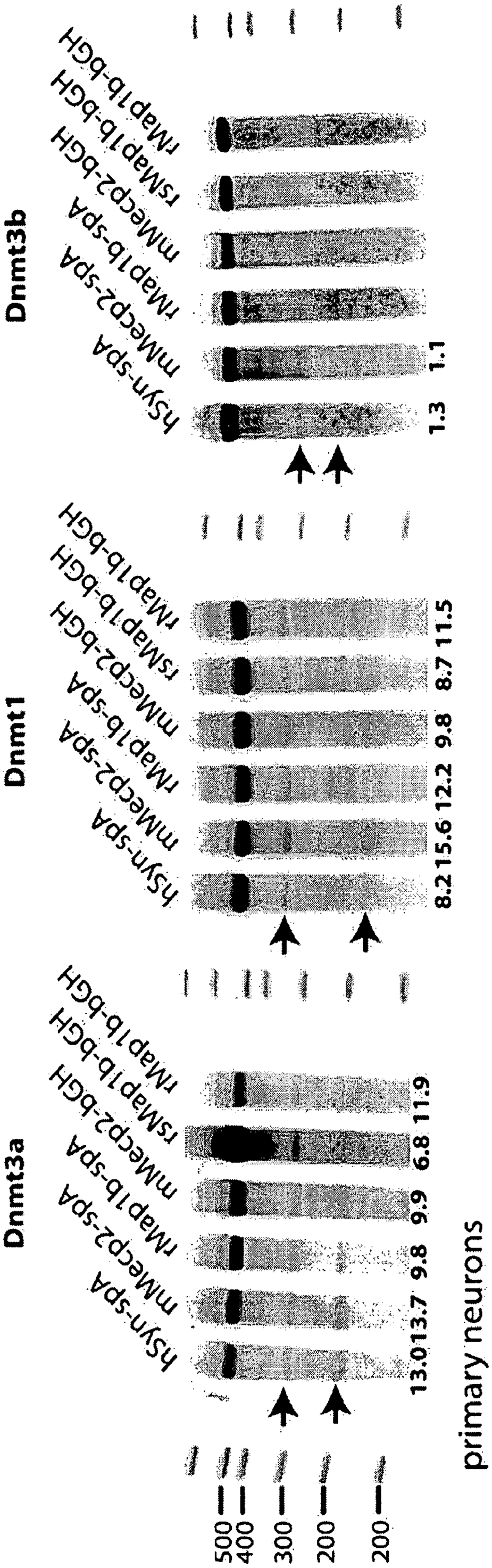

Dnmt3b
rMap1b-bGH
rSMap1b-bGH
rMeCP2-bGH
mMap1b-SPA
mMeCP2-SPA
hSyn-SPA
1.1
1.3
Dnmt1
rMap1b-bGH
rSMap1b-bGH
rMeCP2-bGH
mMap1b-SPA
mMeCP2-SPA
hSyn-SPA
11.5
8.7
9.8
12.2
15.6
8.2
Dnmt3a
rMap1b-bGH
rSMap1b-bGH
rMeCP2-bGH
mMap1b-SPA
mMeCP2-SPA
hSyn-SPA
11.9
6.8
9.9
9.8
13.7
13.0
500
400
300
200
200
primary neurons

FIG. 46

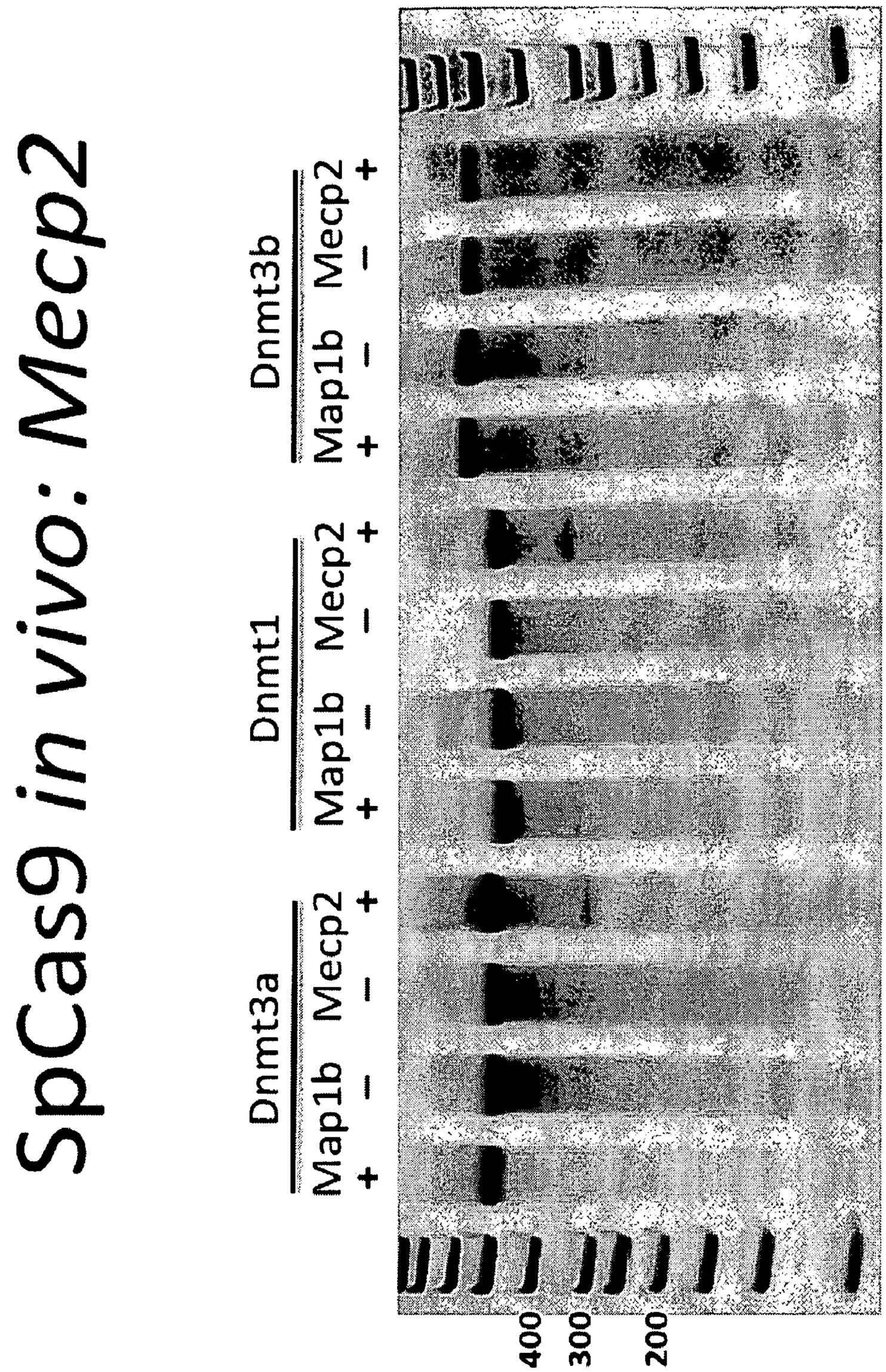

SpCas9 in vivo: Mecp2
Dnmt1
Map1b Mecp2
+ – – +
Dnmt3b
Mecp2
– +
Map1b
+ –
Dnmt3a
Map1b Mecp2
+ – – +
400
300
200
17.0%
12.0%

FIG. 47

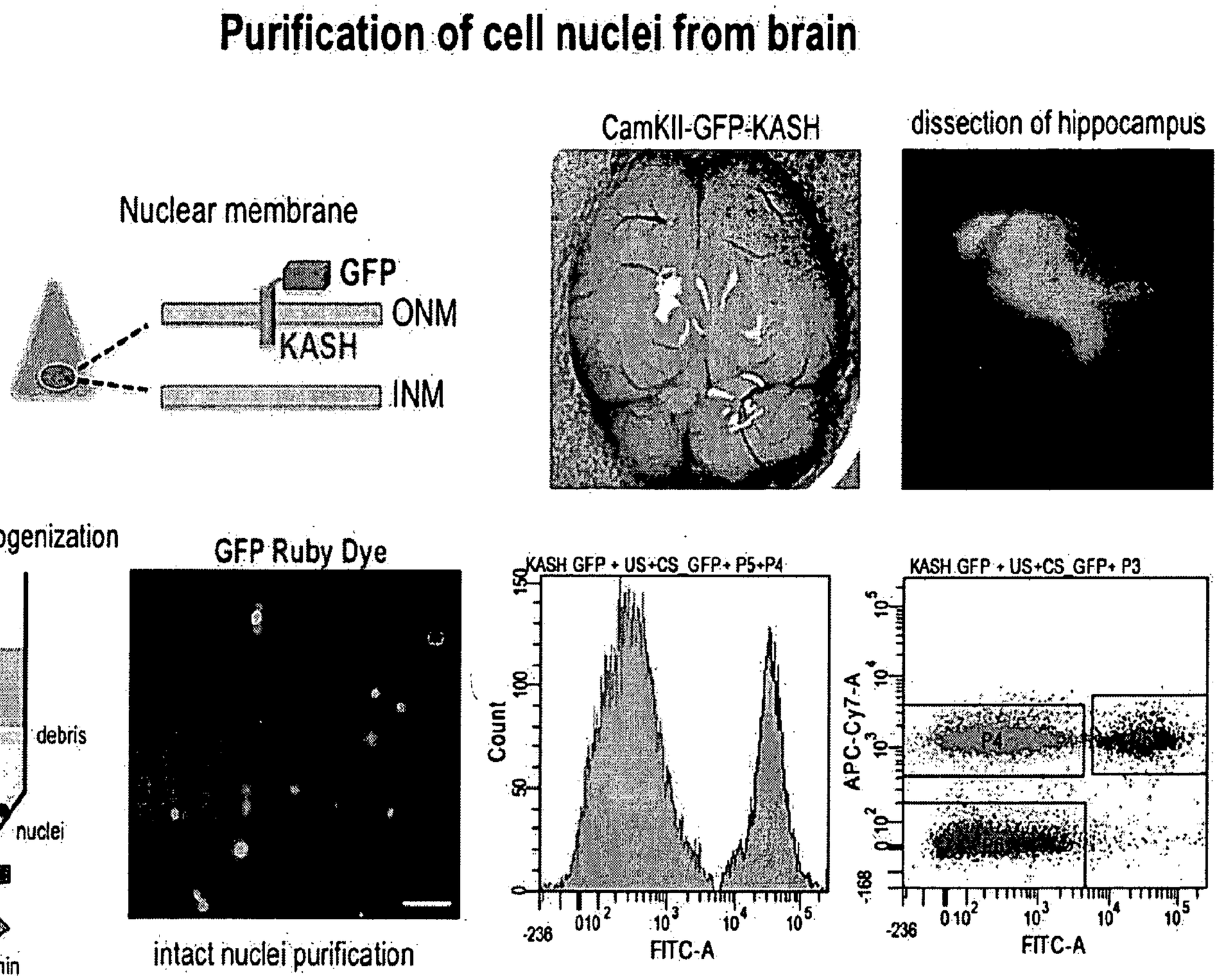
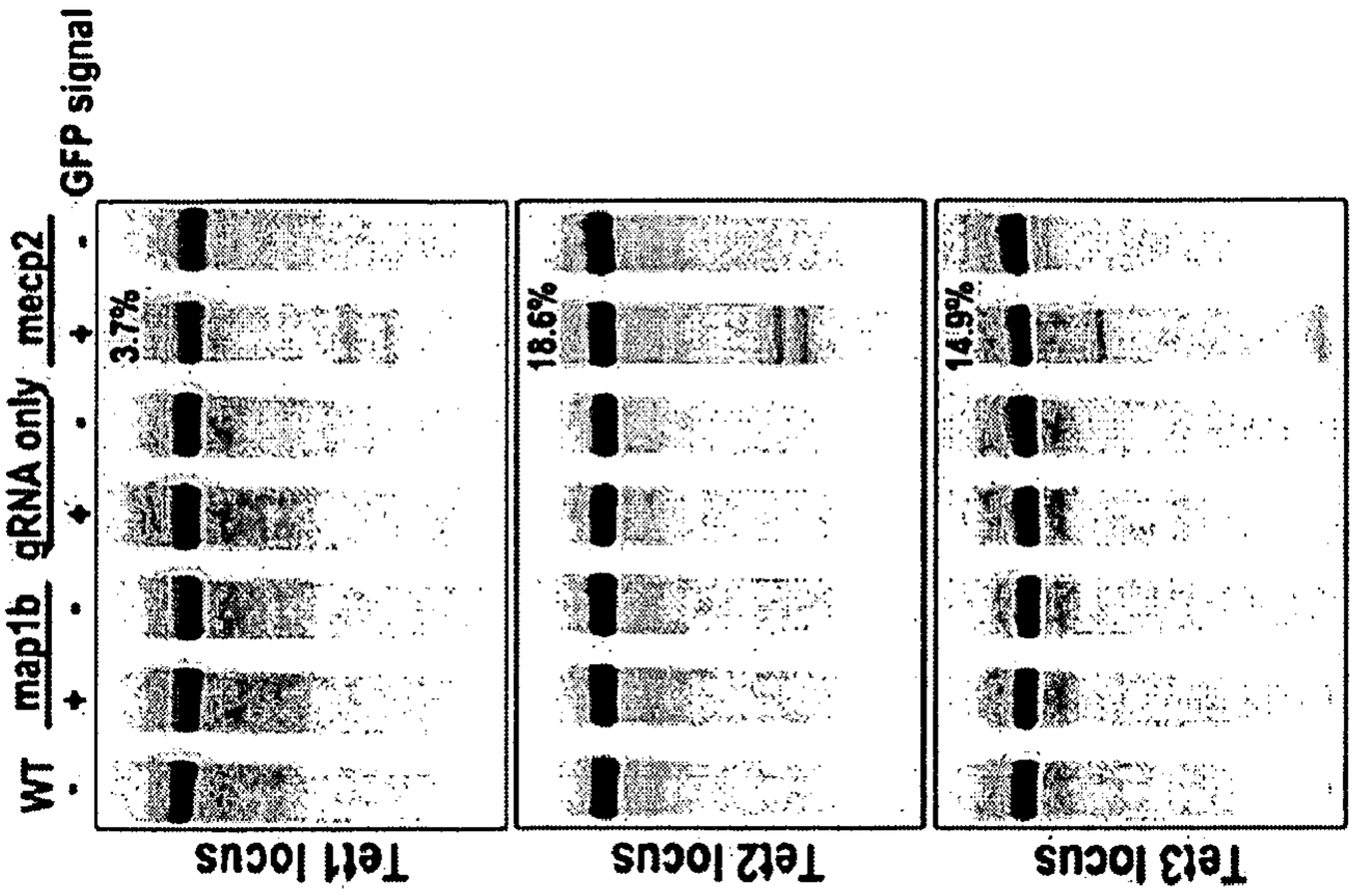

Purification of cell nuclei from brain
Nuclear membrane
GFP
ONM
KASH
INM
CamKII-GFP-KASH
dissection of hippocampus
tissue homogenization
debris
nuclei
30 min
density gradient centrifugation
GFP Ruby Dye
intact nuclei purification
KASH GFP + US+CS_GFP+ P5+P4
Count
FITC-A
KASH GFP + US+CS_GFP+ P3
APC-Cy7-A
P4
FITC-A
FACS sorting

FIG. 48


WT
map1b
gRNA only
GFP signal
3.7%
18.6%
14.9%
Tet1 locus
Tet2 locus
Tet3 locus

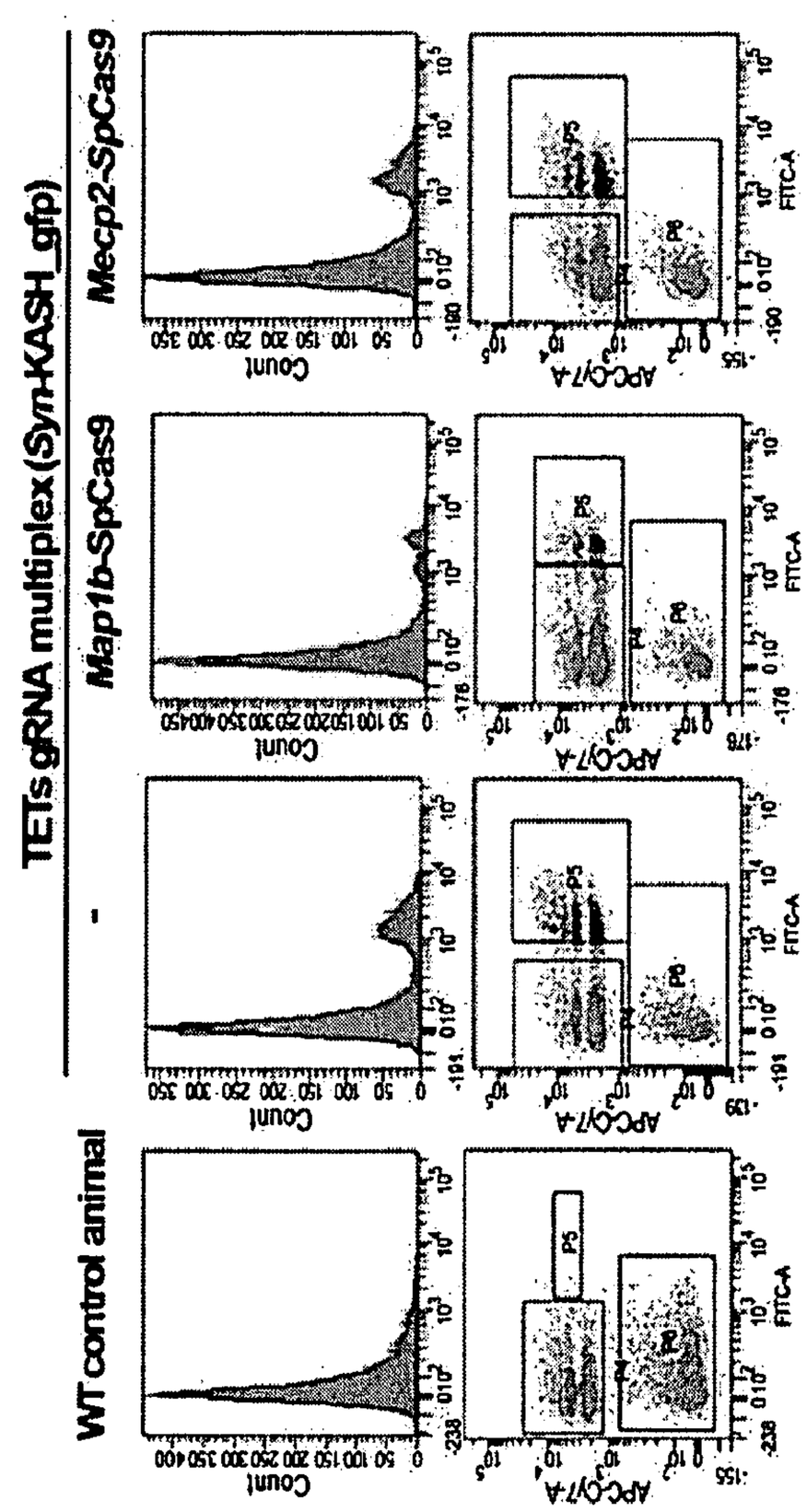

TETs gRNA multiplex (Syn-H1-KASH_gfp)
Mecp2-SpCas9
Map1b-SpCas9
-
+
WT control animal
Count
APC-Cy7-A
FITC-A
P5
P6

FIG. 49

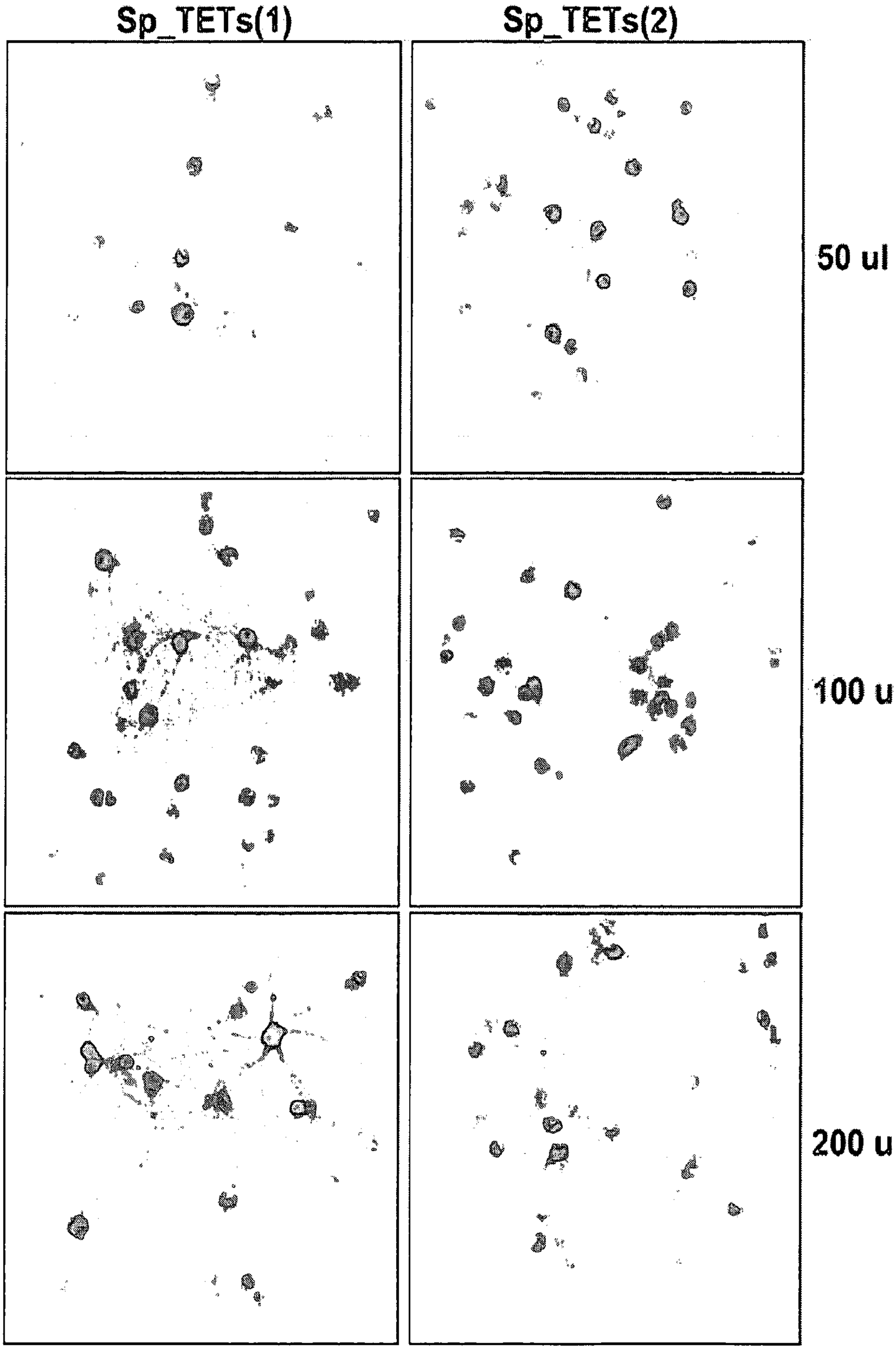


FIG. 50

	pattern	overhang
1	CCnNNNNNNNNNNNNNNNnnnnnnnnnnnnnNNNNNNNNNNNNNNNNnGG	38
2	CCnNNNNNNNNNNNNNNNnnnnnnnnnNNNNNNNNNNNNNNNNnGG	34
3	CCnNNNNNNNNNNNNNNNnnnnNNNNNNNNNNNNNNNNnGG	30
4	CCnNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNnGG	26
5	CCnNNNNNNNNNNNNNNNNNNNNNNNNNNNNnGG	22
6	CCnNNNNNNNNNNNNNNNNNNNNNNNNnGG	18
7	CCnNNNNNNNNNNNNNNNNNNnGG	14
8	CCnNNNNNNNNNNNNNNNnGG	10
9	CCnNNNNNNNNNNNnGG	6
10	CCnNNNNNNNNNnGG	4
11	CCnNNNNNNNNnGG	3
12	CCnNNNNNNNNnGG	2
13	CCnNNNNNNNnGG	1
14	CCnNNNNNNnGG	0
15	CCnNNNNNnGG	1
16	CCnNNNNnGG	2
17	CCnNNNnGG	3
18	CCnnGG	4
19	CCGG	8
20	GGCC	10
21	GGNNCC	12
22	GGNNNCC	14
23	GGNNNNCC	16
24	GGNNNNNNNCC	20

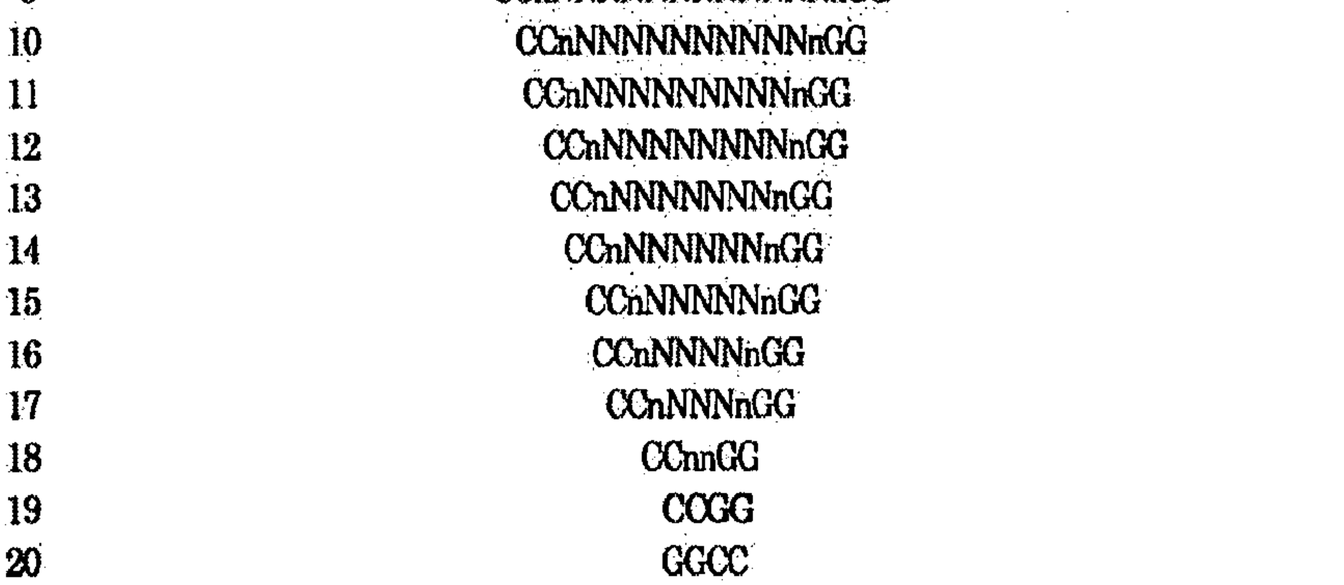


FIG. 51

Reverse PCR primers for generating U6-guide RNA expression Cassette (pair with U6 forward primer)

Z0260	AAAAAAAGCACCGACTCGGTGCCACTTTTTCAAGTTGATAACGGACTAGCCTTATTTTAACTTGCTATTTCTAGCTCTAAAACTCACATCAACCGGTGGCGCAGGTGTTTCGTCCTTTCCACAAG	Left guide RNA 1
Z0261	AAAAAAAGCACCGACTCGGTGCCACTTTTTCAAGTTGATAACGGACTAGCCTTATTTTAACTTGCTATTTCTAGCTCTAAAACTCACATCAACCGGTGGCGCAGGTGTTTCGTCCTTTCCACAAG	Left guide RNA 2
Z0262	AAAAAAAGCACCGACTCGGTGCCACTTTTTCAAGTTGATAACGGACTAGCCTTATTTTAACTTGCTATTTCTAGCTCTAAAACGAGGACAAAGTACAAACGGCGGTGTTTCGTCCTTTCCACAAG	Left guide RNA 3
Z0263	AAAAAAAGCACCGACTCGGTGCCACTTTTTCAAGTTGATAACGGACTAGCCTTATTTTAACTTGCTATTTCTAGCTCTAAAACGAGGACAAAGTACAAACGGCGGTGTTTCGTCCTTTCCACAAG	Left guide RNA 4
Z0264	AAAAAAAGCACCGACTCGGTGCCACTTTTTCAAGTTGATAACGGACTAGCCTTATTTTAACTTGCTATTTCTAGCTCTAAAACGTGGCGCATTGCCACGAAGCGGTGTTTCGTCCTTTCCACAAG	Left guide RNA 5
Z0265	AAAAAAAGCACCGACTCGGTGCCACTTTTTCAAGTTGATAACGGACTAGCCTTATTTTAACTTGCTATTTCTAGCTCTAAAACCGAGGGCAGAGTGCTGCTTGGGTGTTTCGTCCTTTCCACAAG	Left guide RNA 6
Z0266	AAAAAAAGCACCGACTCGGTGCCACTTTTTCAAGTTGATAACGGACTAGCCTTATTTTAACTTGCTATTTCTAGCTCTAAAACGAGTCCGAGCAGAAGAAGAAGGTGTTTCGTCCTTTCCACAAG	Left guide RNA 7
Z0267	AAAAAAAGCACCGACTCGGTGCCACTTTTTCAAGTTGATAACGGACTAGCCTTATTTTAACTTGCTATTTCTAGCTCTAAAACGAGGACAAAGTACAAACGGCGGTGTTTCGTCCTTTCCACAAG	Left guide RNA 8
Z0268	AAAAAAAGCACCGACTCGGTGCCACTTTTTCAAGTTGATAACGGACTAGCCTTATTTTAACTTGCTATTTCTAGCTCTAAAACAGCAGAAGAAGAAGGGCTCCGGTGTTTCGTCCTTTCCACAAG	Left guide RNA 9
Z0269	AAAAAAAGCACCGACTCGGTGCCACTTTTTCAAGTTGATAACGGACTAGCCTTATTTTAACTTGCTATTTCTAGCTCTAAAACTCACATCAACCGGTGGCGCAGGTGTTTCGTCCTTTCCACAAG	Left guide RNA 10
Z0270	AAAAAAAGCACCGACTCGGTGCCACTTTTTCAAGTTGATAACGGACTAGCCTTATTTTAACTTGCTATTTCTAGCTCTAAAACCCCTGGCCCAGGTGAAGGTGGGTGTTTCGTCCTTTCCACAAG	Left guide RNA 11
Z0271	AAAAAAAGCACCGACTCGGTGCCACTTTTTCAAGTTGATAACGGACTAGCCTTATTTTAACTTGCTATTTCTAGCTCTAAAACTCCCTCCCTGGCCCAGGTGAGGTGTTTCGTCCTTTCCACAAG	Left guide RNA 12
Z0272	AAAAAAAGCACCGACTCGGTGCCACTTTTTCAAGTTGATAACGGACTAGCCTTATTTTAACTTGCTATTTCTAGCTCTAAAACGAACCGGAGGACAAAGTACAGGTGTTTCGTCCTTTCCACAAG	Left guide RNA 13
Z0273	AAAAAAAGCACCGACTCGGTGCCACTTTTTCAAGTTGATAACGGACTAGCCTTATTTTAACTTGCTATTTCTAGCTCTAAAACAGGTGAAGGTGTGGTTCCAGGGTGTTTCGTCCTTTCCACAAG	Left guide RNA 14
Z0274	AAAAAAAGCACCGACTCGGTGCCACTTTTTCAAGTTGATAACGGACTAGCCTTATTTTAACTTGCTATTTCTAGCTCTAAAACGGTGAAGGTGTGGTTCCAGAGGTGTTTCGTCCTTTCCACAAG	Left guide RNA 15
Z0275	AAAAAAAGCACCGACTCGGTGCCACTTTTTCAAGTTGATAACGGACTAGCCTTATTTTAACTTGCTATTTCTAGCTCTAAAACGAACCGGAGGACAAAGTACAGGTGTTTCGTCCTTTCCACAAG	Left guide RNA 16
Z0276	AAAAAAAGCACCGACTCGGTGCCACTTTTTCAAGTTGATAACGGACTAGCCTTATTTTAACTTGCTATTTCTAGCTCTAAAACCCCTGGCCCAGGTGAAGGTGGGTGTTTCGTCCTTTCCACAAG	Left guide RNA 17
Z0277	AAAAAAAGCACCGACTCGGTGCCACTTTTTCAAGTTGATAACGGACTAGCCTTATTTTAACTTGCTATTTCTAGCTCTAAAACAGGTGAAGGTGTGGTTCCAGGGTGTTTCGTCCTTTCCACAAG	Left guide RNA 18
Z0278	AAAAAAAGCACCGACTCGGTGCCACTTTTTCAAGTTGATAACGGACTAGCCTTATTTTAACTTGCTATTTCTAGCTCTAAAACGAGGACAAAGTACAAACGGCGGTGTTTCGTCCTTTCCACAAG	Left guide RNA 19
Z0279	AAAAAAAGCACCGACTCGGTGCCACTTTTTCAAGTTGATAACGGACTAGCCTTATTTTAACTTGCTATTTCTAGCTCTAAAACGGGAGGGAGGGGCACAGATGGGTGTTTCGTCCTTTCCACAAG	Left guide RNA 20
Z0280	AAAAAAAGCACCGACTCGGTGCCACTTTTTCAAGTTGATAACGGACTAGCCTTATTTTAACTTGCTATTTCTAGCTCTAAAACCACCTTCACCTGGGCCAGGGGGTGTTTCGTCCTTTCCACAAG	Left guide RNA 21
Z0281	AAAAAAAGCACCGACTCGGTGCCACTTTTTCAAGTTGATAACGGACTAGCCTTATTTTAACTTGCTATTTCTAGCTCTAAAACACCCTAGTCATTGGAGGTGAGGTGTTTCGTCCTTTCCACAAG	Left guide RNA 22
Z0282	AAAAAAAGCACCGACTCGGTGCCACTTTTTCAAGTTGATAACGGACTAGCCTTATTTTAACTTGCTATTTCTAGCTCTAAAACCAGAGCAGCCACTGGGGCCTGGTGTTTCGTCCTTTCCACAAG	Left guide RNA 23
Z0283	AAAAAAAGCACCGACTCGGTGCCACTTTTTCAAGTTGATAACGGACTAGCCTTATTTTAACTTGCTATTTCTAGCTCTAAAACCACCTTCACCTGGGCCAGGGGGTGTTTCGTCCTTTCCACAAG	Left guide RNA 24
Z0284	AAAAAAAGCACCGACTCGGTGCCACTTTTTCAAGTTGATAACGGACTAGCCTTATTTTAACTTGCTATTTCTAGCTCTAAAACCCCCATTGGCCTGCTTCGTGGGTGTTTCGTCCTTTCCACAAG	Right guide RNA 1
Z0285	AAAAAAAGCACCGACTCGGTGCCACTTTTTCAAGTTGATAACGGACTAGCCTTATTTTAACTTGCTATTTCTAGCTCTAAAACATTGGCCTGCTTCGTGGCAAGGTGTTTCGTCCTTTCCACAAG	Right guide RNA 2
Z0286	AAAAAAAGCACCGACTCGGTGCCACTTTTTCAAGTTGATAACGGACTAGCCTTATTTTAACTTGCTATTTCTAGCTCTAAAACTCCTCCTCCAGCTTCTGCCGGGTGTTTCGTCCTTTCCACAAG	Right guide RNA 3
Z0287	AAAAAAAGCACCGACTCGGTGCCACTTTTTCAAGTTGATAACGGACTAGCCTTATTTTAACTTGCTATTTCTAGCTCTAAAACCCTCCAGCTTCTGCCGTTTGGGTGTTTCGTCCTTTCCACAAG	Right guide RNA 4
Z0288	AAAAAAAGCACCGACTCGGTGCCACTTTTTCAAGTTGATAACGGACTAGCCTTATTTTAACTTGCTATTTCTAGCTCTAAAACATTGGCCTGCTTCGTGGCAAGGTGTTTCGTCCTTTCCACAAG	Right guide RNA 5
Z0289	AAAAAAAGCACCGACTCGGTGCCACTTTTTCAAGTTGATAACGGACTAGCCTTATTTTAACTTGCTATTTCTAGCTCTAAAACGCAGCAAGCAGCACTCTGCCGGTGTTTCGTCCTTTCCACAAG	Right guide RNA 6
Z0290	AAAAAAAGCACCGACTCGGTGCCACTTTTTCAAGTTGATAACGGACTAGCCTTATTTTAACTTGCTATTTCTAGCTCTAAAACTTCTTCTTCTGCTCGGACTCGGTGTTTCGTCCTTTCCACAAG	Right guide RNA 7
Z0291	AAAAAAAGCACCGACTCGGTGCCACTTTTTCAAGTTGATAACGGACTAGCCTTATTTTAACTTGCTATTTCTAGCTCTAAAACACCGGAGGACAAAGTACAAAGGTGTTTCGTCCTTTCCACAAG	Right guide RNA 8
Z0292	AAAAAAAGCACCGACTCGGTGCCACTTTTTCAAGTTGATAACGGACTAGCCTTATTTTAACTTGCTATTTCTAGCTCTAAAACTCTTCTTCTGCTCGGACTCAGGTGTTTCGTCCTTTCCACAAG	Right guide RNA 9
Z0293	AAAAAAAGCACCGACTCGGTGCCACTTTTTCAAGTTGATAACGGACTAGCCTTATTTTAACTTGCTATTTCTAGCTCTAAAACGTTGATGTGATGGGAGCCCTGGTGTTTCGTCCTTTCCACAAG	Right guide RNA 10
Z0294	AAAAAAAGCACCGACTCGGTGCCACTTTTTCAAGTTGATAACGGACTAGCCTTATTTTAACTTGCTATTTCTAGCTCTAAAACGGGCCAGGGAGGGAGGGGCAGGTGTTTCGTCCTTTCCACAAG	Right guide RNA 11
Z0295	AAAAAAAGCACCGACTCGGTGCCACTTTTTCAAGTTGATAACGGACTAGCCTTATTTTAACTTGCTATTTCTAGCTCTAAAACGGGAGGGAGGGGCACAGATGGGTGTTTCGTCCTTTCCACAAG	Right guide RNA 12
Z0296	AAAAAAAGCACCGACTCGGTGCCACTTTTTCAAGTTGATAACGGACTAGCCTTATTTTAACTTGCTATTTCTAGCTCTAAAACCCGGTTCTGGAACCACACCTGGTGTTTCGTCCTTTCCACAAG	Right guide RNA 13
Z0297	AAAAAAAGCACCGACTCGGTGCCACTTTTTCAAGTTGATAACGGACTAGCCTTATTTTAACTTGCTATTTCTAGCTCTAAAACTCACCTGGGCCAGGGAGGGAGGTGTTTCGTCCTTTCCACAAG	Right guide RNA 14
Z0298	AAAAAAAGCACCGACTCGGTGCCACTTTTTCAAGTTGATAACGGACTAGCCTTATTTTAACTTGCTATTTCTAGCTCTAAAACTCACCTGGGCCAGGGAGGGAGGTGTTTCGTCCTTTCCACAAG	Right guide RNA 15
Z0299	AAAAAAAGCACCGACTCGGTGCCACTTTTTCAAGTTGATAACGGACTAGCCTTATTTTAACTTGCTATTTCTAGCTCTAAAACGTTCTGGAACCACACCTTCAGGTGTTTCGTCCTTTCCACAAG	Right guide RNA 16
Z0300	AAAAAAAGCACCGACTCGGTGCCACTTTTTCAAGTTGATAACGGACTAGCCTTATTTTAACTTGCTATTTCTAGCTCTAAAACGGGAGGGAGGGGCACAGATGGGTGTTTCGTCCTTTCCACAAG	Right guide RNA 17
Z0301	AAAAAAAGCACCGACTCGGTGCCACTTTTTCAAGTTGATAACGGACTAGCCTTATTTTAACTTGCTATTTCTAGCTCTAAAACGGGCCAGGGAGGGAGGGGCAGGTGTTTCGTCCTTTCCACAAG	Right guide RNA 18
Z0302	AAAAAAAGCACCGACTCGGTGCCACTTTTTCAAGTTGATAACGGACTAGCCTTATTTTAACTTGCTATTTCTAGCTCTAAAACGTTCTGGAACCACACCTTCAGGTGTTTCGTCCTTTCCACAAG	Right guide RNA 19
Z0303	AAAAAAAGCACCGACTCGGTGCCACTTTTTCAAGTTGATAACGGACTAGCCTTATTTTAACTTGCTATTTCTAGCTCTAAAACAGGTGAAGGTGTGGTTCCAGGGTGTTTCGTCCTTTCCACAAG	Right guide RNA 20
Z0304	AAAAAAAGCACCGACTCGGTGCCACTTTTTCAAGTTGATAACGGACTAGCCTTATTTTAACTTGCTATTTCTAGCTCTAAAACGAACCGGAGGACAAAGTACAGGTGTTTCGTCCTTTCCACAAG	Right guide RNA 21
Z0305	AAAAAAAGCACCGACTCGGTGCCACTTTTTCAAGTTGATAACGGACTAGCCTTATTTTAACTTGCTATTTCTAGCTCTAAAACCAAACCCACGAGGGCAGAGTGGTGTTTCGTCCTTTCCACAAG	Right guide RNA 22
Z0306	AAAAAAAGCACCGACTCGGTGCCACTTTTTCAAGTTGATAACGGACTAGCCTTATTTTAACTTGCTATTTCTAGCTCTAAAACGAGTTTCTCATCTGTGCCCCGGTGTTTCGTCCTTTCCACAAG	Right guide RNA 23
Z0307	AAAAAAAGCACCGACTCGGTGCCACTTTTTCAAGTTGATAACGGACTAGCCTTATTTTAACTTGCTATTTCTAGCTCTAAAACGAGGACAAAGTACAAACGGCGGTGTTTCGTCCTTTCCACAAG	Right guide RNA 24

FIG. 52

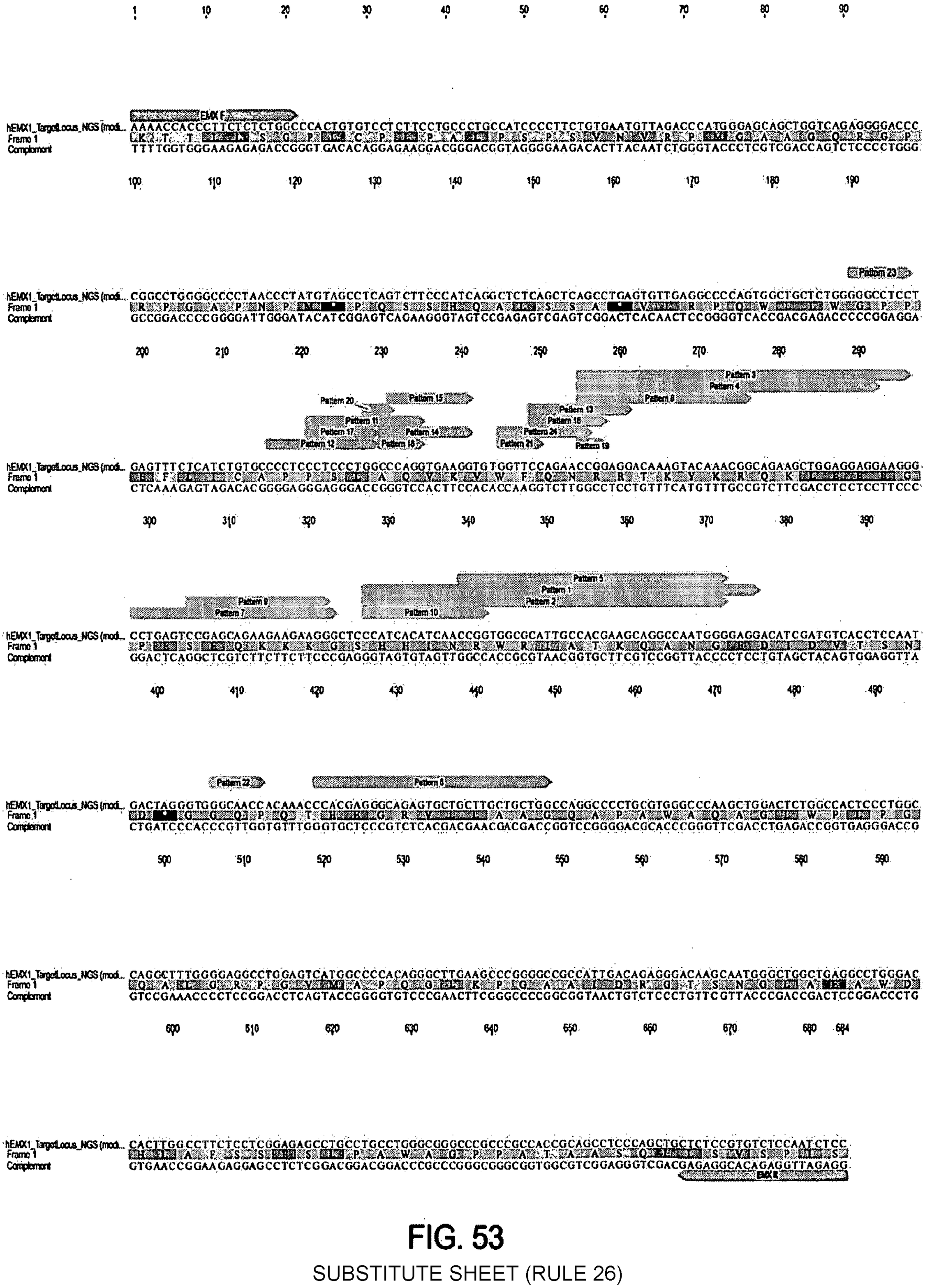


FIG. 53

SUBSTITUTE SHEET (RULE 26)

lane #	Cas9 to use	Left gRNA	Right gRNA	Overhang (D10A) - number of bases protruding from 5'
1	D10A	left 23	left 12	-36
2	D10A	right 4	left 9	-25
3	D10A	left 23	right 23	-16
4	D10A	right 7	left 10	-15
5	D10A	right 16	left 3	-8
6	D10A	right 22	right 6	26
7	D10A	left 12	right 16	31
8	D10A	left 12	right 13	34
9	D10A	left 10	right 1	38
10	D10A	right 23	right 16	51
11	D10A	right 23	right 13	54
12	D10A	left 3	right 7	57
13	D10A	left 12	right 4	65
14	D10A	left 12	right 3	69
15	D10A	left 3	right 10	76
16	D10A	right 23	right 4	85
17	D10A	left 12	right 9	95
18	D10A	left 12	right 10	115
19	D10A	right 23	right 10	135
20	D10A	left 12	right 2	145
21	D10A	left 12	left 22	181
22	D10A	right 23	left 22	201
23	D10A	left 12	right 6	222
24	D10A	right 23	right 6	242

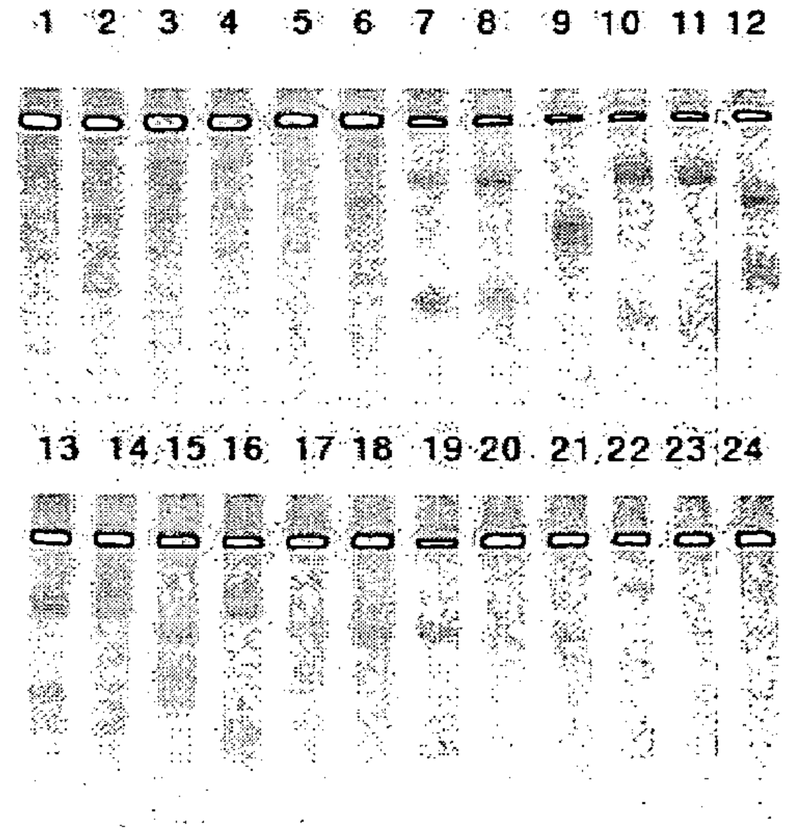


FIG. 54

CA 02894681 2015-06-10

SUBSTITUTE SHEET (RULE 26)

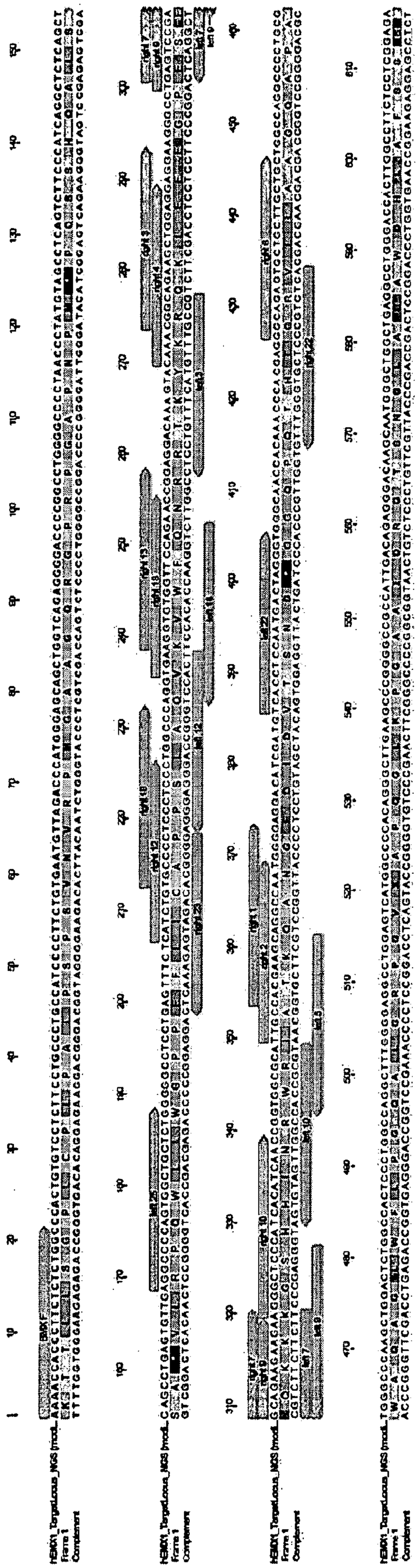


FIG. 55

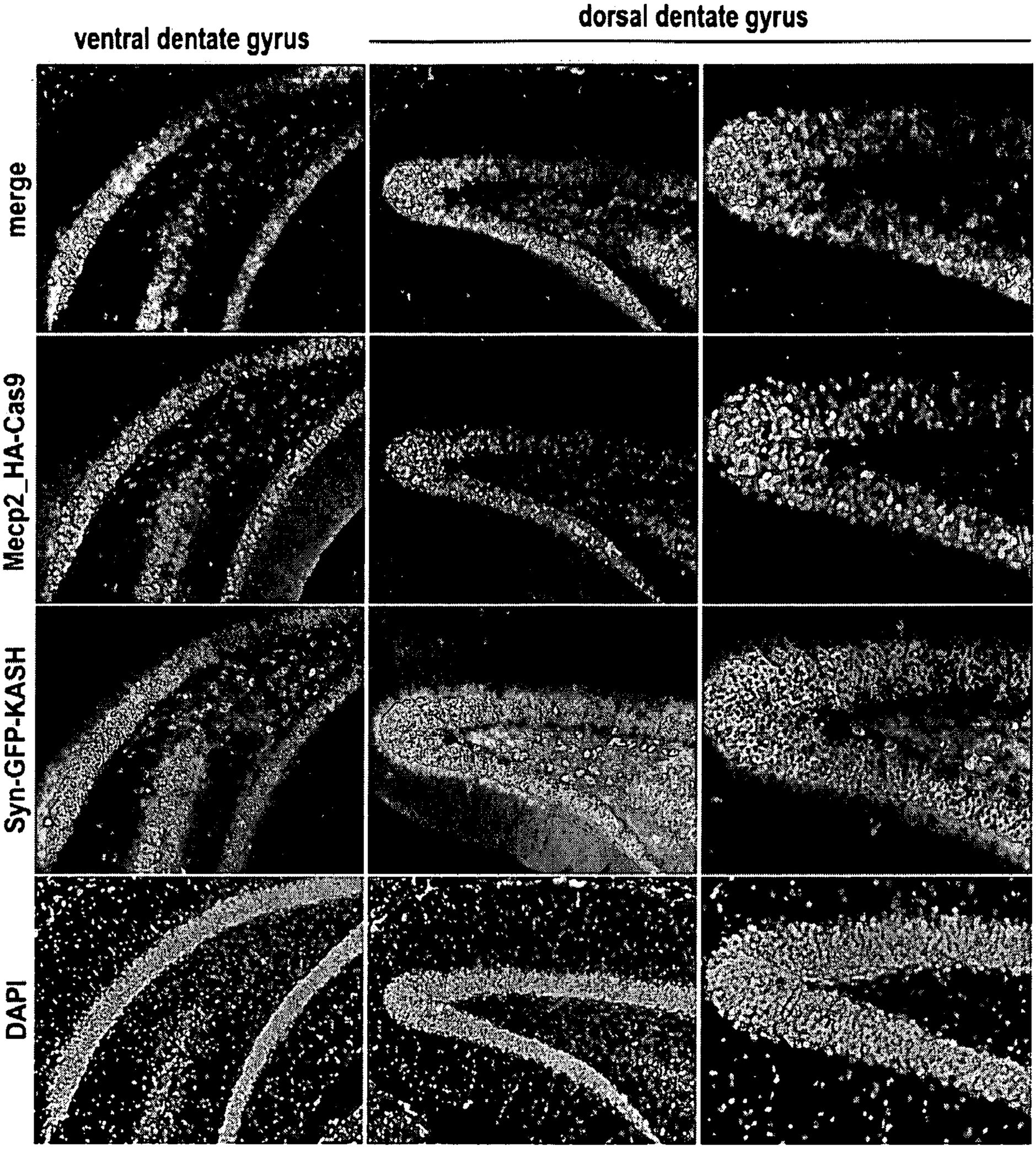

ventral dentate gyrus
dorsal dentate gyrus
merge
Mecp2_HA-Cas9
Syn-GFP-KASH
DAPI

FIG. 56

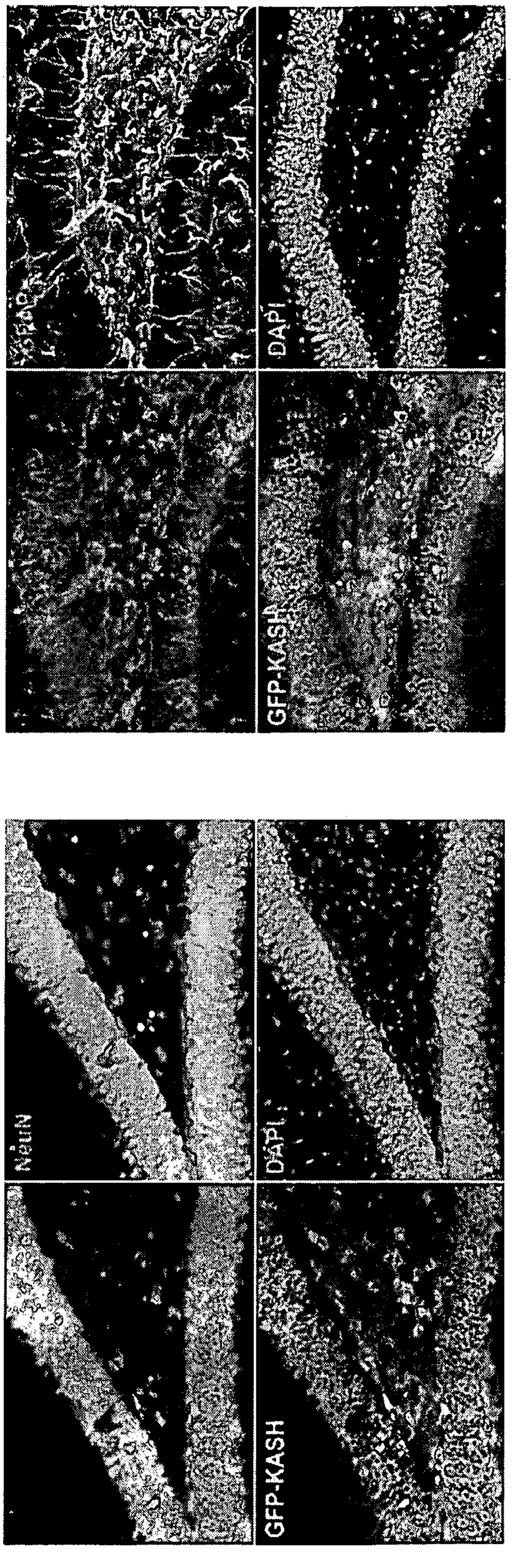
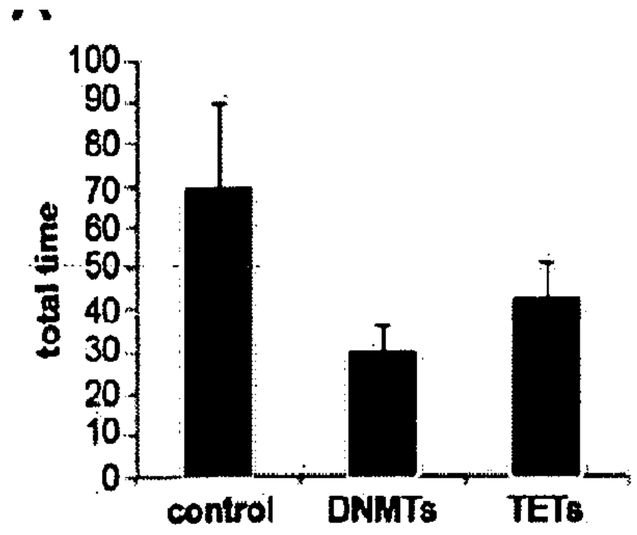
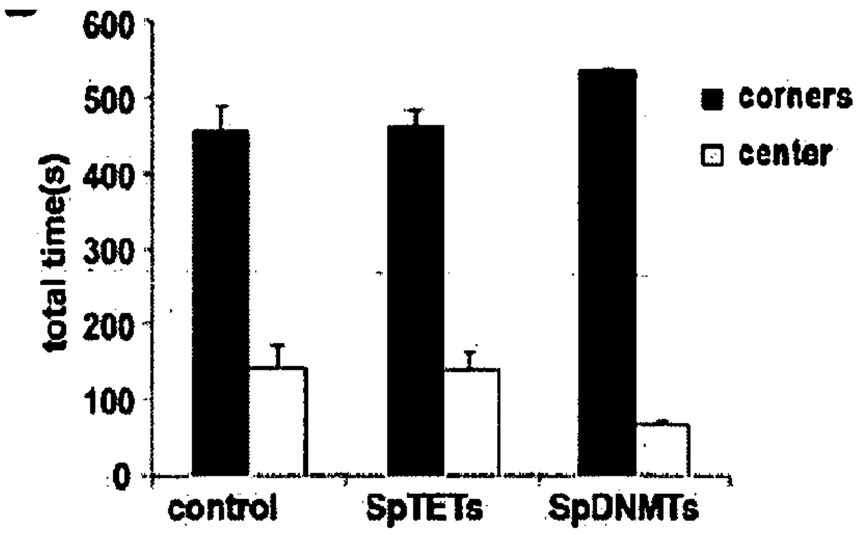
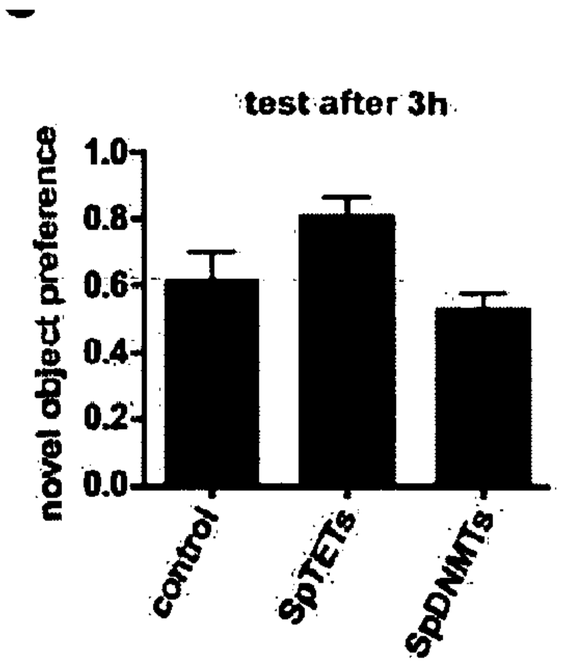


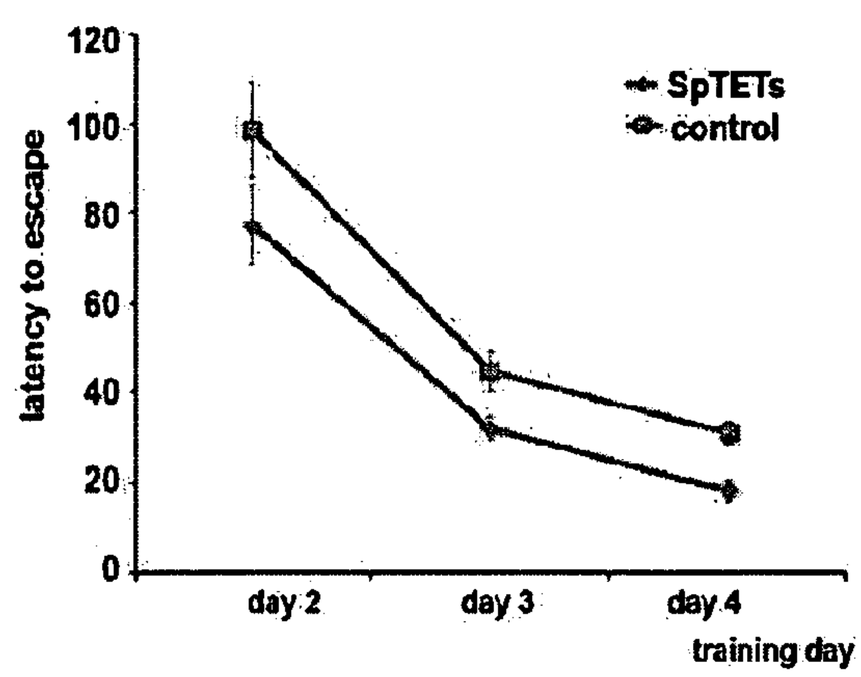
FIG. 57



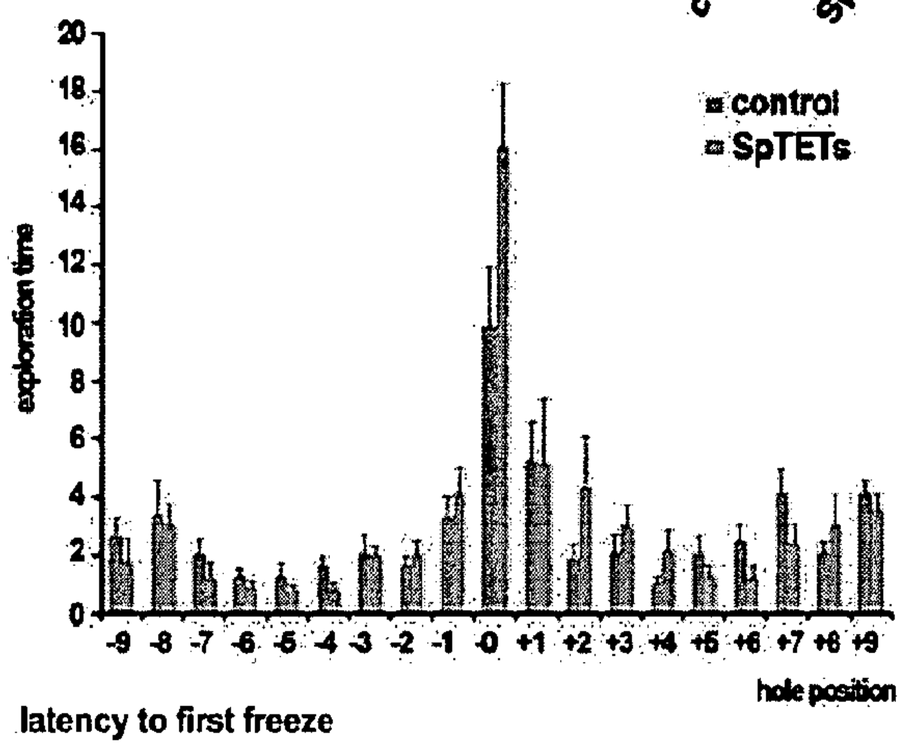




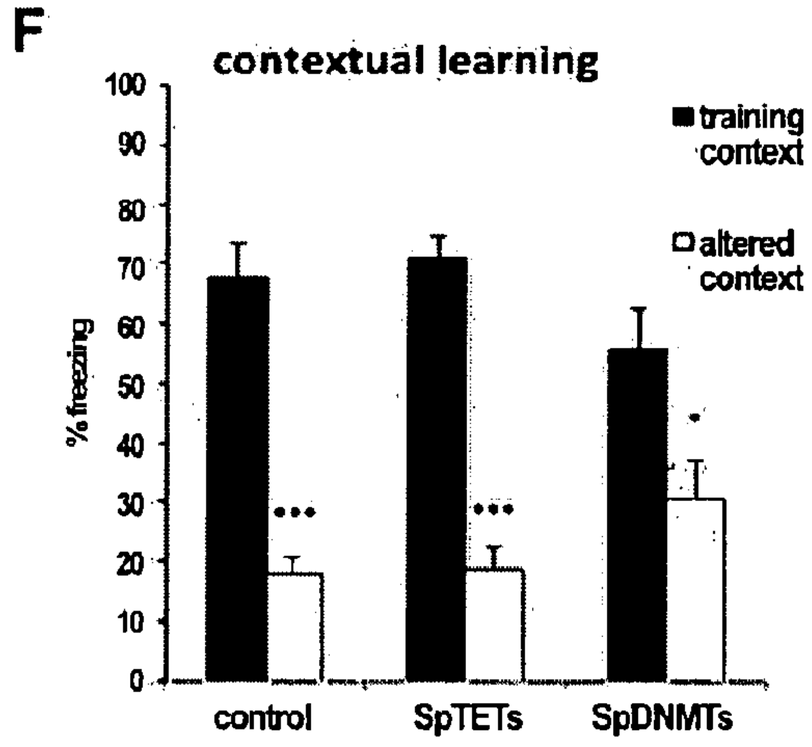
D



E

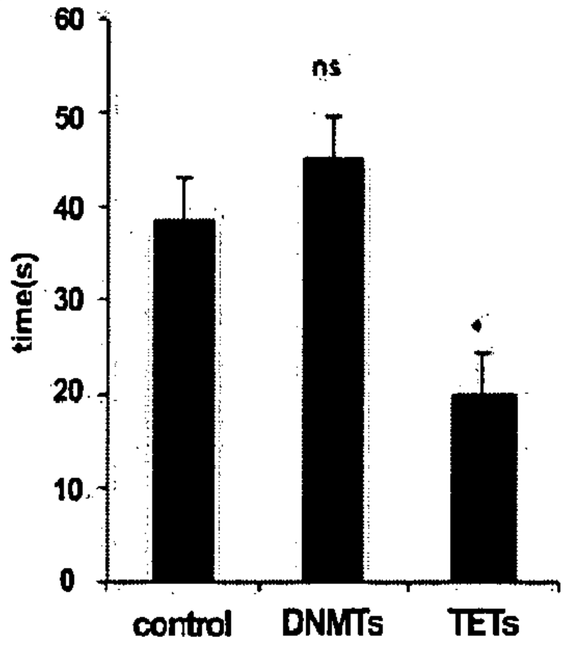


F

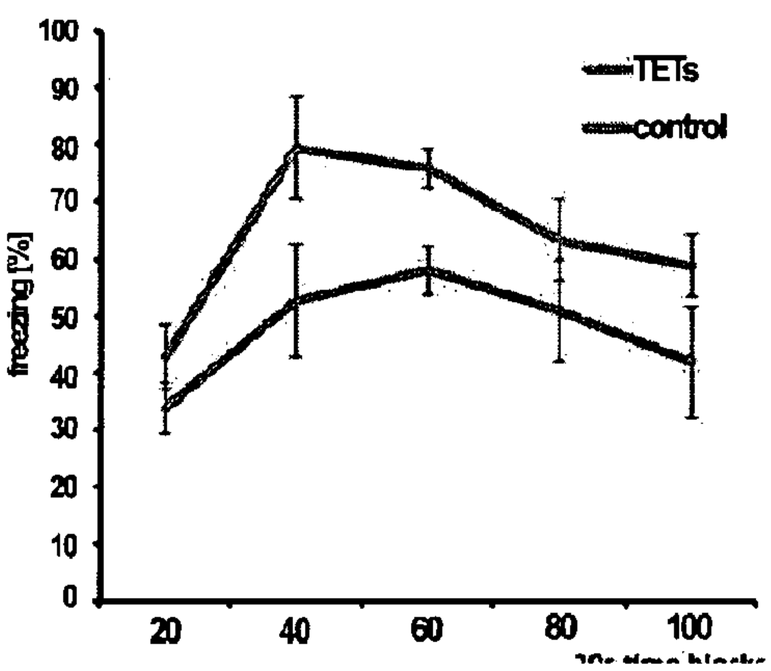


latency to first freeze

G



H



I

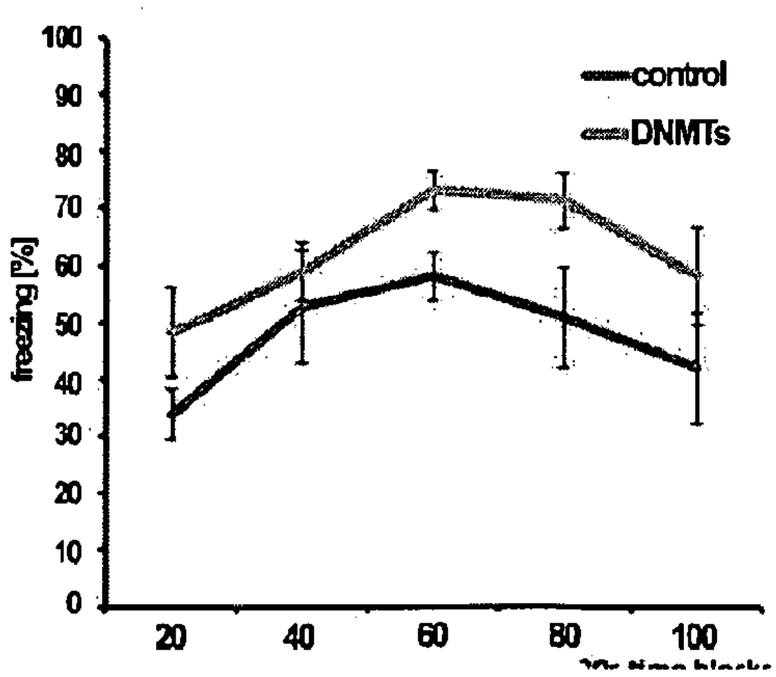


FIG. 58

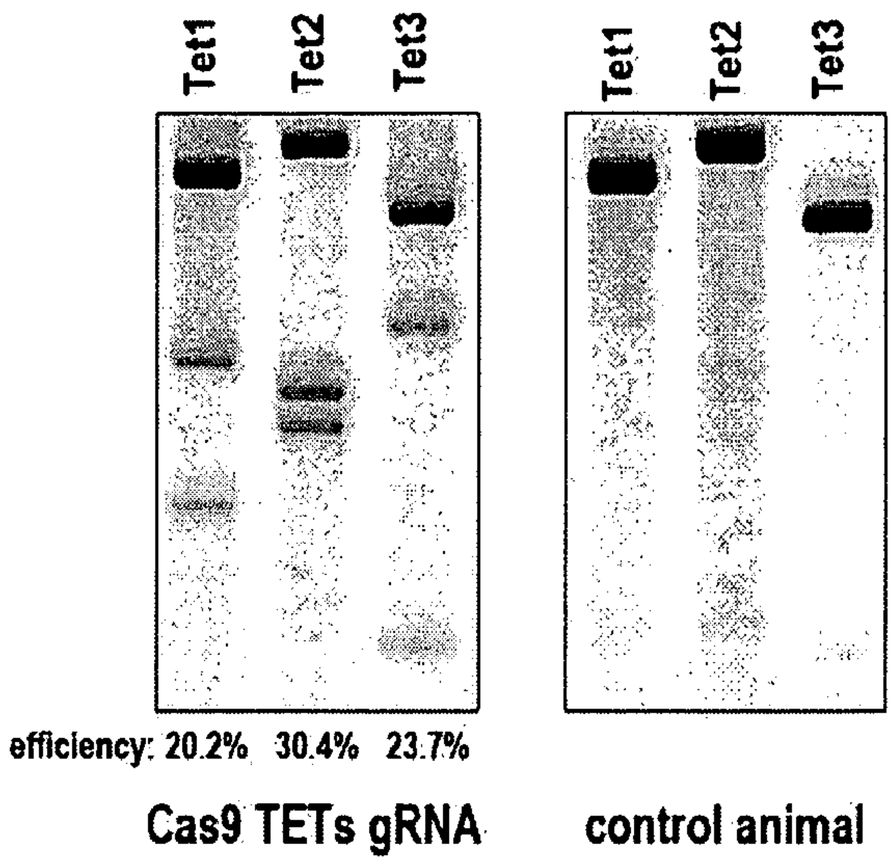

Tet1
Tet2
Tet3
Tet1
Tet2
Tet3
efficiency: 20.2% 30.4% 23.7%
Cas9 TETs gRNA
control animal

FIG. 59

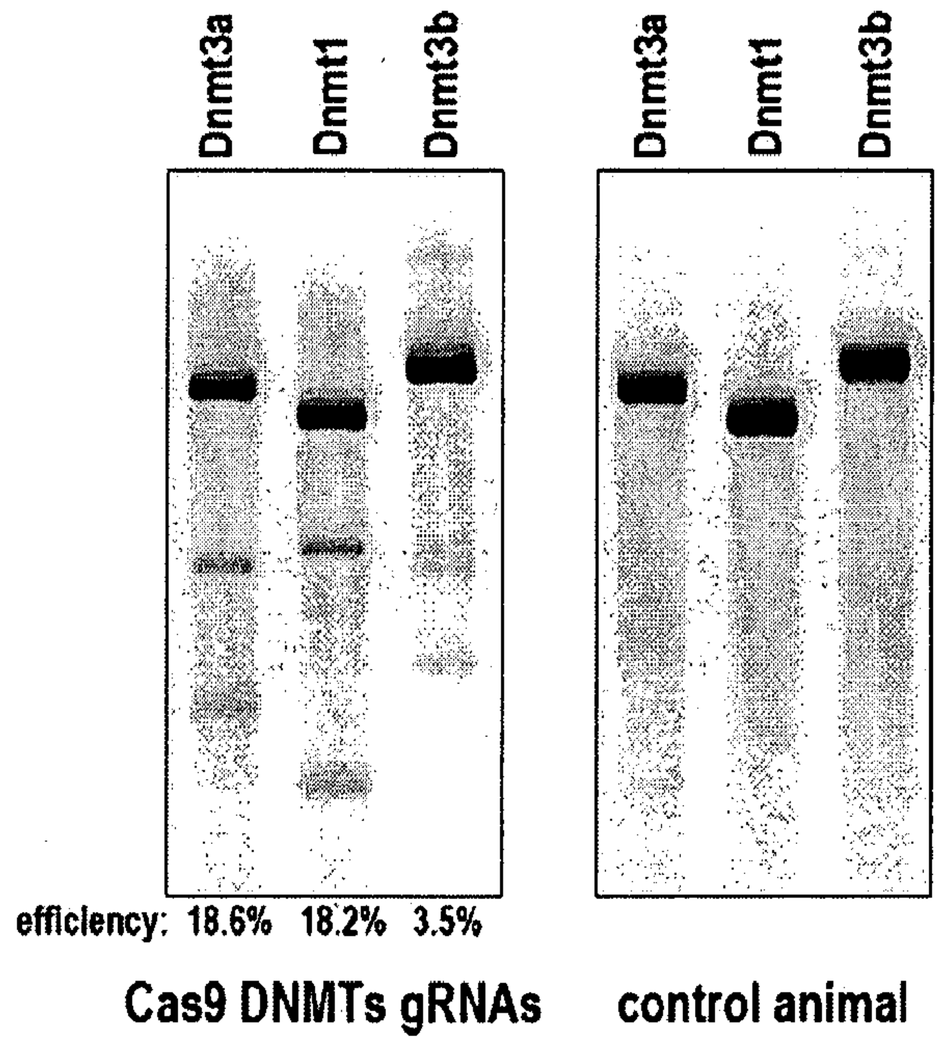

Dnmt3a
Dnmt1
Dnmt3b
Dnmt3a
Dnmt1
Dnmt3b
efficiency: 18.6% 18.2% 3.5%
Cas9 DNMTs gRNAs
control animal

FIG. 60

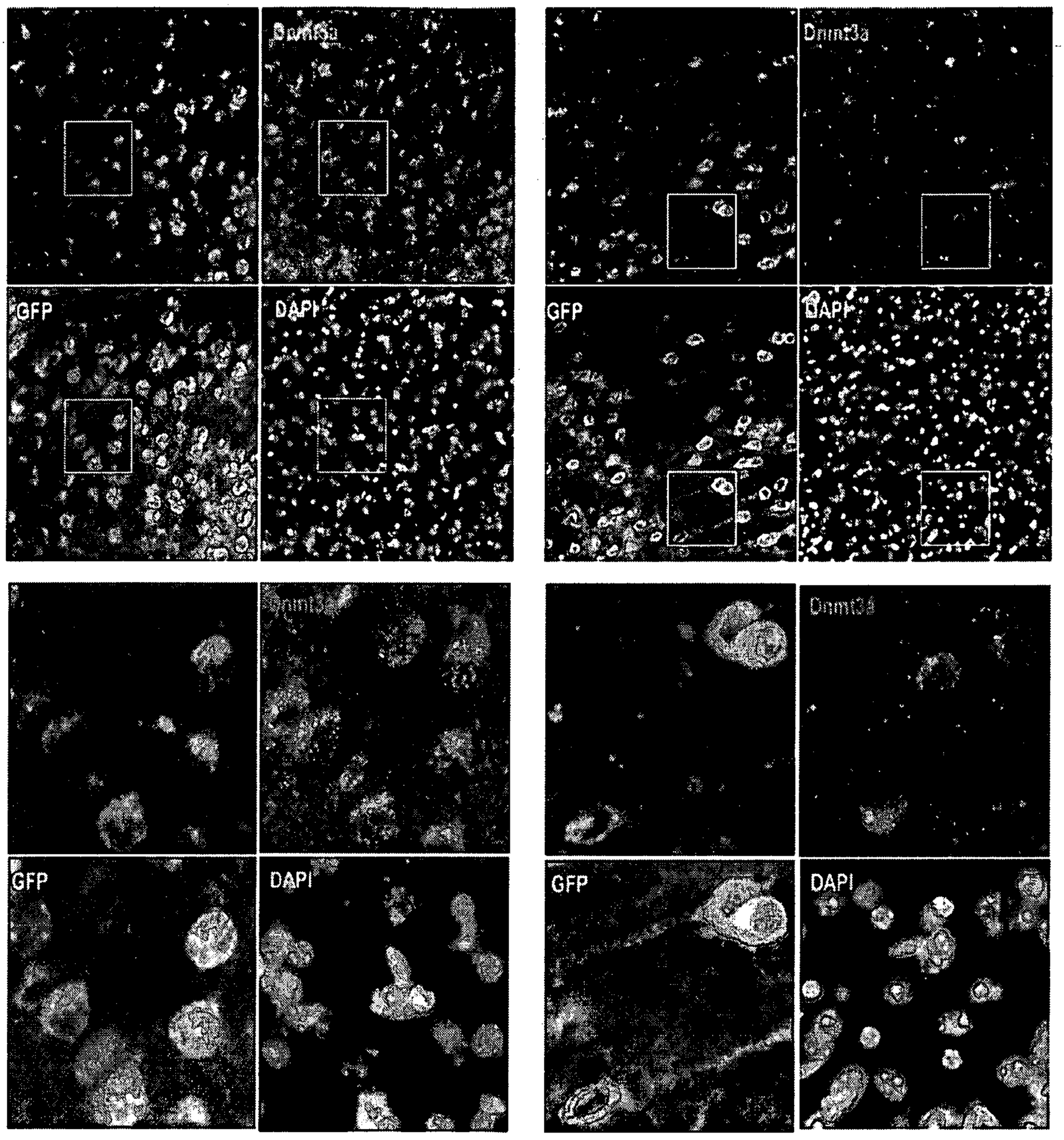

Mecp2_Cas9 / Syn_GFP-KASH
Mecp2_Cas9 / Syn_GFP-KASH_U6-DNMTs gRNAs
Dnmt3a
GFP
DAPI
Dnmt3a
GFP
DAPI
Dnmt3a
GFP
DAPI
Dnmt3a
GFP
DAPI

FIG. 61

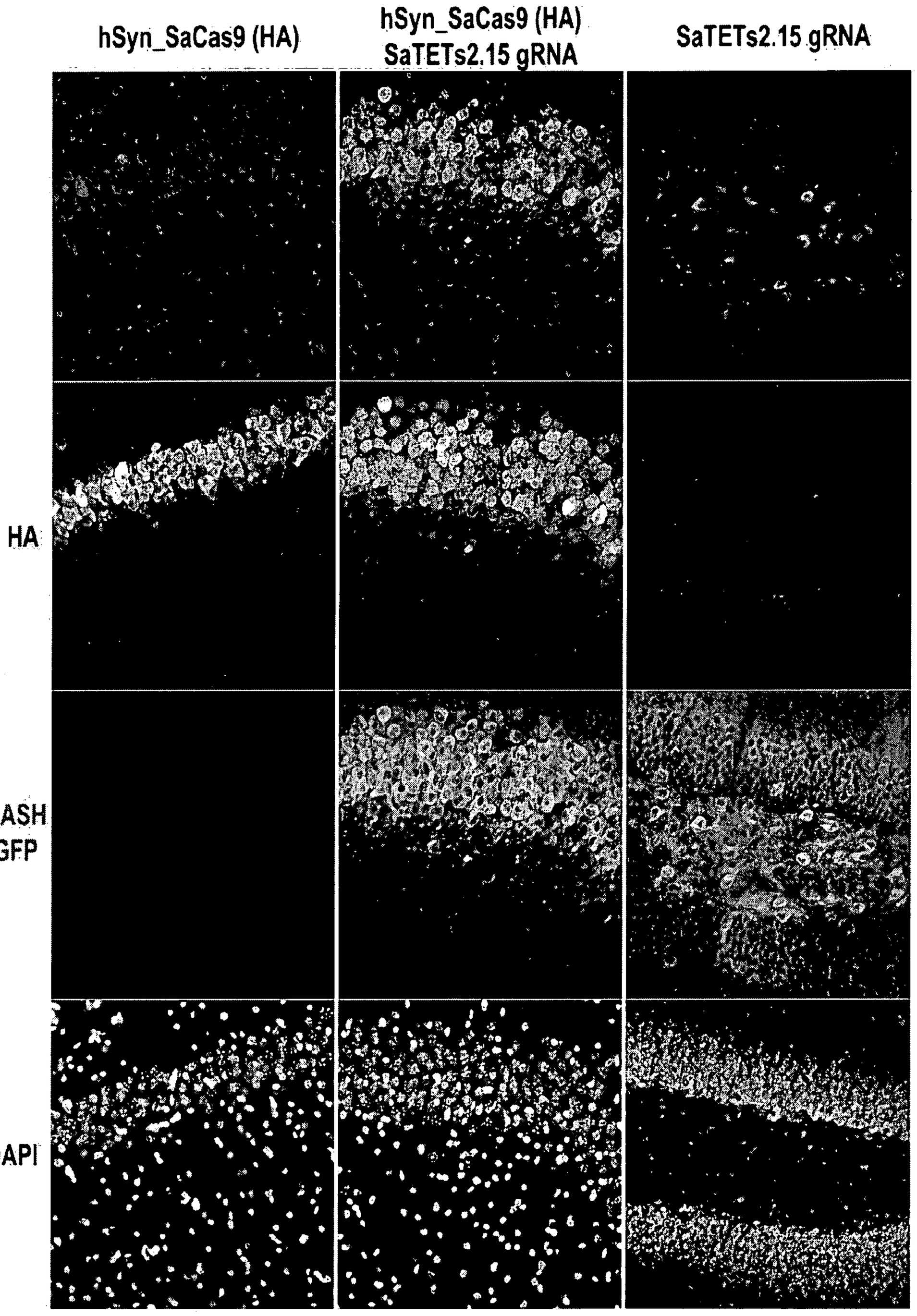

hSyn_SaCas9 (HA)
hSyn_SaCas9 (HA)
SaTETs2.15 gRNA
SaTETs2.15 gRNA
HA
KASH
GFP
DAPI

FIG. 62

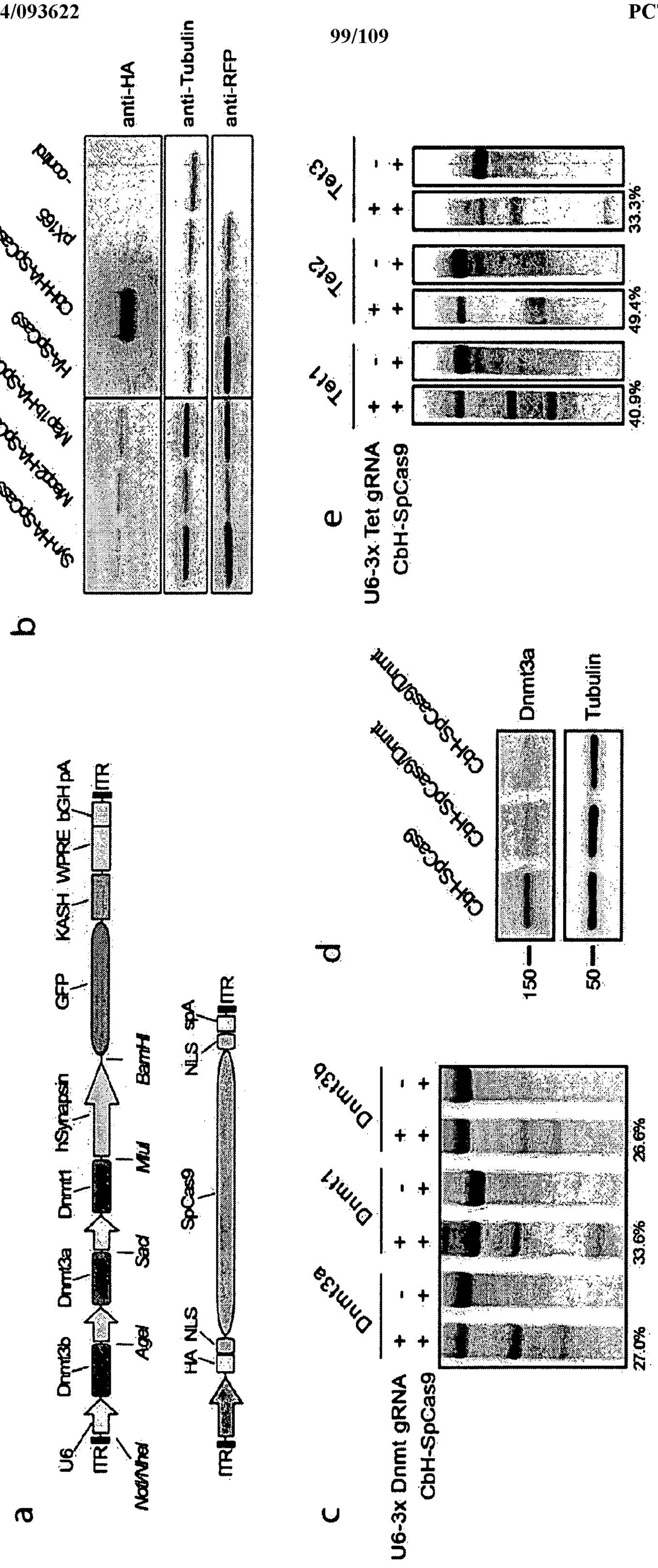

a
U6
ITR
NotI/NheI
Dnmt3b
AgeI
Dnmt3a
SacI
Dnmt1
MluI
hSynapsin
BamHI
GFP
KASH
WPRE
bGH pA
ITR
HA NLS
SpCas9
NLS spA
ITR
b
anti-HA
anti-Tubulin
anti-RFP
control
pX165
CbH-SpCas9
Hsp70A-SpCas9
Mapt1bHA-SpCas9
Mapt2HA-SpCas9
SynHA-SpCas9
c
U6-3x Dnmt gRNA
CbH-SpCas9
Dnmt3a
Dnmt3b
Dnmt1
33.6%
27.0%
26.6%
d
CbH-SpCas9
CbH-SpCas9/Dnmt
CbH-SpCas9/Dnmt
Dnmt3a
Tubulin
150
50
e
U6-3x Tet gRNA
CbH-SpCas9
Tet1
Tet2
Tet3
40.9%
49.4%
33.3%

FIG. 63

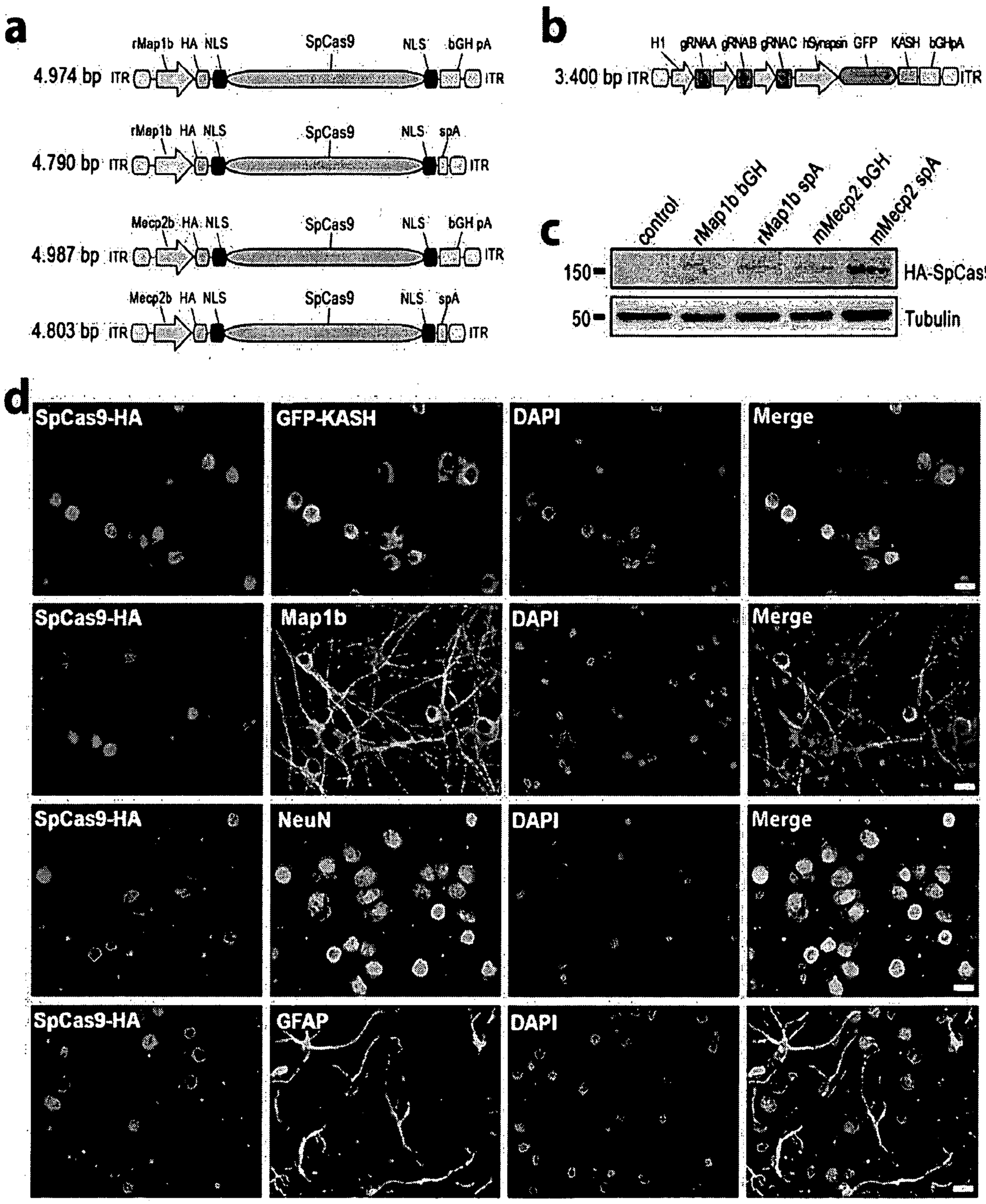

a
rMap1b HA NLS SpCas9 NLS bGH pA
4.974 bp ITR ITR
rMap1b HA NLS SpCas9 NLS spA
4.790 bp ITR ITR
Mecp2b HA NLS SpCas9 NLS bGH pA
4.987 bp ITR ITR
Mecp2b HA NLS SpCas9 NLS spA
4.803 bp ITR ITR
b
H1 gRNAA gRNAB gRNAC hSynapsin GFP KASH bGHpA
3.400 bp ITR ITR
c
control
rMap1b bGH
rMap1b spA
mMecp2 bGH
mMecp2 spA
150
HA-SpCas9
50
Tubulin
d
SpCas9-HA
GFP-KASH
DAPI
Merge
SpCas9-HA
Map1b
DAPI
Merge
SpCas9-HA
NeuN
DAPI
Merge
SpCas9-HA
GFAP
DAPI

FIG. 64

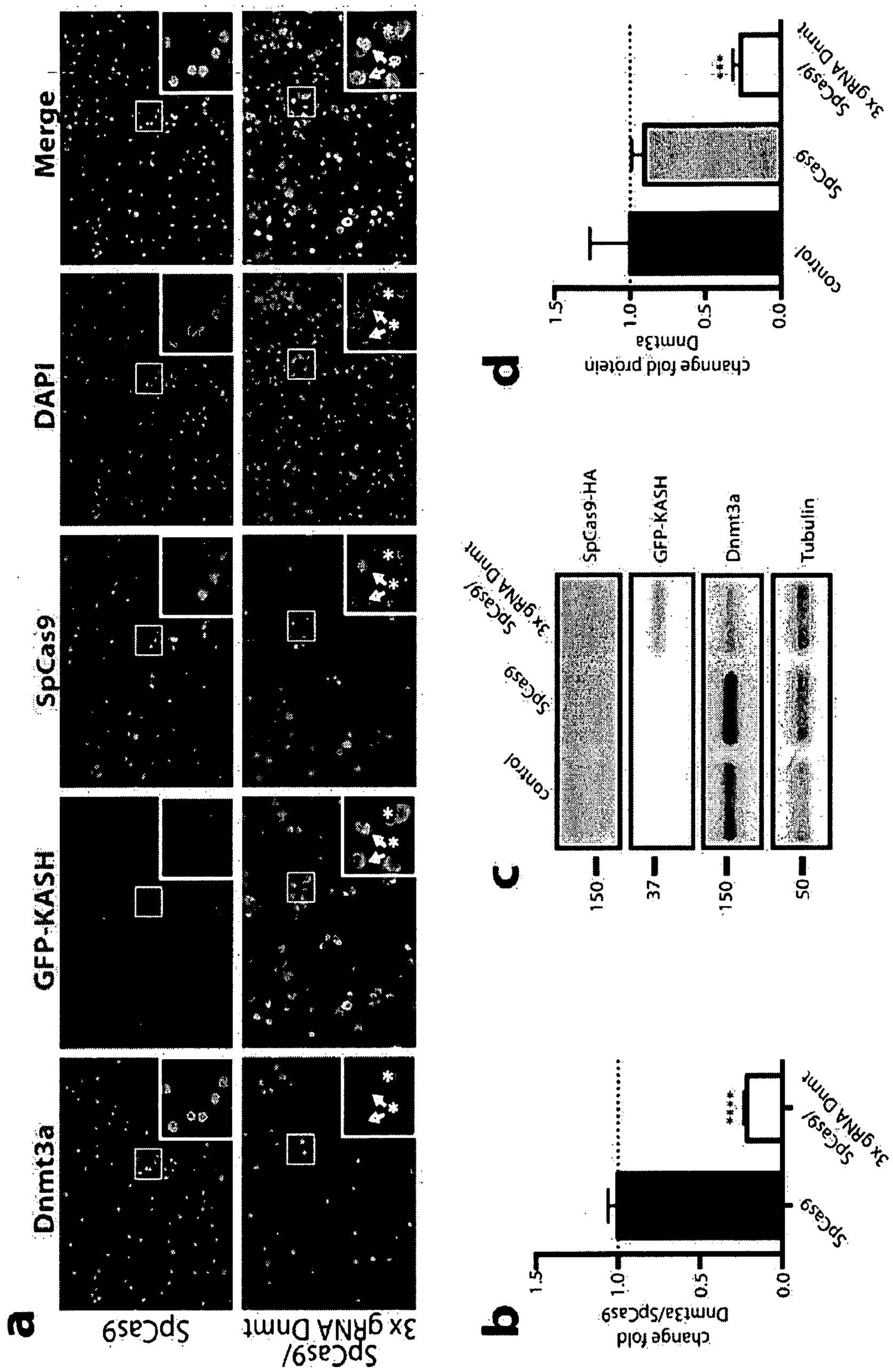

a
Dnmt3a
GFP-KASH
Merge
DAPI
SpCas9
3x gRNA Dnmt
SpCas9/
3x gRNA Dnmt
b
change fold
Dnmt3a/SpCas9
1.5
1.0
0.5
0.0
****
SpCas9
SpCas9/
3x gRNA Dnmt
c
control
SpCas9
SpCas9/
3x gRNA Dnmt
SpCas9-HA
GFP-KASH
Dnmt3a
Tubulin
150
37
150
50
d
change fold protein
Dnmt3a
1.5
1.0
0.5
0.0
control
SpCas9
SpCas9/
3x gRNA Dnmt

FIG. 65

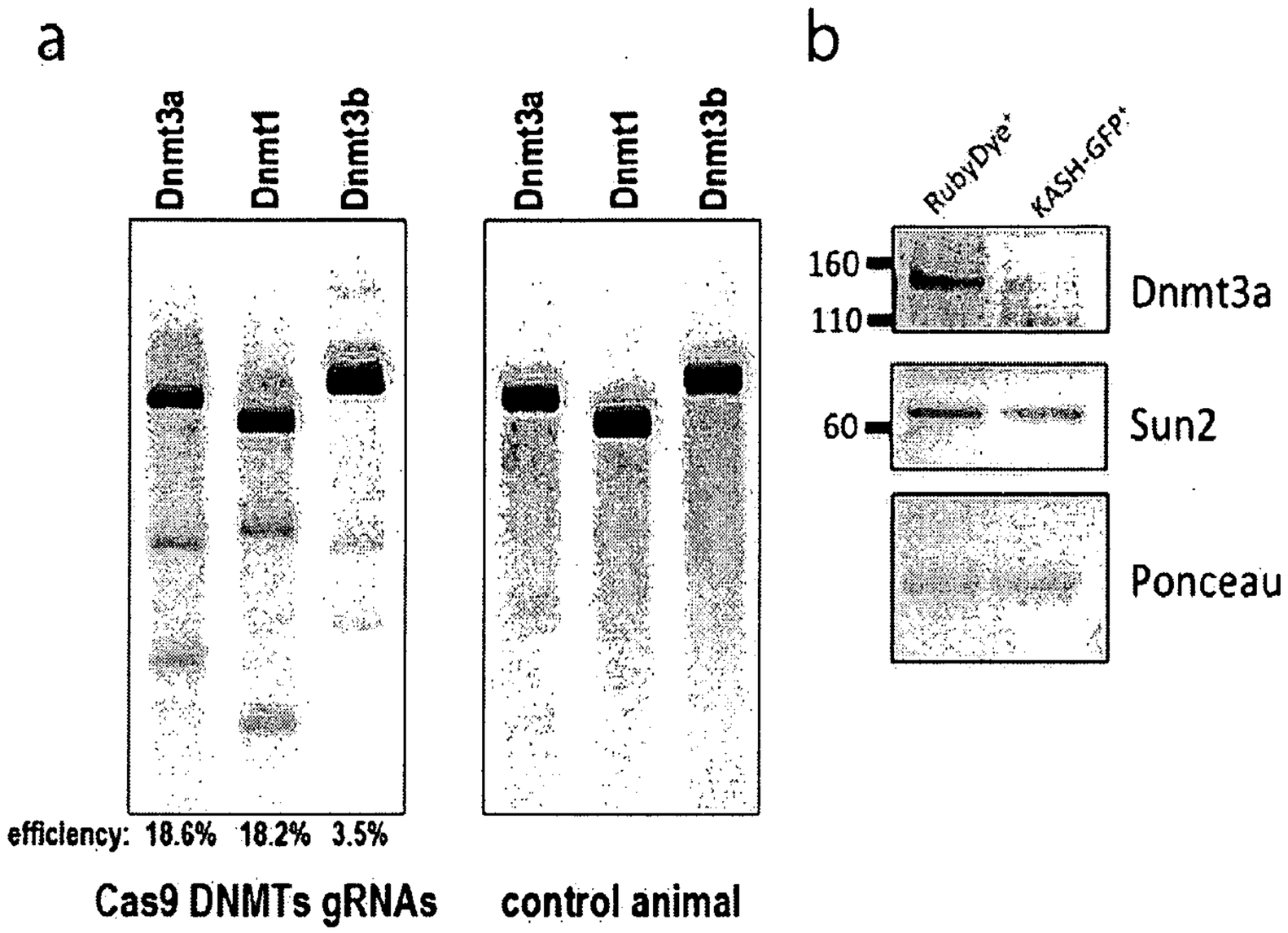
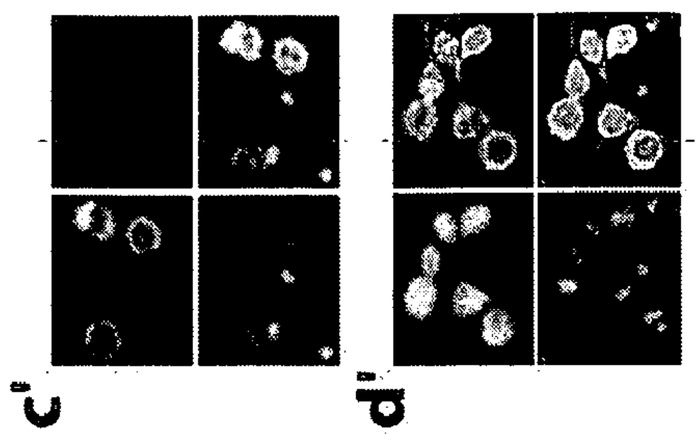

a
Dnmt3a
Dnmt1
Dnmt3b
Dnmt3a
Dnmt1
Dnmt3b
efficiency: 18.6% 18.2% 3.5%
Cas9 DNMTs gRNAs
control animal
b
RubyDye+
KASH-GFP+
160
110
Dnmt3a
60
Sun2
Ponceau

FIG. 66


c
d

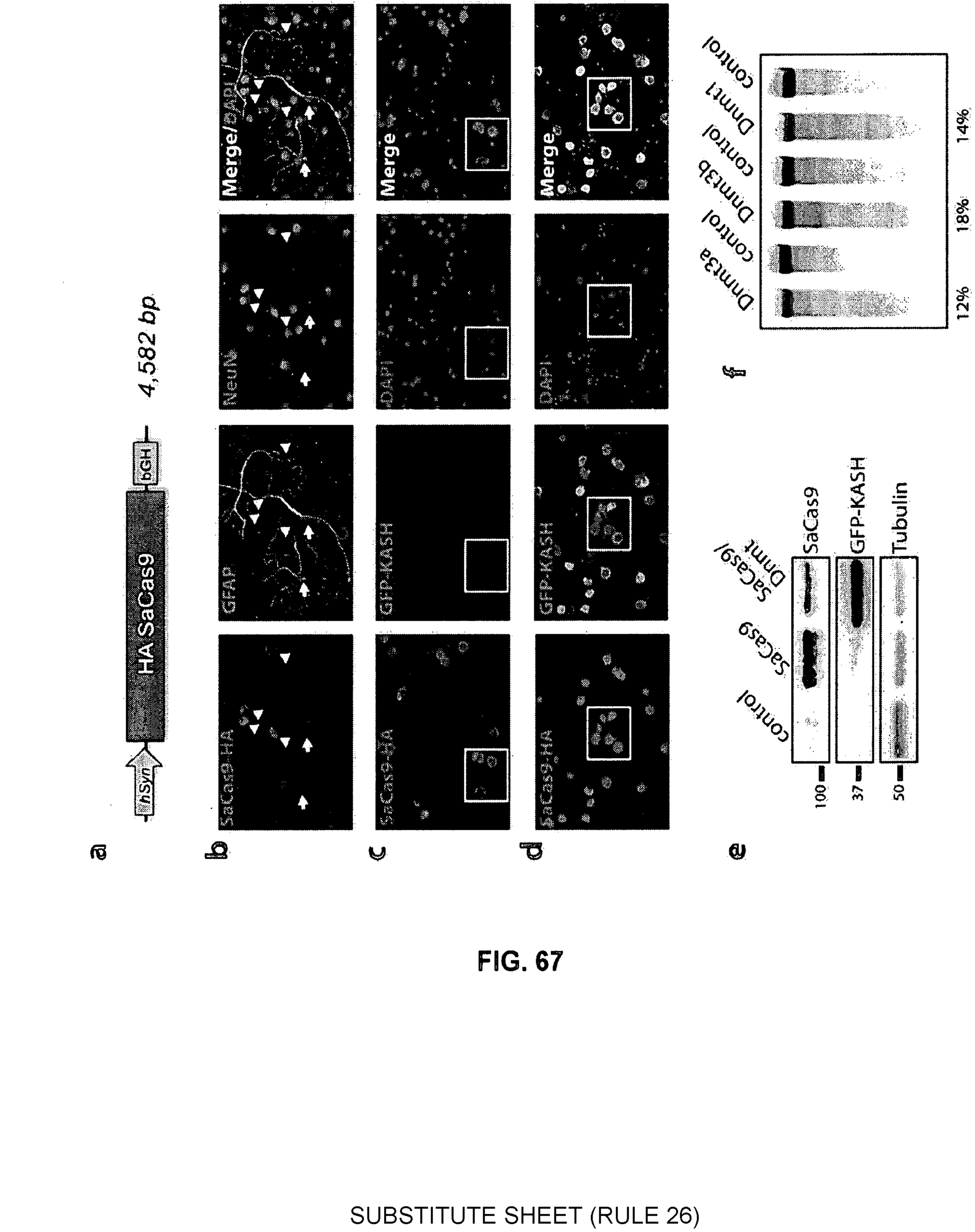
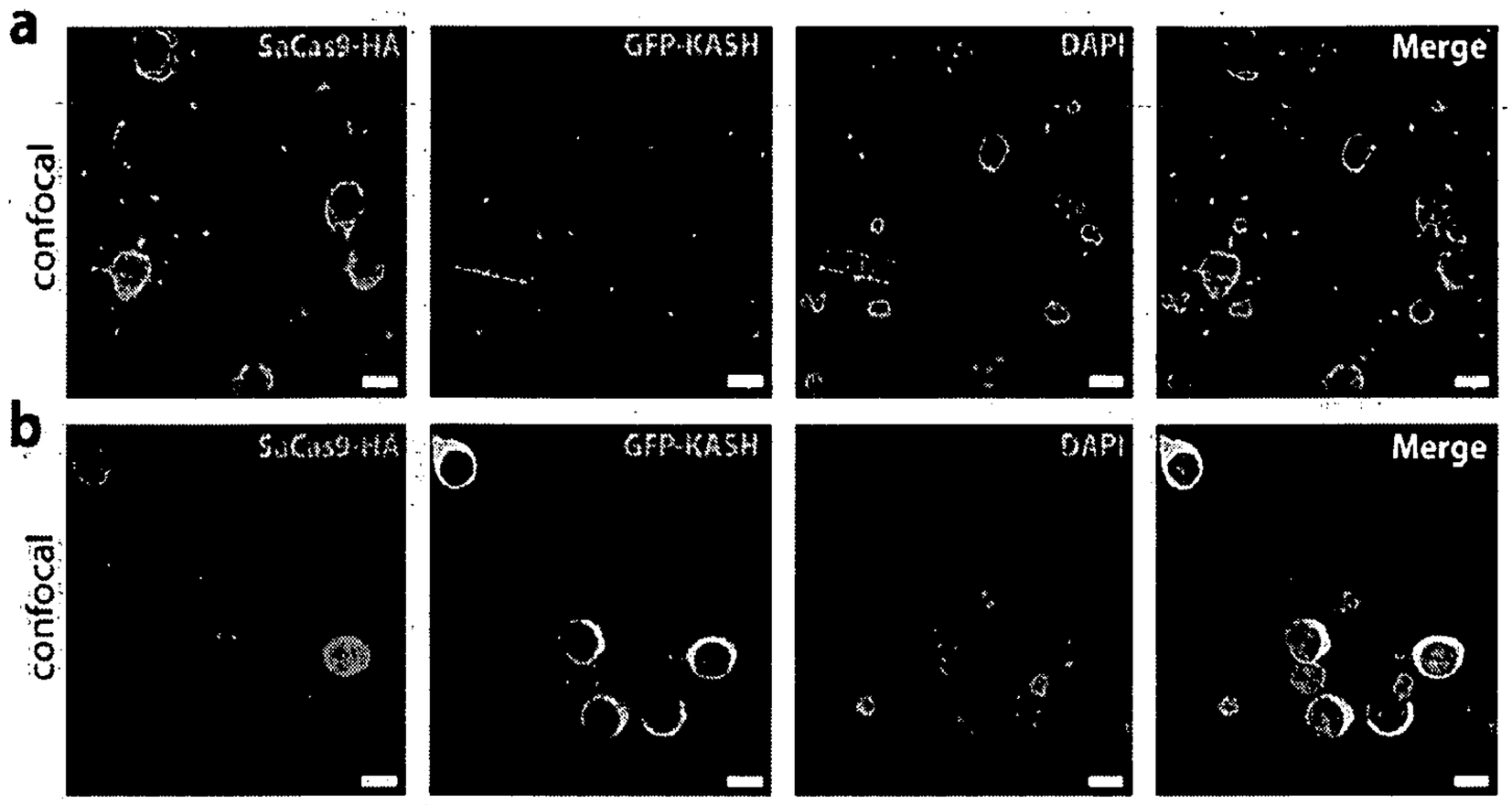
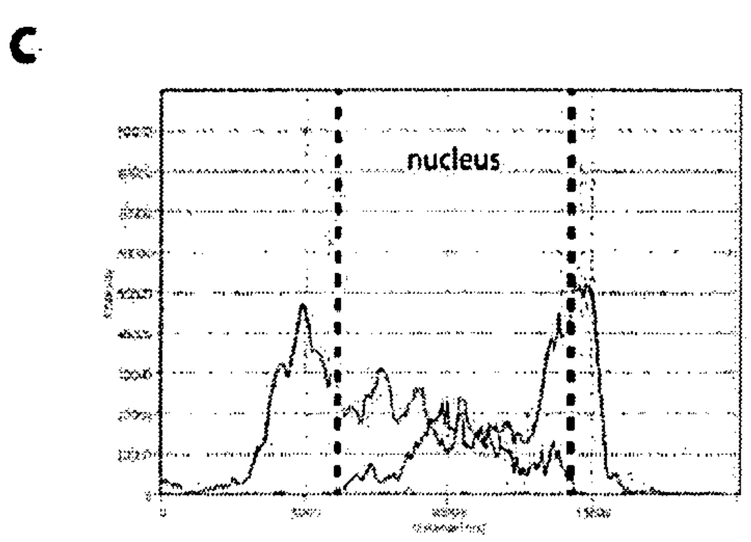
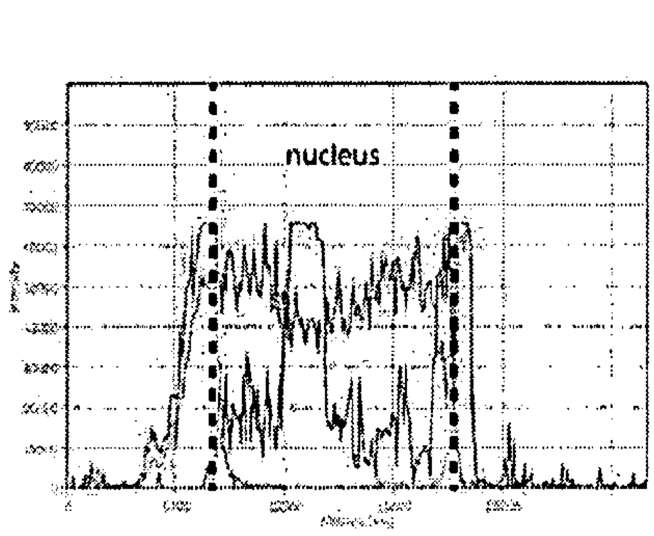

4,582 bp
NeuN
Merge/DAPI
DAPI
Merge
f
Dnmt3a control
Dnmt3b control
Dnmt control
12%
18%
14%
a
hSyn
HA SaCas9
bGH
b
SaCas9-HA
GFAP
c
SaCas9-HA
GFP-KASH
d
SaCas9-HA
GFP-KASH
e
control
SaCas9
SaCas9 Dnmt
SaCas9
GFP-KASH
Tubulin
100
37
50

FIG. 67


a
SaCas9-HA
GFP-KASH
DAPI
Merge
confocal
b
SaCas9-HA
GFP-KASH
DAPI
Merge
confocal


c
nucleus


d
nucleus

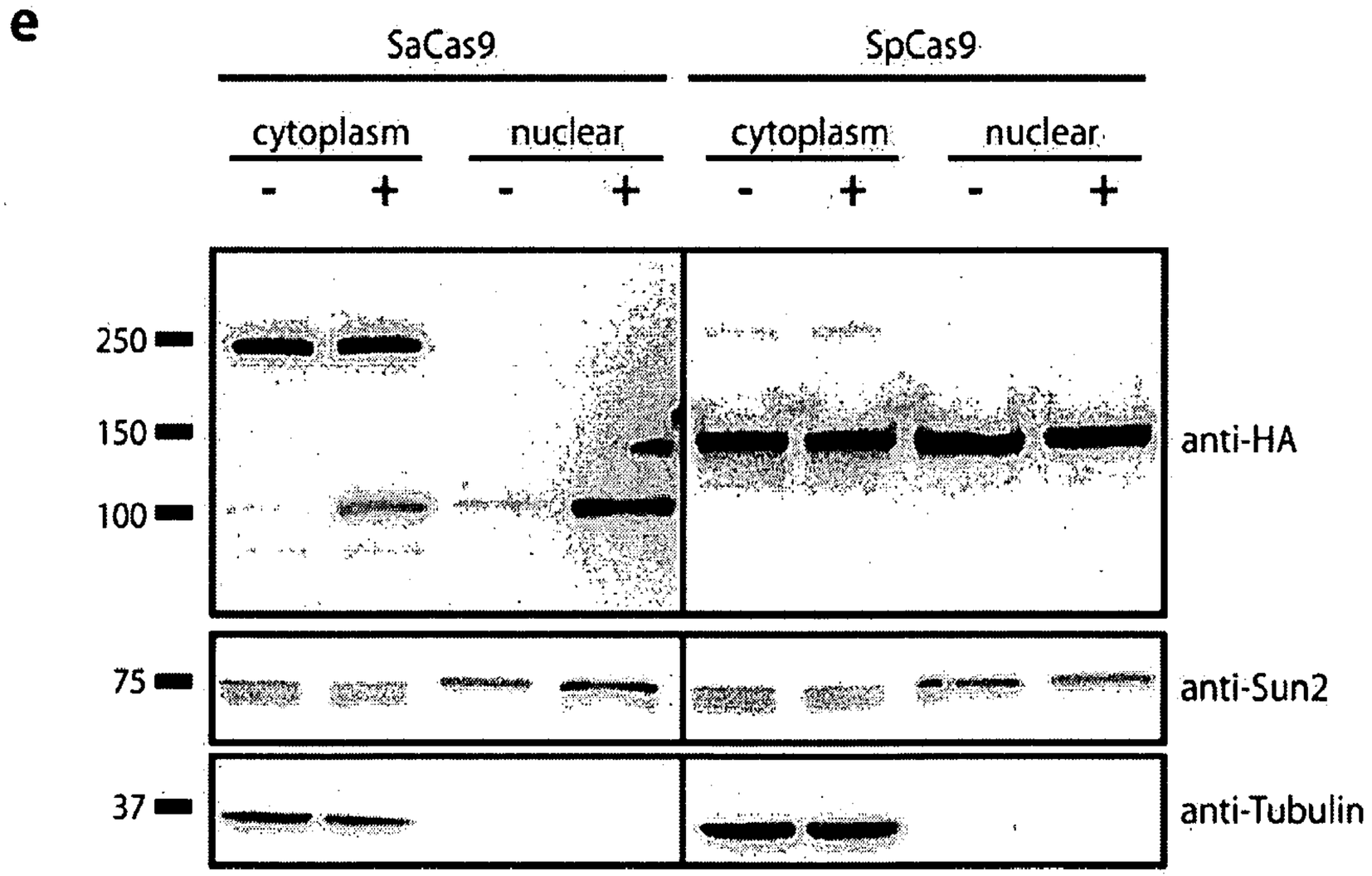
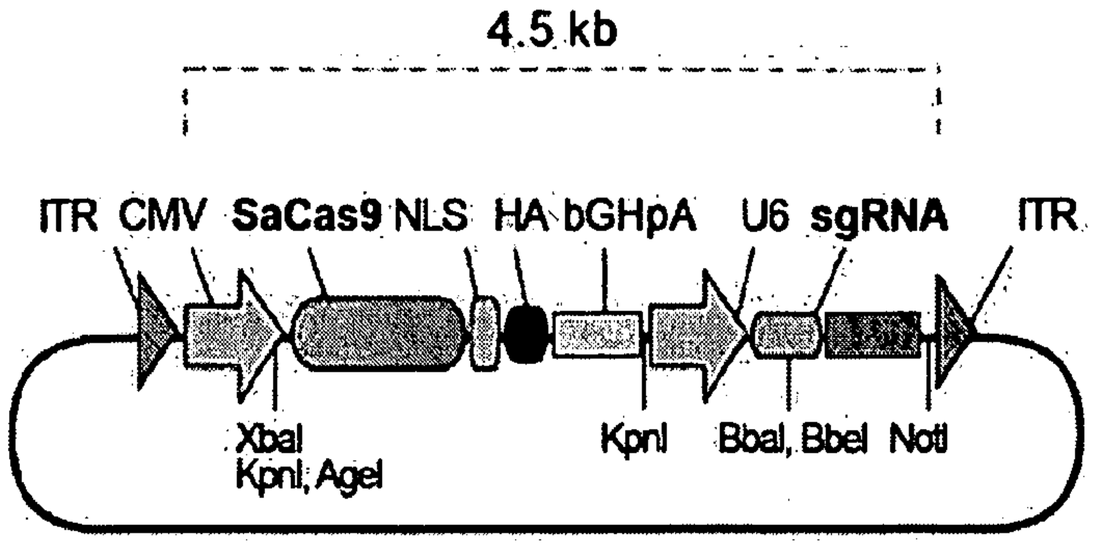
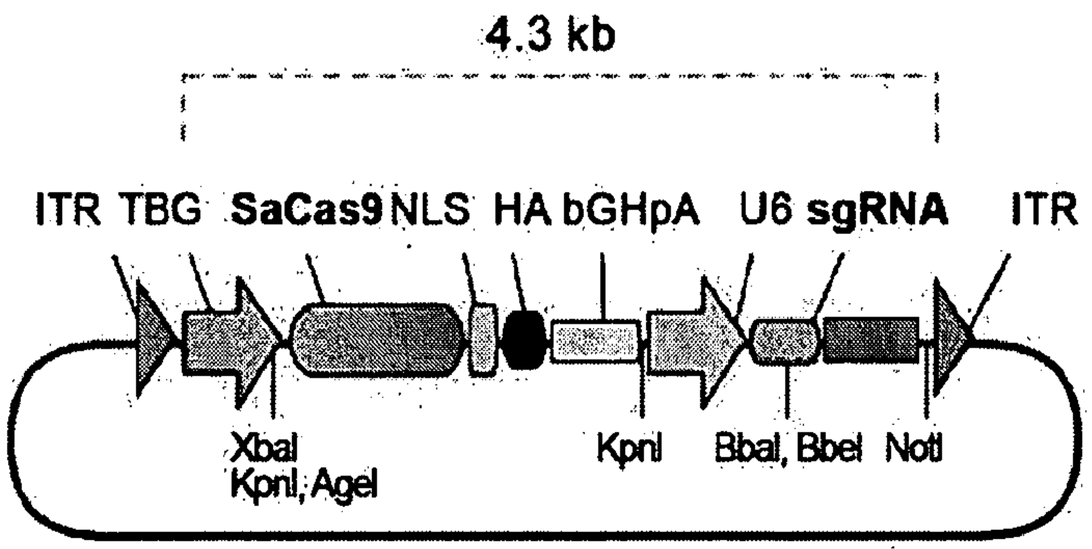

e
SaCas9
SpCas9
cytoplasm
nuclear
cytoplasm
nuclear
- + - + - + - +
250
150
100
anti-HA
75
anti-Sun2
37
anti-Tubulin

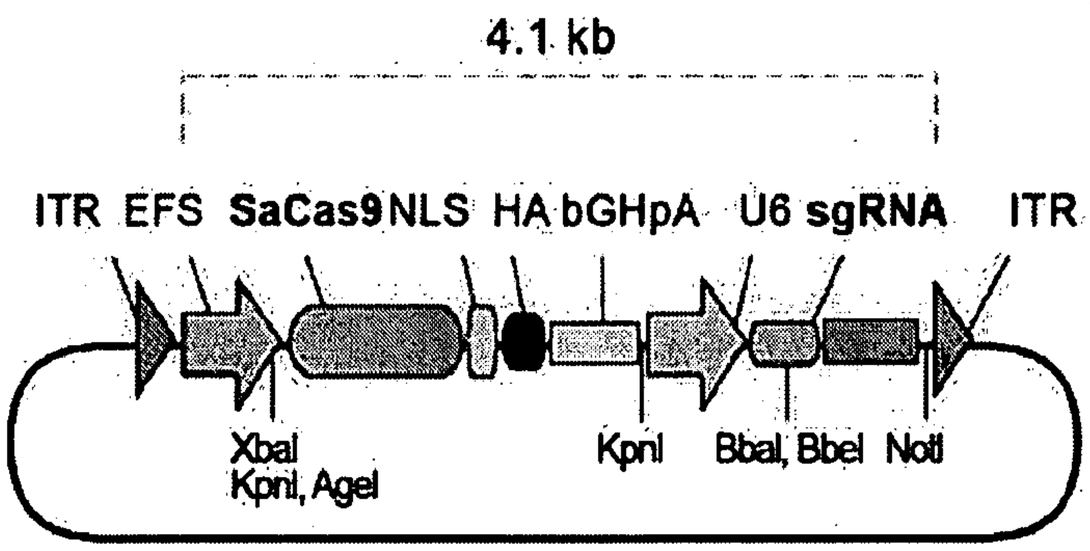
FIG. 68



AAV-Sa-Cas9 vector:
CMV-SaCas9-NLS-HA-U6-sgRNA



Liver-specific AAV-Sa-Cas9 vector:
TBG-SaCas9-NLS-HA-U6-sgRNA



Alternate AAV-Sa-Cas9 vector:
EFS-SaCas9-NLS-HA-U6-sgRNA

FIG. 69

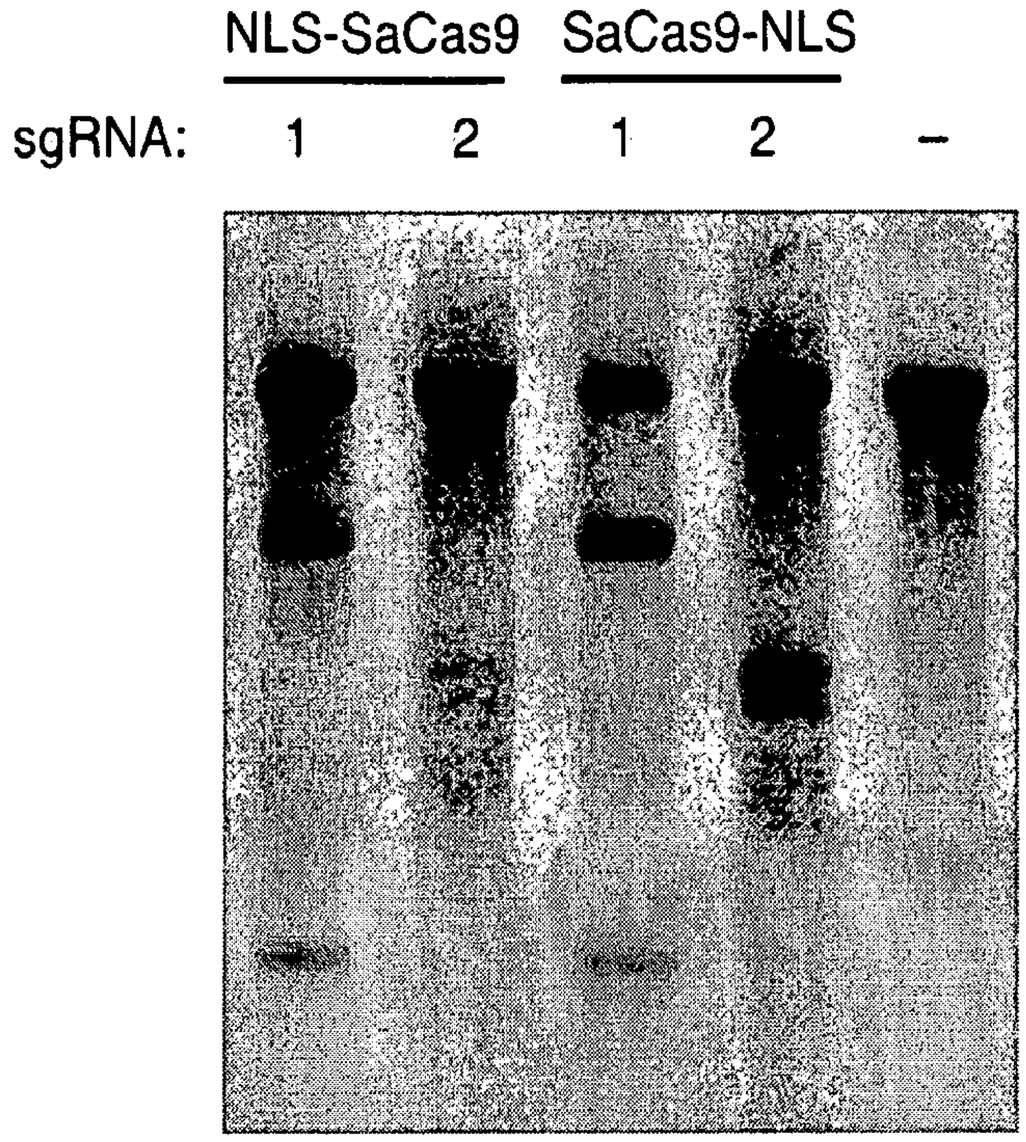


FIG. 70

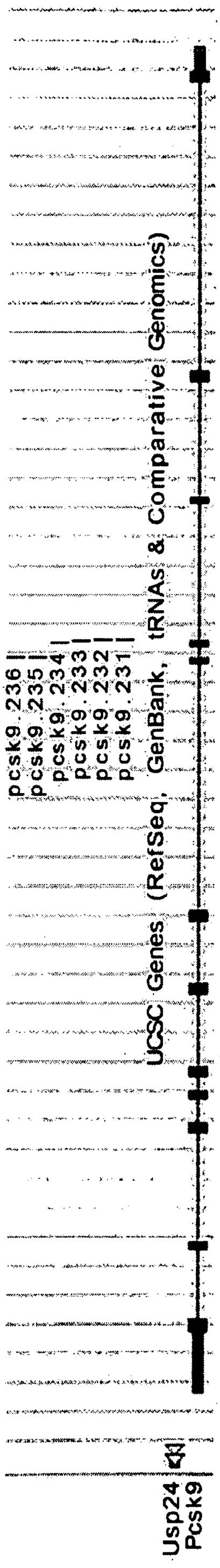

Usp24
Pcsk9
UCSC Genes (RefSeq, GenBank, tRNAs & Comparative Genomics)
pcsk9_236
pcsk9_235
pcsk9_234
pcsk9_233
pcsk9_232
pcsk9_231

FIG. 71

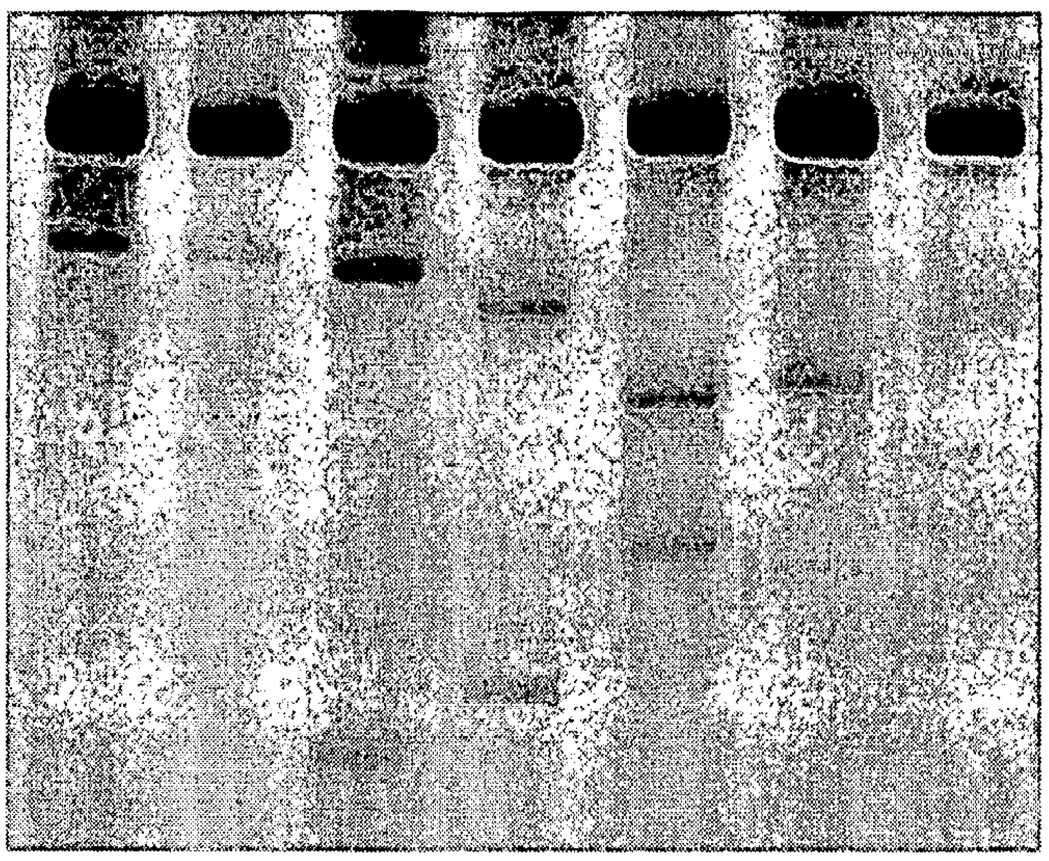


FIG. 72

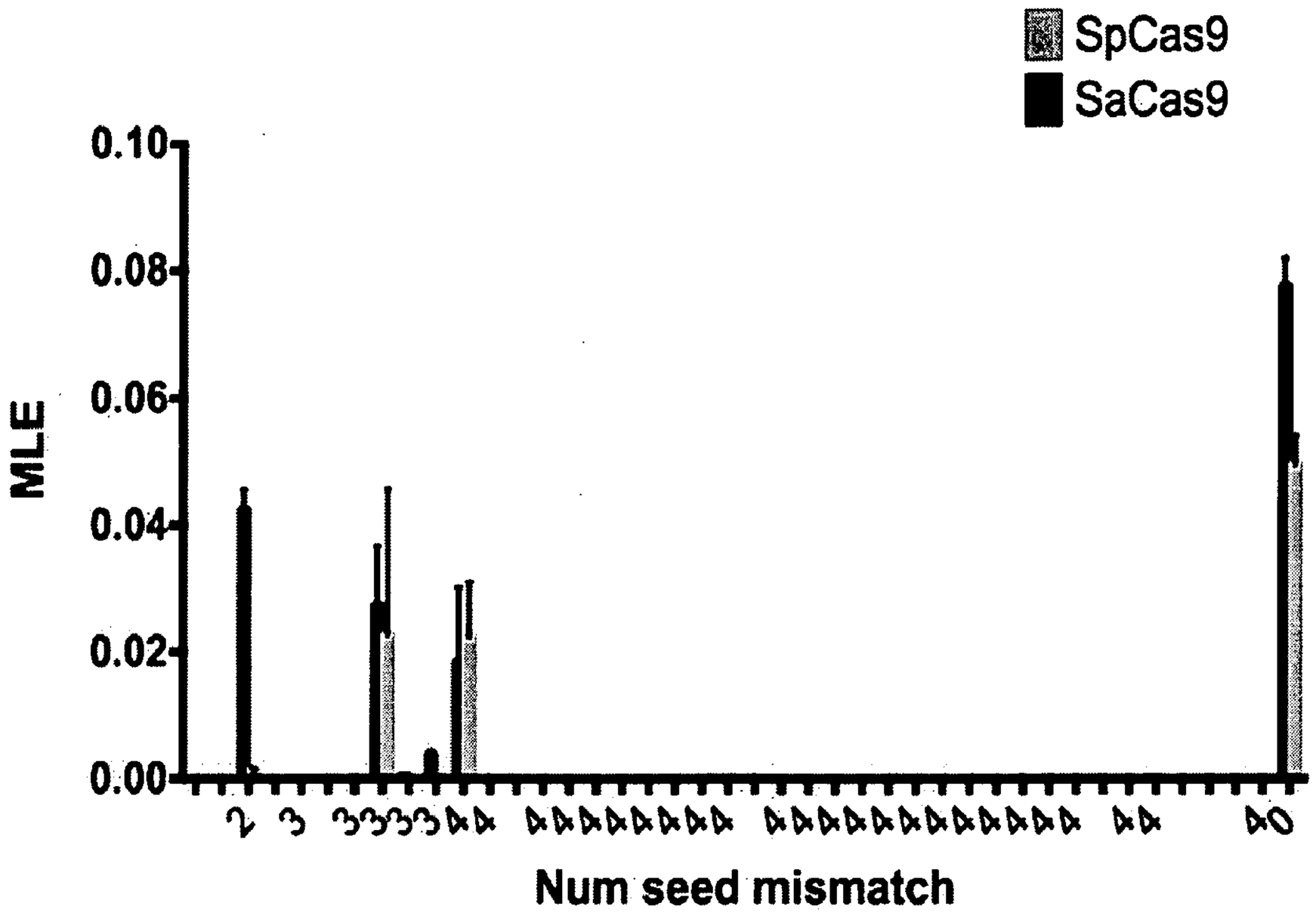

SpCas9 vs SaCas9 Genome Wide Off Target
SpCas9
SaCas9
MLE
0.10
0.08
0.06
0.04
0.02
0.00
Num seed mismatch

FIG. 73

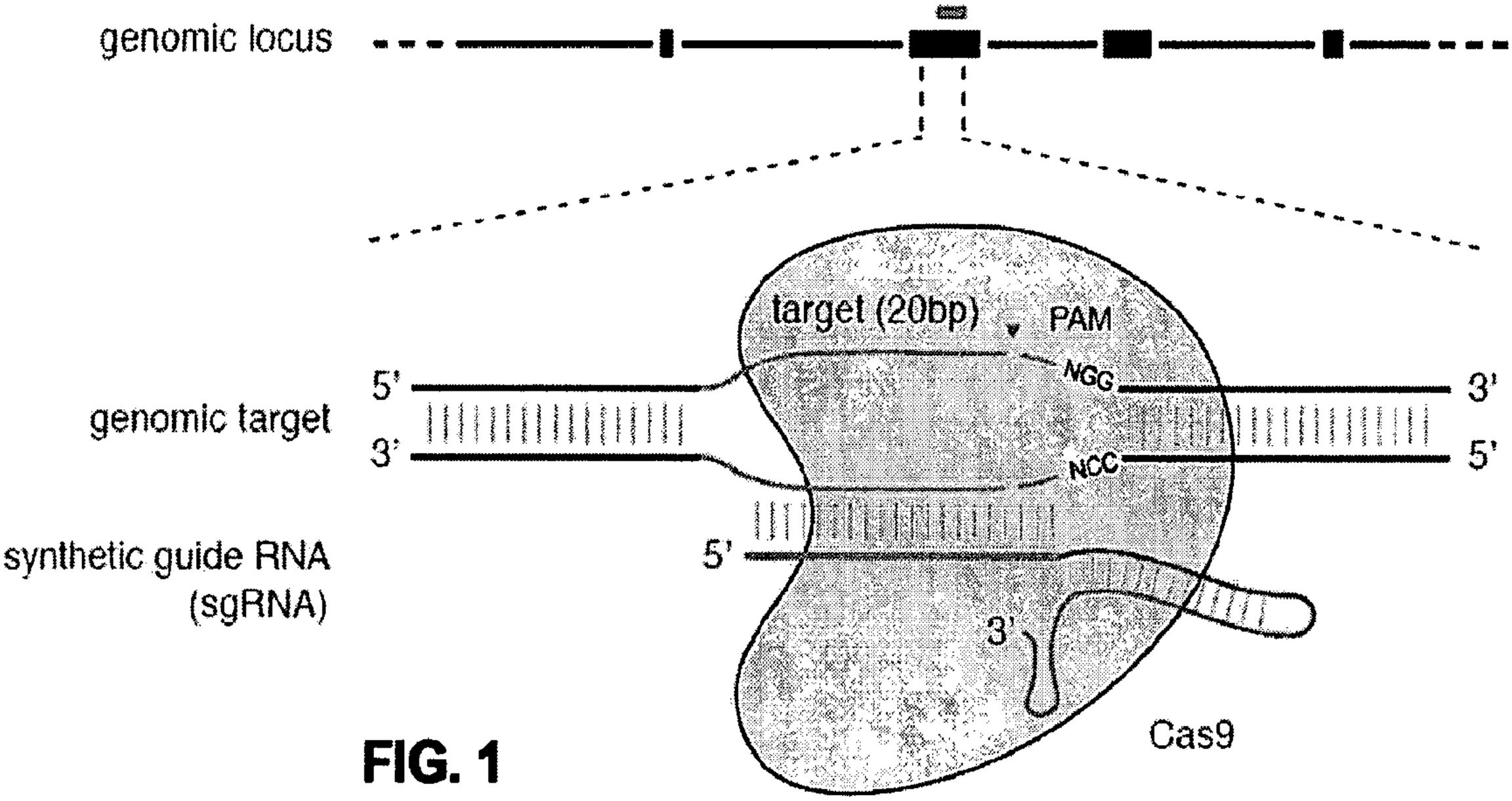

genomic locus
genomic target
synthetic guide RNA
(sgRNA)
target (20bp)
PAM
NGG
NCC
5'
3'
5'
3'
5'
3'
Cas9

FIG. 1