

RNA-GUIDED NUCLEASES AND ACTIVE FRAGMENTS AND VARIANTS THEREOF AND METHODS OF USE

5 FIELD OF THE INVENTION

The present invention relates to the field of molecular biology and gene editing.

REFERENCE TO A SEQUENCE LISTING SUBMITTED AS A TEXT FILE VIA EFS-WEB

10 The instant application contains a Sequence Listing which has been submitted in ASCII format via EFS-Web and is hereby incorporated by reference in its entirety. Said ASCII copy, created on August 11, 2020, is named L103438_1170WO_0049_2_Seq_List.txt, and is 489,326 bytes in size.

BACKGROUND OF THE INVENTION

15 Targeted genome editing or modification is rapidly becoming an important tool for basic and applied research. Initial methods involved engineering nucleases such as meganucleases, zinc finger fusion proteins or TALENs, requiring the generation of chimeric nucleases with engineered, programmable, sequence-specific DNA-binding domains specific for each particular target sequence. RNA-guided nucleases, such as the Clustered Regularly Interspaced Short Palindromic Repeats (CRISPR)-associated (Cas) proteins of the
20 CRISPR-Cas bacterial system, allow for the targeting of specific sequences by complexing the nucleases with guide RNA that specifically hybridizes with a particular target sequence. Producing target-specific guide RNAs is less costly and more efficient than generating chimeric nucleases for each target sequence. Such RNA-guided nucleases can be used to edit genomes optionally through the introduction of a sequence-specific, double-stranded break that is repaired via error-prone non-homologous end-joining (NHEJ) to
25 introduce a mutation at a specific genomic location. Alternatively, heterologous DNA may be introduced into the genomic site via homology-directed repair.

BRIEF SUMMARY OF THE INVENTION

Compositions and methods for binding a target sequence of interest are provided. The compositions
30 find use in cleaving or modifying a target sequence of interest, detection of a target sequence of interest, and modifying the expression of a sequence of interest. Compositions comprise RNA-guided nuclease (RGN) polypeptides, CRISPR RNAs (crRNAs), trans-activating CRISPR RNAs (tracrRNAs), guide RNAs (gRNAs), nucleic acid molecules encoding the same, and vectors and host cells comprising the nucleic acid molecules. Also provided are CRISPR systems for binding a target sequence of interest, wherein the
35 CRISPR system comprises an RNA-guided nuclease polypeptide and one or more guide RNAs. Thus, methods disclosed herein are drawn to binding a target sequence of interest, and in some embodiments, cleaving or modifying the target sequence of interest. The target sequence of interest can be modified, for

example, as a result of non-homologous end joining or homology-directed repair with an introduced donor sequence. Further provided are methods and kits for detecting a target DNA sequence of a DNA molecule using detector single-stranded DNA.

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DETAILED DESCRIPTION

Many modifications and other embodiments of the inventions set forth herein will come to mind to one skilled in the art to which these inventions pertain having the benefit of the teachings presented in the foregoing descriptions and the associated drawings. Therefore, it is to be understood that the inventions are not to be limited to the specific embodiments disclosed and that modifications and other embodiments are intended to be included within the scope of the appended embodiments. Although specific terms are employed herein, they are used in a generic and descriptive sense only and not for purposes of limitation.

I. Overview

RNA-guided nucleases (RGNs) allow for the targeted manipulation of a single site within a genome and are useful in the context of gene targeting for therapeutic and research applications. In a variety of organisms, including mammals, RNA-guided nucleases have been used for genome engineering by stimulating non-homologous end joining and homologous recombination, for example. The compositions and methods described herein are useful for creating single- or double-stranded breaks in polynucleotides, modifying polynucleotides, detecting a particular site within a polynucleotide, or modifying the expression of a particular gene.

The RNA-guided nucleases disclosed herein can alter gene expression by modifying a target sequence. In specific embodiments, the RNA-guided nucleases are directed to the target sequence by a guide RNA (gRNA) as part of a Clustered Regularly Interspaced Short Palindromic Repeats (CRISPR) RNA-guided nuclease system. The RGNs are considered “RNA-guided” because guide RNAs form a complex with the RNA-guided nucleases to direct the RNA-guided nuclease to bind to a target sequence and in some embodiments, introduce a single-stranded or double-stranded break at the target sequence. After the target sequence has been cleaved, the break can be repaired such that the DNA sequence of the target sequence is modified during the repair process. Thus, provided herein are methods for using the RNA-guided nucleases to modify a target sequence in the DNA of host cells. For example, RNA-guided nucleases can be used to modify a target sequence at a genomic locus of eukaryotic cells or prokaryotic cells.

II. RNA-guided nucleases

Provided herein are RNA-guided nucleases. The term RNA-guided nuclease (RGN) refers to a polypeptide that binds to a particular target nucleotide sequence in a sequence-specific manner and is directed to the target nucleotide sequence by a guide RNA molecule that is complexed with the polypeptide and hybridizes with the target sequence. Although an RNA-guided nuclease can be capable of cleaving the target sequence upon binding, the term RNA-guided nuclease also encompasses nuclease-dead RNA-guided

nucleases that are capable of binding to, but not cleaving, a target sequence. Cleavage of a target sequence by an RNA-guided nuclease can result in a single- or double-stranded break. RNA-guided nucleases only capable of cleaving a single strand of a double-stranded nucleic acid molecule are referred to herein as nickases.

The RNA-guided nucleases disclosed herein include the APG05733.1, APG06207.1, APG01647.1, APG08032.1, APG05712.1, APG01658.1, APG06498.1, APG09106.1, APG09882.1, APG02675.1, APG01405.1, APG06250.1, APG06877.1, APG09053.1, APG04293.1, APG01308.1, APG06646.1, APG09748, and APG07433.1 RNA-guided nucleases, the amino acid sequences of which are set forth, respectively, as SEQ ID NO: 1, 9, 16, 23, 30, 38, 46, 54, 61, 69, 75, 82, 89, 95, 103, 110, 117, 137 or 235, and active fragments or variants thereof that retain the ability to bind to a target nucleotide sequence in an RNA-guided sequence-specific manner. In some of these embodiments, the active fragment or variant of the APG05733.1, APG06207.1, APG01647.1, APG08032.1, APG05712.1, APG01658.1, APG06498.1, APG09106.1, APG09882.1, APG02675.1, APG01405.1, APG06250.1, APG06877.1, APG09053.1, APG04293.1, APG01308.1, APG06646.1, APG09748, or APG07433.1 RGN is capable of cleaving a single- or double-stranded target sequence. In some embodiments, an active variant of the APG05733.1, APG06207.1, APG01647.1, APG08032.1, APG05712.1, APG01658.1, APG06498.1, APG09106.1, APG09882.1, APG02675.1, APG01405.1, APG06250.1, APG06877.1, APG09053.1, APG04293.1, APG01308.1, APG06646.1, APG09748, or APG07433.1 RGN comprises an amino acid sequence having at least 40%, 45%, 50%, 55%, 60%, 65%, 70%, 75%, 80%, 85%, 90%, 91%, 92%, 93%, 94%, 95%, 96%, 97%, 98%, 99% or more sequence identity to the amino acid sequence set forth as SEQ ID NO: 1, 9, 16, 23, 30, 38, 46, 54, 61, 69, 75, 82, 89, 95, 103, 110, 117, 137 or 235. In certain embodiments, an active fragment of the APG05733.1, APG06207.1, APG01647.1, APG08032.1, APG05712.1, APG01658.1, APG06498.1, APG09106.1, APG09882.1, APG02675.1, APG01405.1, APG06250.1, APG06877.1, APG09053.1, APG04293.1, APG01308.1, APG06646.1, APG09748, or APG07433.1 RGN comprises at least 50, 100, 150, 200, 250, 300, 350, 400, 450, 500, 550, 600, 650, 700, 750, 800, 850, 900, 950, 1000, 1050 or more contiguous amino acid residues of the amino acid sequence set forth as SEQ ID NO: 1, 9, 16, 23, 30, 38, 46, 54, 61, 69, 75, 82, 89, 95, 103, 110, 117, 137, or 235. RNA-guided nucleases provided herein can comprise at least one nuclease domain (*e.g.*, DNase, RNase domain) and at least one RNA recognition and/or RNA binding domain to interact with guide RNAs. Further domains that can be found in RNA-guided nucleases provided herein include, but are not limited to: DNA binding domains, helicase domains, protein-protein interaction domains, and dimerization domains. In specific embodiments, the RNA-guided nucleases provided herein can comprise at least 70%, 75%, 80%, 85%, 90%, 91%, 92%, 93%, 94%, 95%, 96%, 97%, 98%, 99% to one or more of a DNA binding domains, helicase domains, protein-protein interaction domains, and dimerization domains.

A target nucleotide sequence is bound by an RNA-guided nuclease provided herein and hybridizes with the guide RNA associated with the RNA-guided nuclease. The target sequence can then be subsequently cleaved by the RNA-guided nuclease if the polypeptide possesses nuclease activity. The terms

“cleave” or “cleavage” refer to the hydrolysis of at least one phosphodiester bond within the backbone of a target nucleotide sequence that can result in either single-stranded or double-stranded breaks within the target sequence. The presently disclosed RGNs can cleave nucleotides within a polynucleotide, functioning as an endonuclease or can be an exonuclease, removing successive nucleotides from the end (the 5' and/or the 3' end) of a polynucleotide. In other embodiments, the disclosed RGNs can cleave nucleotides of a target sequence within any position of a polynucleotide and thus function as both an endonuclease and exonuclease. The cleavage of a target polynucleotide by the presently disclosed RGNs can result in staggered breaks or blunt ends.

The presently disclosed RNA-guided nucleases can be wild-type sequences derived from bacterial or archaeal species. Alternatively, the RNA-guided nucleases can be variants or fragments of wild-type polypeptides. The wild-type RGN can be modified to alter nuclease activity or alter PAM specificity, for example. In some embodiments, the RNA-guided nuclease is not naturally-occurring.

In certain embodiments, the RNA-guided nuclease functions as a nickase, only cleaving a single strand of the target nucleotide sequence. Such RNA-guided nucleases have a single functioning nuclease domain. In some of these embodiments, additional nuclease domains have been mutated such that the nuclease activity is reduced or eliminated.

In other embodiments, the RNA-guided nuclease lacks nuclease activity altogether or exhibits reduced nuclease activity, and is referred to herein as nuclease-dead or nuclease inactive. Any method known in the art for introducing mutations into an amino acid sequence, such as PCR-mediated mutagenesis and site-directed mutagenesis, can be used for generating nickases or nuclease-dead RGNs. See, e.g., U.S. Publ. No. 2014/0068797 and U.S. Pat. No. 9,790,490; each of which is incorporated by reference in its entirety.

RNA-guided nucleases that lack nuclease activity can be used to deliver a fused polypeptide, polynucleotide, or small molecule payload to a particular genomic location. In some of these embodiments, the RGN polypeptide or guide RNA can be fused to a detectable label to allow for detection of a particular sequence. As a non-limiting example, a nuclease-dead RGN can be fused to a detectable label (e.g., fluorescent protein) and targeted to a particular sequence associated with a disease to allow for detection of the disease-associated sequence.

Alternatively, nuclease-dead RGNs can be targeted to particular genomic locations to alter the expression of a desired sequence. In some embodiments, the binding of a nuclease-dead RNA-guided nuclease to a target sequence results in the repression of expression of the target sequence or a gene under transcriptional control by the target sequence by interfering with the binding of RNA polymerase or transcription factors within the targeted genomic region. In other embodiments, the RGN (e.g., a nuclease-dead RGN) or its complexed guide RNA further comprises an expression modulator that, upon binding to a target sequence, serves to either repress or activate the expression of the target sequence or a gene under transcriptional control by the target sequence. In some of these embodiments, the expression modulator modulates the expression of the target sequence or regulated gene through epigenetic mechanisms.

In other embodiments, the nuclease-dead RGNs or a RGN with only nickase activity can be targeted to particular genomic locations to modify the sequence of a target polynucleotide through fusion to a base-editing polypeptide, for example a deaminase polypeptide or active variant or fragment thereof that deaminates a nucleotide base, resulting in conversion from one nucleotide base to another. The base-editing polypeptide can be fused to the RGN at its N-terminal or C-terminal end. Additionally, the base-editing polypeptide may be fused to the RGN via a peptide linker. A non-limiting example of a deaminase polypeptide that is useful for such compositions and methods include a cytidine deaminase or an adenosine deaminase (such as the adenosine deaminase base editor described in Gaudelli *et al.* (2017) *Nature* 551:464-471, U.S. Publ. Nos. 2017/0121693 and 2018/0073012, and International Publ. No. WO/2018/027078, or any of the deaminases disclosed in International Appl. No. PCT/US2019/068079, each of which is herein incorporated by reference in its entirety). Further, it is known in the art that certain fusion proteins between an RGN and a base-editing enzyme may also comprise at least one uracil stabilizing polypeptide that increases the mutation rate of a cytidine, deoxycytidine, or cytosine to a thymidine, deoxythymidine, or thymine in a nucleic acid molecule by a deaminase. Non-limiting examples of uracil stabilizing polypeptides include those disclosed in U.S. Provisional Appl. No. 63/052,175, filed July 15, 2020, and a uracil glycosylase inhibitor (UGI) domain (SEQ ID NO: 261), which may increase base editing efficiency (U.S. Patent No. 10,167,547, herein incorporated by reference). Therefore, a fusion protein may comprise an RGN described herein or a variant thereof, a deaminase, and optionally at least one uracil stabilizing polypeptides, such as UGI.

RNA-guided nucleases that are fused to a polypeptide or domain can be separated or joined by a linker. The term "linker," as used herein, refers to a chemical group or a molecule linking two molecules or moieties, *e.g.*, a binding domain and a cleavage domain of a nuclease. In some embodiments, a linker joins a gRNA binding domain of an RNA guided nuclease and a base-editing polypeptide, such as a deaminase. In some embodiments, a linker joins a nuclease-dead RGN and a deaminase. Typically, the linker is positioned between, or flanked by, two groups, molecules, or other moieties and connected to each one via a covalent bond, thus connecting the two. In some embodiments, the linker is an amino acid or a plurality of amino acids (*e.g.*, a peptide or protein). In some embodiments, the linker is an organic molecule, group, polymer, or chemical moiety. In some embodiments, the linker is 5-100 amino acids in length, for example, 5, 6, 7, 8, 9, 10, 11, 12, 13, 14, 15, 16, 17, 18, 19, 20, 21, 22, 23, 24, 25, 26, 27, 28, 29, 30, 30-35, 35-40, 40-45, 45-50, 50-60, 60-70, 70-80, 80-90, 90-100, 100-150, or 150-200 amino acids in length. Longer or shorter linkers are also contemplated.

The presently disclosed RNA-guided nucleases can comprise at least one nuclear localization signal (NLS) to enhance transport of the RGN to the nucleus of a cell. Nuclear localization signals are known in the art and generally comprise a stretch of basic amino acids (see, *e.g.*, Lange *et al.*, *J. Biol. Chem.* (2007) 282:5101-5105). In particular embodiments, the RGN comprises 2, 3, 4, 5, 6 or more nuclear localization signals. The nuclear localization signal(s) can be a heterologous NLS. Non-limiting examples of nuclear localization signals useful for the presently disclosed RGNs are the nuclear localization signals of SV40

Large T-antigen, nucleopasmin, and c-Myc (*see, e.g.,* Ray et al. (2015) *Bioconjug Chem* 26(6):1004-7). In particular embodiments, the RGN comprises the NLS sequence set forth as SEQ ID NO: 125 or 127. The RGN can comprise one or more NLS sequences at its N-terminus, C-terminus, or both the N-terminus and C-terminus. For example, the RGN can comprise two NLS sequences at the N-terminal region and four NLS sequences at the C-terminal region.

Other localization signal sequences known in the art that localize polypeptides to particular subcellular location(s) can also be used to target the RGNs, including, but not limited to, plastid localization sequences, mitochondrial localization sequences, and dual-targeting signal sequences that target to both the plastid and mitochondria (*see, e.g.,* Nassoury and Morse (2005) *Biochim Biophys Acta* 1743:5-19; Kunze and Berger (2015) *Front Physiol* dx.doi.org/10.3389/fphys.2015.00259; Herrmann and Neupert (2003) *IUBMB Life* 55:219-225; Soll (2002) *Curr Opin Plant Biol* 5:529-535; Carrie and Small (2013) *Biochim Biophys Acta* 1833:253-259; Carrie et al. (2009) *FEBS J* 276:1187-1195; Silva-Filho (2003) *Curr Opin Plant Biol* 6:589-595; Peeters and Small (2001) *Biochim Biophys Acta* 1541:54-63; Murcha et al. (2014) *J Exp Bot* 65:6301-6335; Mackenzie (2005) *Trends Cell Biol* 15:548-554; Glaser et al. (1998) *Plant Mol Biol* 38:311-338).

In certain embodiments, the presently disclosed RNA-guided nucleases comprise at least one cell-penetrating domain that facilitates cellular uptake of the RGN. Cell-penetrating domains are known in the art and generally comprise stretches of positively charged amino acid residues (*i.e.,* polycationic cell-penetrating domains), alternating polar amino acid residues and non-polar amino acid residues (*i.e.,* amphipathic cell-penetrating domains), or hydrophobic amino acid residues (*i.e.,* hydrophobic cell-penetrating domains) (*see, e.g.,* Milletti F. (2012) *Drug Discov Today* 17:850-860). A non-limiting example of a cell-penetrating domain is the trans-activating transcriptional activator (TAT) from the human immunodeficiency virus 1.

The nuclear localization signal, plastid localization signal, mitochondrial localization signal, dual-targeting localization signal, and/or cell-penetrating domain can be located at the amino-terminus (N-terminus), the carboxyl-terminus (C-terminus), or in an internal location of the RNA-guided nuclease.

The presently disclosed RGNs can be fused to an effector domain, such as a cleavage domain, a deaminase domain, or an expression modulator domain, either directly or indirectly via a linker peptide. Such a domain can be located at the N-terminus, the C-terminus, or an internal location of the RNA-guided nuclease. In some of these embodiments, the RGN component of the fusion protein is a nuclease-dead RGN.

In some embodiments, the RGN fusion protein comprises a cleavage domain, which is any domain that is capable of cleaving a polynucleotide (*i.e.,* RNA, DNA, or RNA/DNA hybrid) and includes, but is not limited to, restriction endonucleases and homing endonucleases, such as Type IIS endonucleases (*e.g., FokI*) (*see, e.g.,* Belfort et al. (1997) *Nucleic Acids Res.* 25:3379-3388; Linn et al. (eds.) *Nucleases*, Cold Spring Harbor Laboratory Press, 1993).

In other embodiments, the RGN fusion protein comprises a deaminase domain that deaminates a nucleotide base, resulting in conversion from one nucleotide base to another, and includes, but is not limited to, a cytidine deaminase or an adenosine deaminase base editor (see, *e.g.*, Gaudelli *et al.* (2017) *Nature* 551:464-471, U.S. Publ. Nos. 2017/0121693 and 2018/0073012, U.S. Patent No. 9,840,699, and International Publ. No. WO/2018/027078).

In some embodiments, the effector domain of the RGN fusion protein can be an expression modulator domain, which is a domain that either serves to upregulate or downregulate transcription. The expression modulator domain can be an epigenetic modification domain, a transcriptional repressor domain or a transcriptional activation domain.

In some of these embodiments, the expression modulator of the RGN fusion protein comprises an epigenetic modification domain that covalently modifies DNA or histone proteins to alter histone structure and/or chromosomal structure without altering the DNA sequence, leading to changes in gene expression (*i.e.*, upregulation or downregulation). Non-limiting examples of epigenetic modifications include acetylation or methylation of lysine residues, arginine methylation, serine and threonine phosphorylation, and lysine ubiquitination and sumoylation of histone proteins, and methylation and hydroxymethylation of cytosine residues in DNA. Non-limiting examples of epigenetic modification domains include histone acetyltransferase domains, histone deacetylase domains, histone methyltransferase domains, histone demethylase domains, DNA methyltransferase domains, and DNA demethylase domains.

In other embodiments, the expression modulator of the fusion protein comprises a transcriptional repressor domain, which interacts with transcriptional control elements and/or transcriptional regulatory proteins, such as RNA polymerases and transcription factors, to reduce or terminate transcription of at least one gene. Transcriptional repressor domains are known in the art and include, but are not limited to, Sp1-like repressors, IκB, and Krüppel associated box (KRAB) domains.

In yet other embodiments, the expression modulator of the fusion protein comprises a transcriptional activation domain, which interacts with transcriptional control elements and/or transcriptional regulatory proteins, such as RNA polymerases and transcription factors, to increase or activate transcription of at least one gene. Transcriptional activation domains are known in the art and include, but are not limited to, a herpes simplex virus VP16 activation domain and an NFAT activation domain.

The presently disclosed RGN polypeptides can comprise a detectable label or a purification tag. The detectable label or purification tag can be located at the N-terminus, the C-terminus, or an internal location of the RNA-guided nuclease, either directly or indirectly via a linker peptide. In some of these embodiments, the RGN component of the fusion protein is a nuclease-dead RGN. In other embodiments, the RGN component of the fusion protein is an RGN with nickase activity.

A detectable label is a molecule that can be visualized or otherwise observed. The detectable label may be fused to the RGN as a fusion protein (*e.g.*, fluorescent protein) or may be a small molecule conjugated to the RGN polypeptide that can be detected visually or by other means. Detectable labels that can be fused to the presently disclosed RGNs as a fusion protein include any detectable protein domain,

including but not limited to, a fluorescent protein or a protein domain that can be detected with a specific antibody. Non-limiting examples of fluorescent proteins include green fluorescent proteins (*e.g.*, GFP, EGFP, ZsGreen1) and yellow fluorescent proteins (*e.g.*, YFP, EYFP, ZsYellow1). Non-limiting examples of small molecule detectable labels include radioactive labels, such as ^3H and ^{35}S .

5 RGN polypeptides can also comprise a purification tag, which is any molecule that can be utilized to isolate a protein or fused protein from a mixture (*e.g.*, biological sample, culture medium). Non-limiting examples of purification tags include biotin, myc, maltose binding protein (MBP), and glutathione-S-transferase (GST).

10 II. Guide RNA

The present disclosure provides guide RNAs and polynucleotides encoding the same. The term “guide RNA” refers to a nucleotide sequence having sufficient complementarity with a target nucleotide sequence to hybridize with the target sequence and direct sequence-specific binding of an associated RNA-guided nuclease to the target nucleotide sequence. Thus, a RGN’s respective guide RNA is one or more
15 RNA molecules (generally, one or two), that can bind to the RGN and guide the RGN to bind to a particular target nucleotide sequence, and in those instances wherein the RGN has nickase or nuclease activity, also cleave the target nucleotide sequence. In general, a guide RNA comprises a CRISPR RNA (crRNA) and in some embodiments, a trans-activating CRISPR RNA (tracrRNA). Native guide RNAs that comprise both a crRNA and a tracrRNA generally comprise two separate RNA molecules that hybridize to each other
20 through the repeat sequence of the crRNA and the anti-repeat sequence of the tracrRNA.

Native direct repeat sequences within a CRISPR array generally range in length from 28 to 37 base pairs, although the length can vary between about 23 bp to about 55 bp. Spacer sequences within a CRISPR array generally range from about 32 to about 38 bp in length, although the length can be between about 21 bp to about 72 bp. Each CRISPR array generally comprises less than 50 units of the CRISPR repeat-spacer
25 sequence. The CRISPRs are transcribed as part of a long transcript termed the primary CRISPR transcript, which comprises much of the CRISPR array. The primary CRISPR transcript is cleaved by Cas proteins to produce crRNAs or in some cases, to produce pre-crRNAs that are further processed by additional Cas proteins into mature crRNAs. Mature crRNAs comprise a spacer sequence and a CRISPR repeat sequence. In some embodiments in which pre-crRNAs are processed into mature (or processed) crRNAs, maturation
30 involves the removal of about one to about six or more 5', 3', or 5' and 3' nucleotides. For the purposes of genome editing or targeting a particular target nucleotide sequence of interest, these nucleotides that are removed during maturation of the pre-crRNA molecule are not necessary for generating or designing a guide RNA.

A CRISPR RNA (crRNA) comprises a spacer sequence and a CRISPR repeat sequence. The
35 “spacer sequence” is the nucleotide sequence that directly hybridizes with the target nucleotide sequence of interest. The spacer sequence is engineered to be fully or partially complementary with the target sequence of interest. In various embodiments, the spacer sequence can comprise from about 8 nucleotides to about 30

nucleotides, or more. For example, the spacer sequence can be about 8, about 9, about 10, about 11, about 12, about 13, about 14, about 15, about 16, about 17, about 18, about 19, about 20, about 21, about 22, about 23, about 24, about 25, about 26, about 27, about 28, about 29, about 30, or more nucleotides in length. In some embodiments, the spacer sequence is about 10 to about 26 nucleotides in length, or about 12 to about 30 nucleotides in length. In particular embodiments, the spacer sequence is about 30 nucleotides in length. In some embodiments, the degree of complementarity between a spacer sequence and its corresponding target sequence, when optimally aligned using a suitable alignment algorithm, is about or more than about 50%, about 60%, about 70%, about 75%, about 80%, about 81%, about 82%, about 83%, about 84%, about 85%, about 86%, about 87%, about 88%, about 89%, about 90%, about 91%, about 92%, about 93%, about 94%, about 95%, about 96%, about 97%, about 98%, about 99%, or more. In particular embodiments, the spacer sequence is free of secondary structure, which can be predicted using any suitable polynucleotide folding algorithm known in the art, including but not limited to mFold (see, *e.g.*, Zuker and Stiegler (1981) *Nucleic Acids Res.* 9:133-148) and RNAfold (see, *e.g.*, Gruber et al. (2008) *Cell* 106(1):23-24).

The CRISPR RNA repeat sequence comprises a nucleotide sequence that comprises a region with sufficient complementarity to hybridize to a tracrRNA. In various embodiments, the CRISPR RNA repeat sequence can comprise from about 8 nucleotides to about 30 nucleotides, or more. For example, the CRISPR repeat sequence can be about 8, about 9, about 10, about 11, about 12, about 13, about 14, about 15, about 16, about 17, about 18, about 19, about 20, about 21, about 22, about 23, about 24, about 25, about 26, about 27, about 28, about 29, about 30, or more nucleotides in length. In some embodiments, the CRISPR repeat sequence is about 21 nucleotides in length. In some embodiments, the degree of complementarity between a CRISPR repeat sequence and its corresponding tracrRNA sequence, when optimally aligned using a suitable alignment algorithm, is about or more than about 50%, about 60%, about 70%, about 75%, about 80%, about 81%, about 82%, about 83%, about 84%, about 85%, about 86%, about 87%, about 88%, about 89%, about 90%, about 91%, about 92%, about 93%, about 94%, about 95%, about 96%, about 97%, about 98%, about 99%, or more. In particular embodiments, the CRISPR repeat sequence comprises the nucleotide sequence of SEQ ID NO: 2, 10, 17, 24, 31, 39, 47, 55, 62, 70, 76, 83, 90, 96, 104, 111, 118, 240, 273, or 287, or an active variant or fragment thereof that when comprised within a guide RNA, is capable of directing the sequence-specific binding of an associated RNA-guided nuclease provided herein to a target sequence of interest. In certain embodiments, an active CRISPR repeat sequence variant of a wild-type sequence comprises a nucleotide sequence having at least 40%, 45%, 50%, 55%, 60%, 65%, 70%, 75%, 80%, 85%, 90%, 91%, 92%, 93%, 94%, 95%, 96%, 97%, 98%, 99% or more sequence identity to the nucleotide sequence set forth as SEQ ID NO: 2, 10, 17, 24, 31, 39, 47, 55, 62, 70, 76, 83, 90, 96, 104, 111, 118, 240, 273 or 287. In certain embodiments, an active CRISPR repeat sequence fragment of a wild-type sequence comprises at least 5, 6, 7, 8, 9, 10, 11, 12, 13, 14, 15, 16, 17, 18, 19, or 20 contiguous nucleotides of the nucleotide sequence set forth as SEQ ID NO: 2, 10, 17, 24, 31, 39, 47, 55, 62, 70, 76, 83, 90, 96, 104, 111, 118, 240, 273, or 287.

In certain embodiments, the crRNA is not naturally-occurring. In some of these embodiments, the specific CRISPR repeat sequence is not linked to the engineered spacer sequence in nature and the CRISPR repeat sequence is considered heterologous to the spacer sequence. In certain embodiments, the spacer sequence is an engineered sequence that is not naturally occurring.

5 A trans-activating CRISPR RNA or tracrRNA molecule comprises a nucleotide sequence comprising a region that has sufficient complementarity to hybridize to a CRISPR repeat sequence of a crRNA, which is referred to herein as the anti-repeat region. In some embodiments, the tracrRNA molecule further comprises a region with secondary structure (*e.g.*, stem-loop) or forms secondary structure upon hybridizing with its corresponding crRNA. In particular embodiments, the region of the tracrRNA that is
10 fully or partially complementary to a CRISPR repeat sequence is at the 5' end of the molecule and the 3' end of the tracrRNA comprises secondary structure. This region of secondary structure generally comprises several hairpin structures, including the nexus hairpin, which is found adjacent to the anti-repeat sequence. The nexus hairpin often has a conserved nucleotide sequence in the base of the hairpin stem, with the motif UNANNA, CNANNG, CNANNU, UNANNG, UNANNC, or CNANNU (SEQ ID NOs: 8, 37, 45, 53, 68, and 102, respectively) found in many nexus hairpins in tracrRNAs. There are often terminal hairpins at the
15 3' end of the tracrRNA that can vary in structure and number, but often comprise a GC-rich Rho-independent transcriptional terminator hairpin followed by a string of U's at the 3' end. See, for example, Briner et al. (2014) *Molecular Cell* 56:333-339, Briner and Barrangou (2016) *Cold Spring Harb Protoc*; doi: 10.1101/pdb.top090902, and U.S. Publication No. 2017/0275648, each of which is herein incorporated by
20 reference in its entirety.

In various embodiments, the anti-repeat region of the tracrRNA that is fully or partially complementary to the CRISPR repeat sequence comprises from about 8 nucleotides to about 30 nucleotides, or more. For example, the region of base pairing between the tracrRNA anti-repeat sequence and the CRISPR repeat sequence can be about 8, about 9, about 10, about 11, about 12, about 13, about 14, about 15,
25 about 16, about 17, about 18, about 19, about 20, about 21, about 22, about 23, about 24, about 25, about 26, about 27, about 28, about 29, about 30, or more nucleotides in length. In particular embodiments, the anti-repeat region of the tracrRNA that is fully or partially complementary to a CRISPR repeat sequence is about 20 nucleotides in length. In some embodiments, the degree of complementarity between a CRISPR repeat sequence and its corresponding tracrRNA anti-repeat sequence, when optimally aligned using a suitable
30 alignment algorithm, is about or more than about 50%, about 60%, about 70%, about 75%, about 80%, about 81%, about 82%, about 83%, about 84%, about 85%, about 86%, about 87%, about 88%, about 89%, about 90%, about 91%, about 92%, about 93%, about 94%, about 95%, about 96%, about 97%, about 98%, about 99%, or more.

In various embodiments, the entire tracrRNA can comprise from about 60 nucleotides to more than
35 about 140 nucleotides. For example, the tracrRNA can be about 60, about 65, about 70, about 75, about 80, about 85, about 90, about 95, about 100, about 105, about 110, about 115, about 120, about 125, about 130, about 135, about 140, or more nucleotides in length. In particular embodiments, the tracrRNA is about 80 to

about 90 nucleotides in length, including about 80, about 81, about 82, about 83, about 84, about 85, about 86, about 87, about 88, about 89, and about 90 nucleotides in length. In certain embodiments, the tracrRNA is about 85 nucleotides in length.

In particular embodiments, the tracrRNA comprises the nucleotide sequence of SEQ ID NO: 3, 11, 18, 25, 32, 40, 48, 56, 63, 71, 77, 84, 91, 97, 105, 112, or 119, or an active variant or fragment thereof that when comprised within a guide RNA is capable of directing the sequence-specific binding of an associated RNA-guided nuclease provided herein to a target sequence of interest. In certain embodiments, an active tracrRNA sequence variant of a wild-type sequence comprises a nucleotide sequence having at least 40%, 45%, 50%, 55%, 60%, 65%, 70%, 75%, 80%, 85%, 90%, 91%, 92%, 93%, 94%, 95%, 96%, 97%, 98%, 99% or more sequence identity to the nucleotide sequence set forth as SEQ ID NO: 3, 11, 18, 25, 32, 40, 48, 56, 63, 71, 77, 84, 91, 97, 105, 112, 119, 241, 274, or 286. In certain embodiments, an active tracrRNA sequence fragment of a wild-type sequence comprises at least 5, 10, 15, 20, 25, 30, 35, 40, 45, 50, 55, 60, 65, 70, 75, 80, or more contiguous nucleotides of the nucleotide sequence set forth as SEQ ID NO: 3, 11, 18, 25, 32, 40, 48, 56, 63, 71, 77, 84, 91, 97, 105, 112, 119, 241, 274, or 286.

Two polynucleotide sequences can be considered to be substantially complementary when the two sequences hybridize to each other under stringent conditions. Likewise, an RGN is considered to bind to a particular target sequence within a sequence-specific manner if the guide RNA bound to the RGN binds to the target sequence under stringent conditions. By "stringent conditions" or "stringent hybridization conditions" is intended conditions under which the two polynucleotide sequences will hybridize to each other to a detectably greater degree than to other sequences (*e.g.*, at least 2-fold over background). Stringent conditions are sequence-dependent and will be different in different circumstances. Typically, stringent conditions will be those in which the salt concentration is less than about 1.5 M Na ion, typically about 0.01 to 1.0 M Na ion concentration (or other salts) at pH 7.0 to 8.3, and the temperature is at least about 30°C for short sequences (*e.g.*, 10 to 50 nucleotides) and at least about 60°C for long sequences (*e.g.*, greater than 50 nucleotides). Stringent conditions may also be achieved with the addition of destabilizing agents such as formamide. Exemplary low stringency conditions include hybridization with a buffer solution of 30 to 35% formamide, 1 M NaCl, 1% SDS (sodium dodecyl sulfate) at 37°C, and a wash in 1X to 2X SSC (20X SSC = 3.0 M NaCl/0.3 M trisodium citrate) at 50 to 55°C. Exemplary moderate stringency conditions include hybridization in 40 to 45% formamide, 1.0 M NaCl, 1% SDS at 37°C, and a wash in 0.5X to 1X SSC at 55 to 60°C. Exemplary high stringency conditions include hybridization in 50% formamide, 1 M NaCl, 1% SDS at 37°C, and a wash in 0.1X SSC at 60 to 65°C. Optionally, wash buffers may comprise about 0.1% to about 1% SDS. Duration of hybridization is generally less than about 24 hours, usually about 4 to about 12 hours. The duration of the wash time will be at least a length of time sufficient to reach equilibrium.

The T_m is the temperature (under defined ionic strength and pH) at which 50% of a complementary target sequence hybridizes to a perfectly matched sequence. For DNA-DNA hybrids, the T_m can be approximated from the equation of Meinkoth and Wahl (1984) *Anal. Biochem.* 138:267-284: $T_m = 81.5^\circ\text{C} + 16.6 (\log M) + 0.41 (\%GC) - 0.61 (\% \text{ form}) - 500/L$; where M is the molarity of monovalent cations, %GC

is the percentage of guanosine and cytosine nucleotides in the DNA, % form is the percentage of formamide in the hybridization solution, and L is the length of the hybrid in base pairs. Generally, stringent conditions are selected to be about 5°C lower than the thermal melting point (T_m) for the specific sequence and its complement at a defined ionic strength and pH. However, severely stringent conditions can utilize a hybridization and/or wash at 1, 2, 3, or 4°C lower than the thermal melting point (T_m); moderately stringent conditions can utilize a hybridization and/or wash at 6, 7, 8, 9, or 10°C lower than the thermal melting point (T_m); low stringency conditions can utilize a hybridization and/or wash at 11, 12, 13, 14, 15, or 20°C lower than the thermal melting point (T_m). Using the equation, hybridization and wash compositions, and desired T_m, those of ordinary skill will understand that variations in the stringency of hybridization and/or wash solutions are inherently described. An extensive guide to the hybridization of nucleic acids is found in Tijssen (1993) *Laboratory Techniques in Biochemistry and Molecular Biology—Hybridization with Nucleic Acid Probes*, Part I, Chapter 2 (Elsevier, New York); and Ausubel et al., eds. (1995) *Current Protocols in Molecular Biology*, Chapter 2 (Greene Publishing and Wiley-Interscience, New York). See Sambrook et al. (1989) *Molecular Cloning: A Laboratory Manual* (2d ed., Cold Spring Harbor Laboratory Press, Plainview, New York).

The guide RNA can be a single guide RNA or a dual-guide RNA system. A single guide RNA comprises the crRNA and tracrRNA on a single molecule of RNA, whereas a dual-guide RNA system comprises a crRNA and a tracrRNA present on two distinct RNA molecules, hybridized to one another through at least a portion of the CRISPR repeat sequence of the crRNA and at least a portion of the tracrRNA, which may be fully or partially complementary to the CRISPR repeat sequence of the crRNA. In some of those embodiments wherein the guide RNA is a single guide RNA, the crRNA and tracrRNA are separated by a linker nucleotide sequence. In general, the linker nucleotide sequence is one that does not include complementary bases in order to avoid the formation of secondary structure within or comprising nucleotides of the linker nucleotide sequence. In some embodiments, the linker nucleotide sequence between the crRNA and tracrRNA is at least 3, at least 4, at least 5, at least 6, at least 7, at least 8, at least 9, at least 10, at least 11, at least 12, or more nucleotides in length. In particular embodiments, the linker nucleotide sequence of a single guide RNA is at least 4 nucleotides in length. In certain embodiments, the linker nucleotide sequence is the nucleotide sequence set forth as SEQ ID NO: 123.

The single guide RNA or dual-guide RNA can be synthesized chemically or via *in vitro* transcription. Assays for determining sequence-specific binding between a RGN and a guide RNA are known in the art and include, but are not limited to, *in vitro* binding assays between an expressed RGN and the guide RNA, which can be tagged with a detectable label (e.g., biotin) and used in a pull-down detection assay in which the guide RNA:RGN complex is captured via the detectable label (e.g., with streptavidin beads). A control guide RNA with an unrelated sequence or structure to the guide RNA can be used as a negative control for non-specific binding of the RGN to RNA. In certain embodiments, the guide RNA is SEQ ID NO: 4, 12, 19, 26, 33, 41, 49, 57, 64, 72, 78, 85, 92, 98, 106, 113, or 120, wherein the spacer sequence can be any sequence and is indicated as a poly-N sequence.

In certain embodiments, the guide RNA can be introduced into a target cell, organelle, or embryo as an RNA molecule. The guide RNA can be transcribed *in vitro* or chemically synthesized. In other embodiments, a nucleotide sequence encoding the guide RNA is introduced into the cell, organelle, or embryo. In some of these embodiments, the nucleotide sequence encoding the guide RNA is operably
 5 linked to a promoter (*e.g.*, an RNA polymerase III promoter). The promoter can be a native promoter or heterologous to the guide RNA-encoding nucleotide sequence.

In various embodiments, the guide RNA can be introduced into a target cell, organelle, or embryo as a ribonucleoprotein complex, as described herein, wherein the guide RNA is bound to an RNA-guided
 10 nuclease polypeptide.

The guide RNA directs an associated RNA-guided nuclease to a particular target nucleotide
 15 sequence of interest through hybridization of the guide RNA to the target nucleotide sequence. A target nucleotide sequence can comprise DNA, RNA, or a combination of both and can be single-stranded or double-stranded. A target nucleotide sequence can be genomic DNA (*i.e.*, chromosomal DNA), plasmid DNA, or an RNA molecule (*e.g.*, messenger RNA, ribosomal RNA, transfer RNA, micro RNA, small
 20 interfering RNA). The target nucleotide sequence can be bound (and in some embodiments, cleaved) by an RNA-guided nuclease *in vitro* or in a cell. The chromosomal sequence targeted by the RGN can be a nuclear, plastid or mitochondrial chromosomal sequence. In some embodiments, the target nucleotide sequence is unique in the target genome.

The target nucleotide sequence is adjacent to a protospacer adjacent motif (PAM). A protospacer
 25 adjacent motif is generally within about 1 to about 10 nucleotides from the target nucleotide sequence, including about 1, about 2, about 3, about 4, about 5, about 6, about 7, about 8, about 9, or about 10 nucleotides from the target nucleotide sequence. The PAM can be 5' or 3' of the target sequence. In some embodiments, the PAM is 3' of the target sequence for the presently disclosed RGNs. Generally, the PAM is a consensus sequence of about 3-4 nucleotides, but in particular embodiments, can be 2, 3, 4, 5, 6, 7, 8, 9, or
 30 more nucleotides in length. In various embodiments, the PAM sequence recognized by the presently disclosed RGNs comprises the consensus sequence set forth as SEQ ID NO: 7, 15, 22, 29, 36, 44, 52, 60, 67, 81, 88, 101, 109, or 116.

In particular embodiments, an RNA-guided nuclease having SEQ ID NO: 1, 9, 16, 23, 30, 38, 46, 54, 61, 69, 75, 82, 89, 95, 103, 110, or 117 or an active variant or fragment thereof binds respectively a
 35 target nucleotide sequence adjacent to a PAM sequence set forth as SEQ ID NO: 7, 15, 22, 29, 36, 44, 52, 60, 67, 81, 88, 101, 109, or 116. In some embodiments, an RNA-guided nuclease having SEQ ID NO: 54 or 137 or an active variant or fragment thereof binds a target nucleotide sequence adjacent to a PAM sequence set forth as SEQ ID NO: 147. In some of these embodiments, the RGN binds to a guide sequence comprising a CRISPR repeat sequence set forth in SEQ ID NO: 2, 10, 17, 24, 31, 39, 47, 55, 62, 70, 76, 83,
 40 90, 96, 104, 111, or 118, respectively, or an active variant or fragment thereof, and a tracrRNA sequence set forth in SEQ ID NO: 3, 11, 18, 25, 32, 40, 48, 56, 63, 71, 77, 84, 91, 97, 105, 112, or 119, respectively, or an

active variant or fragment thereof. The RGN systems are described further in Example 1 and Tables 1 and 2 of the present specification.

It is well-known in the art that PAM sequence specificity for a given nuclease enzyme is affected by enzyme concentration (*see, e.g.,* Karvelis *et al.* (2015) *Genome Biol* 16:253), which may be modified by
 5 altering the promoter used to express the RGN, or the amount of ribonucleoprotein complex delivered to the cell, organelle, or embryo.

Upon recognizing its corresponding PAM sequence, the RGN can cleave the target nucleotide sequence at a specific cleavage site. As used herein, a cleavage site is made up of the two particular nucleotides within a target nucleotide sequence between which the nucleotide sequence is cleaved by an
 10 RGN. The cleavage site can comprise the 1st and 2nd, 2nd and 3rd, 3rd and 4th, 4th and 5th, 5th and 6th, 7th and 8th, or 8th and 9th nucleotides from the PAM in either the 5' or 3' direction. In some embodiments, the cleavage site may be over 10, 11, 12, 13, 14, 15, 16, 17, 18, 19, or 20 nucleotides from the PAM in either the 5' or 3' direction. In some embodiments, the cleavage site is 4 nucleotides away from the PAM. In other
 15 embodiments, the cleavage site is at least 15 nucleotides away from the PAM. As RGNs can cleave a target nucleotide sequence resulting in staggered ends, in some embodiments, the cleavage site is defined based on the distance of the two nucleotides from the PAM on the positive (+) strand of the polynucleotide and the distance of the two nucleotides from the PAM on the negative (-) strand of the polynucleotide.

The PAM is regarded as a hallmark of the RNA-guided nucleases of Type II CRISPR systems (Szczelkun *et al.*, PNAS, 111: 9798-9803, 2014; Sternberg *et al.*, Nature 507: 62-67, 2014). Interestingly,
 20 although APG06646.1 and APG04293.1 function as RNA-guided nucleases and possess many of the same domains as Type II CRISPR Cas9 nucleases, they each lack the typical PAM-Interacting domain (PID; Interpro: IPR032237; Pfam: PF16595). Accordingly, APG06646.1 and APG04293.1 also do not possess the typical PAM requirement, which is a motif of 2-5 nucleotides as described above. Instead, these proteins have unique DNA recognition domains at their C-termini (residues 821-1092 of APG06646.1 (full length
 25 sequence is SEQ ID NO: 117); residues 1064-1401 of APG04293.1 (full-length sequence is SEQ ID NO: 103). These unique DNA recognition domains enable the nucleases to cleave at a genomic target site based on a single-nucleotide motif in the vicinity of the genomic target sequence (SEQ ID NO: 109; see Table 2).

APG04293.1 also possesses a unique signature domain of 133 amino acid residues proximal to its N-terminus (residues 144-276). The function of this domain is not known in either Type II CRISPR Cas9
 30 nucleases or generally in the art.

III. Nucleotides Encoding RNA-guided nucleases, CRISPR RNA, and/or tracrRNA

The present disclosure provides polynucleotides comprising the presently disclosed CRISPR RNAs, tracrRNAs, and/or sgRNAs and polynucleotides comprising a nucleotide sequence encoding the presently
 35 disclosed RNA-guided nucleases, CRISPR RNAs, tracrRNAs, and/or sgRNAs. Presently disclosed polynucleotides include those comprising or encoding a CRISPR repeat sequence comprising the nucleotide sequence of SEQ ID NO: 2, 10, 17, 24, 31, 39, 47, 55, 62, 70, 76, 83, 90, 96, 104, 111, 118, 240, 273, or

287, or an active variant or fragment thereof that when comprised within a guide RNA is capable of directing the sequence-specific binding of an associated RNA-guided nuclease to a target sequence of interest. Also disclosed are polynucleotides comprising or encoding a tracrRNA comprising the nucleotide sequence of SEQ ID NO: 3, 11, 18, 25, 32, 40, 48, 56, 63, 71, 77, 84, 91, 97, 105, 112, 119, 241, 274, or 286, or an active variant or fragment thereof that when comprised within a guide RNA is capable of directing the sequence-specific binding of an associated RNA-guided nuclease to a target sequence of interest. Polynucleotides are also provided that encode an RNA-guided nuclease comprising the amino acid sequence set forth as SEQ ID NO: 1, 9, 16, 23, 30, 38, 46, 54, 61, 69, 75, 82, 89, 95, 103, 110, 117, 137, or 235, and active fragments or variants thereof that retain the ability to bind to a target nucleotide sequence in an RNA-guided sequence-specific manner.

The use of the term "polynucleotide" is not intended to limit the present disclosure to polynucleotides comprising DNA. Those of ordinary skill in the art will recognize that polynucleotides can comprise ribonucleotides (RNA) and combinations of ribonucleotides and deoxyribonucleotides. Such deoxyribonucleotides and ribonucleotides include both naturally occurring molecules and synthetic analogues. These include peptide nucleic acids (PNAs), PNA-DNA chimers, locked nucleic acids (LNAs), and phosphothiorate linked sequences. The polynucleotides disclosed herein also encompass all forms of sequences including, but not limited to, single-stranded forms, double-stranded forms, DNA-RNA hybrids, triplex structures, stem-and-loop structures, and the like.

The nucleic acid molecules encoding RGNs can be codon optimized for expression in an organism of interest. A "codon-optimized" coding sequence is a polynucleotide coding sequence having its frequency of codon usage designed to mimic the frequency of preferred codon usage or transcription conditions of a particular host cell. Expression in the particular host cell or organism is enhanced as a result of the alteration of one or more codons at the nucleic acid level such that the translated amino acid sequence is not changed. Nucleic acid molecules can be codon optimized, either wholly or in part. Codon tables and other references providing preference information for a wide range of organisms are available in the art (*see, e.g.,* Campbell and Gowri (1990) *Plant Physiol.* 92:1-11 for a discussion of plant-preferred codon usage). Methods are available in the art for synthesizing plant-preferred genes. *See, for example, U.S. Patent Nos. 5,380,831, and 5,436,391, and Murray et al. (1989) Nucleic Acids Res. 17:477-498, herein incorporated by reference.*

Polynucleotides encoding the RGNs, crRNAs, tracrRNAs, and/or sgRNAs provided herein can be provided in expression cassettes for *in vitro* expression or expression in a cell, organelle, embryo, or organism of interest. The cassette will include 5' and 3' regulatory sequences operably linked to a polynucleotide encoding an RGN, crRNA, tracrRNAs, and/or sgRNAs provided herein that allows for expression of the polynucleotide. The cassette may additionally contain at least one additional gene or genetic element to be cotransformed into the organism. Where additional genes or elements are included, the components are operably linked. The term "operably linked" is intended to mean a functional linkage between two or more elements. For example, an operable linkage between a promoter and a coding region of interest (*e.g.,* region coding for an RGN, crRNA, tracrRNAs, and/or sgRNAs) is a functional link that

allows for expression of the coding region of interest. Operably linked elements may be contiguous or non-contiguous. When used to refer to the joining of two protein coding regions, by operably linked is intended that the coding regions are in the same reading frame. Alternatively, the additional gene(s) or element(s) can be provided on multiple expression cassettes. For example, the nucleotide sequence encoding a presently disclosed RGN can be present on one expression cassette, whereas the nucleotide sequence encoding a crRNA, tracrRNA, or complete guide RNA can be on a separate expression cassette. Such an expression cassette is provided with a plurality of restriction sites and/or recombination sites for insertion of the polynucleotides to be under the transcriptional regulation of the regulatory regions. The expression cassette may additionally contain a selectable marker gene.

The expression cassette will include in the 5'-3' direction of transcription, a transcriptional (and, in some embodiments, translational) initiation region (*i.e.*, a promoter), an RGN-, crRNA-, tracrRNA-and/or sgRNA- encoding polynucleotide of the invention, and a transcriptional (and in some embodiments, translational) termination region (*i.e.*, termination region) functional in the organism of interest. The promoters of the invention are capable of directing or driving expression of a coding sequence in a host cell. The regulatory regions (*e.g.*, promoters, transcriptional regulatory regions, and translational termination regions) may be endogenous or heterologous to the host cell or to each other. As used herein, "heterologous" in reference to a sequence is a sequence that originates from a foreign species, or, if from the same species, is substantially modified from its native form in composition and/or genomic locus by deliberate human intervention. As used herein, a chimeric gene comprises a coding sequence operably linked to a transcription initiation region that is heterologous to the coding sequence.

Convenient termination regions are available from the Ti-plasmid of *A. tumefaciens*, such as the octopine synthase and nopaline synthase termination regions. See also Guerineau *et al.* (1991) *Mol. Gen. Genet.* 262:141-144; Proudfoot (1991) *Cell* 64:671-674; Sanfacon *et al.* (1991) *Genes Dev.* 5:141-149; Mogen *et al.* (1990) *Plant Cell* 2:1261-1272; Munroe *et al.* (1990) *Gene* 91:151-158; Ballas *et al.* (1989) *Nucleic Acids Res.* 17:7891-7903; and Joshi *et al.* (1987) *Nucleic Acids Res.* 15:9627-9639.

Additional regulatory signals include, but are not limited to, transcriptional initiation start sites, operators, activators, enhancers, other regulatory elements, ribosomal binding sites, an initiation codon, termination signals, and the like. See, for example, U.S. Pat. Nos. 5,039,523 and 4,853,331; EPO 0480762A2; Sambrook *et al.* (1992) *Molecular Cloning: A Laboratory Manual*, ed. Maniatis *et al.* (Cold Spring Harbor Laboratory Press, Cold Spring Harbor, N.Y.), hereinafter "Sambrook 11"; Davis *et al.*, eds. (1980) *Advanced Bacterial Genetics* (Cold Spring Harbor Laboratory Press), Cold Spring Harbor, N.Y., and the references cited therein.

In preparing the expression cassette, the various DNA fragments may be manipulated, so as to provide for the DNA sequences in the proper orientation and, as appropriate, in the proper reading frame. Toward this end, adapters or linkers may be employed to join the DNA fragments or other manipulations may be involved to provide for convenient restriction sites, removal of superfluous DNA, removal of

restriction sites, or the like. For this purpose, *in vitro* mutagenesis, primer repair, restriction, annealing, resubstitutions, *e.g.*, transitions and transversions, may be involved.

A number of promoters can be used in the practice of the invention. The promoters can be selected based on the desired outcome. The nucleic acids can be combined with constitutive, inducible, growth stage-specific, cell type-specific, tissue-preferred, tissue-specific, or other promoters for expression in the organism of interest. See, for example, promoters set forth in WO 99/43838 and in US Patent Nos: 8,575,425; 7,790,846; 8,147,856; 8,586,832; 7,772,369; 7,534,939; 6,072,050; 5,659,026; 5,608,149; 5,608,144; 5,604,121; 5,569,597; 5,466,785; 5,399,680; 5,268,463; 5,608,142; and 6,177,611; herein incorporated by reference.

For expression in plants, constitutive promoters also include CaMV 35S promoter (Odell *et al.* (1985) *Nature* 313:810-812); rice actin (McElroy *et al.* (1990) *Plant Cell* 2:163-171); ubiquitin (Christensen *et al.* (1989) *Plant Mol. Biol.* 12:619-632 and Christensen *et al.* (1992) *Plant Mol. Biol.* 18:675-689); pEMU (Last *et al.* (1991) *Theor. Appl. Genet.* 81:581-588); and MAS (Velten *et al.* (1984) *EMBO J.* 3:2723-2730).

Examples of inducible promoters are the Adh1 promoter which is inducible by hypoxia or cold stress, the Hsp70 promoter which is inducible by heat stress, the PPDK promoter and the pepcarboxylase promoter which are both inducible by light. Also useful are promoters which are chemically inducible, such as the In2-2 promoter which is safener induced (U.S. Pat. No. 5,364,780), the Axig1 promoter which is auxin induced and tapetum specific but also active in callus (PCT US01/22169), the steroid-responsive promoters (see, for example, the ERE promoter which is estrogen induced, and the glucocorticoid-inducible promoter in Schena *et al.* (1991) *Proc. Natl. Acad. Sci. USA* 88:10421-10425 and McNellis *et al.* (1998) *Plant J.* 14(2):247-257) and tetracycline-inducible and tetracycline-repressible promoters (see, for example, Gatz *et al.* (1991) *Mol. Gen. Genet.* 227:229-237, and U.S. Pat. Nos. 5,814,618 and 5,789,156), herein incorporated by reference.

Tissue-specific or tissue-preferred promoters can be utilized to target expression of an expression construct within a particular tissue. In certain embodiments, the tissue-specific or tissue-preferred promoters are active in plant tissue. Examples of promoters under developmental control in plants include promoters that initiate transcription preferentially in certain tissues, such as leaves, roots, fruit, seeds, or flowers. A "tissue specific" promoter is a promoter that initiates transcription only in certain tissues. Unlike constitutive expression of genes, tissue-specific expression is the result of several interacting levels of gene regulation. As such, promoters from homologous or closely related plant species can be preferable to use to achieve efficient and reliable expression of transgenes in particular tissues. In some embodiments, the expression comprises a tissue-preferred promoter. A "tissue preferred" promoter is a promoter that initiates transcription preferentially, but not necessarily entirely or solely in certain tissues.

In some embodiments, the nucleic acid molecules encoding a RGN, crRNA, and/or tracrRNA comprise a cell type-specific promoter. A "cell type specific" promoter is a promoter that primarily drives expression in certain cell types in one or more organs. Some examples of plant cells in which cell type specific promoters functional in plants may be primarily active include, for example, BETL cells, vascular

cells in roots, leaves, stalk cells, and stem cells. The nucleic acid molecules can also include cell type preferred promoters. A "cell type preferred" promoter is a promoter that primarily drives expression mostly, but not necessarily entirely or solely in certain cell types in one or more organs. Some examples of plant cells in which cell type preferred promoters functional in plants may be preferentially active include, for example, BETL cells, vascular cells in roots, leaves, stalk cells, and stem cells.

The nucleic acid sequences encoding the RGNs, crRNAs, tracrRNAs, and/or sgRNAs can be operably linked to a promoter sequence that is recognized by a phage RNA polymerase for example, for *in vitro* mRNA synthesis. In such embodiments, the *in vitro*-transcribed RNA can be purified for use in the methods described herein. For example, the promoter sequence can be a T7, T3, or SP6 promoter sequence or a variation of a T7, T3, or SP6 promoter sequence. In such embodiments, the expressed protein and/or RNAs can be purified for use in the methods of genome modification described herein.

In certain embodiments, the polynucleotide encoding the RGN, crRNA, tracrRNA, and/or sgRNA also can be linked to a polyadenylation signal (*e.g.*, SV40 polyA signal and other signals functional in plants) and/or at least one transcriptional termination sequence. Additionally, the sequence encoding the RGN also can be linked to sequence(s) encoding at least one nuclear localization signal, at least one cell-penetrating domain, and/or at least one signal peptide capable of trafficking proteins to particular subcellular locations, as described elsewhere herein.

The polynucleotide encoding the RGN, crRNA, tracrRNA, and/or sgRNA can be present in a vector or multiple vectors. A "vector" refers to a polynucleotide composition for transferring, delivering, or introducing a nucleic acid into a host cell. Suitable vectors include plasmid vectors, phagemids, cosmids, artificial/mini-chromosomes, transposons, and viral vectors (*e.g.*, lentiviral vectors, adeno-associated viral vectors, baculoviral vector). The vector can comprise additional expression control sequences (*e.g.*, enhancer sequences, Kozak sequences, polyadenylation sequences, transcriptional termination sequences), selectable marker sequences (*e.g.*, antibiotic resistance genes), origins of replication, and the like. Additional information can be found in "Current Protocols in Molecular Biology" Ausubel *et al.*, John Wiley & Sons, New York, 2003 or "Molecular Cloning: A Laboratory Manual" Sambrook & Russell, Cold Spring Harbor Press, Cold Spring Harbor, N.Y., 3rd edition, 2001.

The vector can also comprise a selectable marker gene for the selection of transformed cells. Selectable marker genes are utilized for the selection of transformed cells or tissues. Marker genes include genes encoding antibiotic resistance, such as those encoding neomycin phosphotransferase II (NEO) and hygromycin phosphotransferase (HPT), as well as genes conferring resistance to herbicidal compounds, such as glufosinate ammonium, bromoxynil, imidazolinones, and 2,4-dichlorophenoxyacetate (2,4-D).

In some embodiments, the expression cassette or vector comprising the sequence encoding the RGN polypeptide can further comprise a sequence encoding a crRNA and/or a tracrRNA, or the crRNA and tracrRNA combined to create a guide RNA. The sequence(s) encoding the crRNA and/or tracrRNA can be operably linked to at least one transcriptional control sequence for expression of the crRNA and/or tracrRNA in the organism or host cell of interest. For example, the polynucleotide encoding the crRNA

and/or tracrRNA can be operably linked to a promoter sequence that is recognized by RNA polymerase III (Pol III). Examples of suitable Pol III promoters include, but are not limited to, mammalian U6, U3, H1, and 7SL RNA promoters and rice U6 and U3 promoters.

As indicated, expression constructs comprising nucleotide sequences encoding the RGNs, crRNA, tracrRNA, and/or sgRNA can be used to transform organisms of interest. Methods for transformation involve introducing a nucleotide construct into an organism of interest. By "introducing" is intended to introduce the nucleotide construct to the host cell in such a manner that the construct gains access to the interior of the host cell. The methods of the invention do not require a particular method for introducing a nucleotide construct to a host organism, only that the nucleotide construct gains access to the interior of at least one cell of the host organism. The host cell can be a eukaryotic or prokaryotic cell. In particular embodiments, the eukaryotic host cell is a plant cell, a mammalian cell, or an insect cell. Methods for introducing nucleotide constructs into plants and other host cells are known in the art including, but not limited to, stable transformation methods, transient transformation methods, and virus-mediated methods.

The methods result in a transformed organism, such as a plant, including whole plants, as well as plant organs (*e.g.*, leaves, stems, roots, etc.), seeds, plant cells, propagules, embryos and progeny of the same. Plant cells can be differentiated or undifferentiated (*e.g.* callus, suspension culture cells, protoplasts, leaf cells, root cells, phloem cells, pollen).

"Transgenic organisms" or "transformed organisms" or "stably transformed" organisms or cells or tissues refers to organisms that have incorporated or integrated a polynucleotide encoding a RGN, crRNA, and/or tracrRNA of the invention. It is recognized that other exogenous or endogenous nucleic acid sequences or DNA fragments may also be incorporated into the host cell. *Agrobacterium*-and biolistic-mediated transformation remain the two predominantly employed approaches for transformation of plant cells. However, transformation of a host cell may be performed by infection, transfection, microinjection, electroporation, microprojection, biolistics or particle bombardment, electroporation, silica/carbon fibers, ultrasound mediated, PEG mediated, calcium phosphate co-precipitation, polycation DMSO technique, DEAE dextran procedure, and viral mediated, liposome mediated and the like. Viral-mediated introduction of a polynucleotide encoding an RGN, crRNA, and/or tracrRNA includes retroviral, lentiviral, adenoviral, and adeno-associated viral mediated introduction and expression, as well as the use of Caulimoviruses, Geminiviruses, and RNA plant viruses.

Transformation protocols as well as protocols for introducing polypeptides or polynucleotide sequences into plants may vary depending on the type of host cell (*e.g.*, monocot or dicot plant cell) targeted for transformation. Methods for transformation are known in the art and include those set forth in US Patent Nos: 8,575,425; 7,692,068; 8,802,934; 7,541,517; each of which is herein incorporated by reference. See, also, Rakoczy-Trojanowska, M. (2002) *Cell Mol Biol Lett.* 7:849-858; Jones *et al.* (2005) *Plant Methods* 1:5; Rivera *et al.* (2012) *Physics of Life Reviews* 9:308-345; Bartlett *et al.* (2008) *Plant Methods* 4:1-12; Bates, G.W. (1999) *Methods in Molecular Biology* 111:359-366; Binns and Thomashow (1988) *Annual Reviews in Microbiology* 42:575-606; Christou, P. (1992) *The Plant Journal* 2:275-281; Christou, P.

(1995) *Euphytica* 85:13-27; Tzfira *et al.* (2004) *TRENDS in Genetics* 20:375-383; Yao *et al.* (2006) *Journal of Experimental Botany* 57:3737-3746; Zupan and Zambryski (1995) *Plant Physiology* 107:1041-1047; Jones *et al.* (2005) *Plant Methods* 1:5;

Transformation may result in stable or transient incorporation of the nucleic acid into the cell.

5 "Stable transformation" is intended to mean that the nucleotide construct introduced into a host cell integrates into the genome of the host cell and is capable of being inherited by the progeny thereof.

"Transient transformation" is intended to mean that a polynucleotide is introduced into the host cell and does not integrate into the genome of the host cell.

Methods for transformation of chloroplasts are known in the art. See, for example, Svab *et al.* (1990) *Proc. Natl. Acad. Sci. USA* 87:8526-8530; Svab and Maliga (1993) *Proc. Natl. Acad. Sci. USA* 90:913-917; Svab and Maliga (1993) *EMBO J.* 12:601-606. The method relies on particle gun delivery of DNA containing a selectable marker and targeting of the DNA to the plastid genome through homologous recombination. Additionally, plastid transformation can be accomplished by transactivation of a silent plastid-borne transgene by tissue-preferred expression of a nuclear-encoded and plastid-directed RNA polymerase. Such a system has been reported in McBride *et al.* (1994) *Proc. Natl. Acad. Sci. USA* 91:7301-7305.

The cells that have been transformed may be grown into a transgenic organism, such as a plant, in accordance with conventional ways. See, for example, McCormick *et al.* (1986) *Plant Cell Reports* 5:81-84. These plants may then be grown, and either pollinated with the same transformed strain or different strains, and the resulting hybrid having constitutive expression of the desired phenotypic characteristic identified. Two or more generations may be grown to ensure that expression of the desired phenotypic characteristic is stably maintained and inherited and then seeds harvested to ensure expression of the desired phenotypic characteristic has been achieved. In this manner, the present invention provides transformed seed (also referred to as "transgenic seed") having a nucleotide construct of the invention, for example, an expression cassette of the invention, stably incorporated into their genome.

Alternatively, cells that have been transformed may be introduced into an organism. These cells could have originated from the organism, wherein the cells are transformed in an *ex vivo* approach.

The sequences provided herein may be used for transformation of any plant species, including, but not limited to, monocots and dicots. Examples of plants of interest include, but are not limited to, corn (maize), sorghum, wheat, sunflower, tomato, crucifers, peppers, potato, cotton, rice, soybean, sugarbeet, sugarcane, tobacco, barley, and oilseed rape, Brassica sp., alfalfa, rye, millet, safflower, peanuts, sweet potato, cassava, coffee, coconut, pineapple, citrus trees, cocoa, tea, banana, avocado, fig, guava, mango, olive, papaya, cashew, macadamia, almond, oats, vegetables, ornamentals, and conifers.

Vegetables include, but are not limited to, tomatoes, lettuce, green beans, lima beans, peas, and members of the genus Curcumins such as cucumber, cantaloupe, and musk melon. Ornamentals include, but are not limited to, azalea, hydrangea, hibiscus, roses, tulips, daffodils, petunias, carnation, poinsettia, and chrysanthemum. Preferably, plants of the present invention are crop plants (for example, maize, sorghum,

wheat, sunflower, tomato, crucifers, peppers, potato, cotton, rice, soybean, sugarbeet, sugarcane, tobacco, barley, oilseed rape, etc.).

As used herein, the term plant includes plant cells, plant protoplasts, plant cell tissue cultures from which plants can be regenerated, plant calli, plant clumps, and plant cells that are intact in plants or parts of plants such as embryos, pollen, ovules, seeds, leaves, flowers, branches, fruit, kernels, ears, cobs, husks, stalks, roots, root tips, anthers, and the like. Grain is intended to mean the mature seed produced by commercial growers for purposes other than growing or reproducing the species. Progeny, variants, and mutants of the regenerated plants are also included within the scope of the invention, provided that these parts comprise the introduced polynucleotides. Further provided is a processed plant product or byproduct that retains the sequences disclosed herein, including for example, soymeal.

The polynucleotides encoding the RGNs, crRNAs, and/or tracrRNAs can also be used to transform any prokaryotic species, including but not limited to, archaea and bacteria (*e.g.*, *Bacillus* sp., *Klebsiella* sp., *Streptomyces* sp., *Rhizobium* sp., *Escherichia* sp., *Pseudomonas* sp., *Salmonella* sp., *Shigella* sp., *Vibrio* sp., *Yersinia* sp., *Mycoplasma* sp., *Agrobacterium*, *Lactobacillus* sp.).

The polynucleotides encoding the RGNs, crRNAs, and/or tracrRNAs can be used to transform any eukaryotic species, including but not limited to animals (*e.g.*, mammals, insects, fish, birds, and reptiles), fungi, amoeba, algae, and yeast.

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 & Feigner, *TIBTECH* 11:211-217 (1993); Mitani & Caskey, *TIBTECH* 11:162-166 (1993); Dillon, *TIBTECH* 11:167-175 (1993); Miller, *Nature* 357:455-460 (1992); Van Brunt, *Biotechnology* 6(10): 1149-1154 (1988); Vigne, *Restorative Neurology and Neuroscience* 8:35-36 (1995); Kremer & Perricaudet, *British Medical Bulletin* 51(1):31-44 (1995); Haddada et al., in *Current Topics in Microbiology and Immunology*, Doerfler and Bohm (eds) (1995); and Yu et al., *Gene Therapy* 1:13-26 (1994).

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

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

The use of RNA or DNA viral based systems for the delivery of nucleic acids takes advantage of highly evolved processes for targeting a virus to specific cells in the body and trafficking the viral payload to the nucleus. Viral vectors can be administered directly to patients (in vivo) or they can be used to treat cells in vitro, and the modified cells may optionally be administered to patients (ex vivo). Conventional viral based systems could include retroviral, lentivirus, adenoviral, adeno-associated and herpes simplex virus vectors for gene transfer. Integration in the host genome is possible with the retrovirus, lentivirus, and adeno-associated virus gene transfer methods, often resulting in long term expression of the inserted transgene. Additionally, high transduction efficiencies have been observed in many different cell types and target tissues.

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

In applications where transient expression is preferred, adenoviral based systems may be used. Adenoviral based vectors are capable of very high transduction efficiency in many cell types and do not require cell division. With such vectors, high titer and levels of expression have been obtained. This vector can be produced in large quantities in a relatively simple system. Adeno-associated virus ("AAV") vectors may also be used to transduce cells with target nucleic acids, e.g., in the in vitro production of nucleic acids and peptides, and for in vivo and ex vivo gene therapy procedures (see, e.g., West et al., *Virology* 160:38-47 (1987); U.S. Pat. No. 4,797,368; WO 93/24641; Katin, *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. Viral.* 63:03822-3828 (1989). Packaging cells are typically used to

form virus particles that are capable of infecting a host cell. Such cells include 293 cells, which package adenovirus, and ψ J2 cells or PA317 cells, which package retrovirus.

Viral vectors used in gene therapy are usually generated by producing a cell line that packages a nucleic acid vector into a viral particle. The vectors typically contain the minimal viral sequences required for packaging and subsequent integration into a host, other viral sequences being replaced by an expression cassette for the polynucleotide(s) to be expressed. The missing viral functions are typically supplied in trans by the packaging cell line. For example, AAV vectors used in gene therapy typically only possess ITR sequences from the AAV genome which are required for packaging and integration into the host genome. Viral DNA is packaged in a cell line, which contains a helper plasmid encoding the other AAV genes, namely rep and cap, but lacking ITR sequences.

The cell line may also be infected with adenovirus as a helper. The helper virus promotes replication of the AAV vector and expression of AAV genes from the helper plasmid. The helper plasmid is not packaged in significant amounts due to a lack of ITR sequences. Contamination with adenovirus can be reduced by, e.g., heat treatment to which adenovirus is more sensitive than AAV. Additional methods for the delivery of nucleic acids to cells are known to those skilled in the art. See, for example, US20030087817, incorporated herein by reference.

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, HeLaS3, Huh1, Huh4, Huh7, HUVEC, HASMC, HEKn, HEKa, MiaPaCell, Panel, PC-3, TFI, CTLL-2, CIR, Rat6, CVI, RPTE, AIO, T24, 182, A375, ARH-77, Calul, SW480, SW620, SKOV3, SK-UT, CaCo2, P388DI, SEM-K2, WEHI-231, HB56, TIB55, lurkat, *145.01*, 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-I 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-L235010, CORL23/ 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, Hepalclc7, HL-60, HMEC, HT-29, lurkat, *1Y* cells, K562 cells, Ku812, KCL22, KGI, KYO1, LNCap, Ma-Mel 1-48, MC-38, MCF-7, MCF-10A, MDA-MB-231, MDA-MB-468, MDA-MB-435, MDCKII, MDCKII, 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, THPI 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) (Manassas, 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.

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.

IV. Variants and Fragments of Polypeptides and Polynucleotides

The present disclosure provides active variants and fragments of a naturally-occurring (*i.e.*, wild-type) RNA-guided nuclease, the amino acid sequence of which is set forth as SEQ ID NO: 1, 9, 16, 23, 30, 38, 46, 54, 61, 69, 75, 82, 89, 95, 103, 110, 117, 137, or 235, as well as active variants and fragments of naturally-occurring CRISPR repeats, such as the sequence set forth as SEQ ID NO: 2, 10, 17, 24, 31, 39, 47, 55, 62, 70, 76, 83, 90, 96, 104, 111, 118, 240, 273, or 287, and active variant and fragments of naturally-occurring tracrRNAs, such as the sequence set forth as SEQ ID NO: 3, 11, 18, 25, 32, 40, 48, 56, 63, 71, 77, 84, 91, 97, 105, 112, 119, 241, 274, or 286, and polynucleotides encoding the same.

While the activity of a variant or fragment may be altered compared to the polynucleotide or polypeptide of interest, the variant and fragment should retain the functionality of the polynucleotide or polypeptide of interest. For example, a variant or fragment may have increased activity, decreased activity, different spectrum of activity or any other alteration in activity when compared to the polynucleotide or polypeptide of interest.

Fragments and variants of naturally-occurring RGN polypeptides, such as those disclosed herein, will retain sequence-specific, RNA-guided DNA-binding activity. In particular embodiments, fragments and variants of naturally-occurring RGN polypeptides, such as those disclosed herein, will retain nuclease activity (single-stranded or double-stranded).

Fragments and variants of naturally-occurring CRISPR repeats, such as those disclosed herein, will retain the ability, when part of a guide RNA (comprising a tracrRNA), to bind to and guide an RNA-guided nuclease (complexed with the guide RNA) to a target nucleotide sequence in a sequence-specific manner.

Fragments and variants of naturally-occurring tracrRNAs, such as those disclosed herein, will retain the ability, when part of a guide RNA (comprising a CRISPR RNA), to guide an RNA-guided nuclease (complexed with the guide RNA) to a target nucleotide sequence in a sequence-specific manner.

The term “fragment” refers to a portion of a polynucleotide or polypeptide sequence of the invention. "Fragments" or "biologically active portions" include polynucleotides comprising a sufficient number of contiguous nucleotides to retain the biological activity (*i.e.*, binding to and directing an RGN in a sequence-specific manner to a target nucleotide sequence when comprised within a guideRNA).

5 "Fragments" or "biologically active portions" include polypeptides comprising a sufficient number of contiguous amino acid residues to retain the biological activity (*i.e.*, binding to a target nucleotide sequence in a sequence-specific manner when complexed with a guide RNA). Fragments of the RGN proteins include those that are shorter than the full-length sequences due to the use of an alternate downstream start site. A biologically active portion of an RGN protein can be a polypeptide that comprises, for example, 10, 25, 50,
 10 100, 150, 200, 250, 300, 350, 400, 450, 500, 550, 600, 650, 700, 750, 800, 850, 900, 950, 1000, 1050 or more contiguous amino acid residues of SEQ ID NO: 1, 9, 16, 23, 30, 38, 46, 54, 61, 69, 75, 82, 89, 95, 103, 110, 117, 137, or 235. Such biologically active portions can be prepared by recombinant techniques and evaluated for sequence-specific, RNA-guided DNA-binding activity. A biologically active fragment of a CRISPR repeat sequence can comprise at least 8 contiguous amino acids of SEQ ID NO: 2, 10, 17, 24, 31,
 15 39, 47, 55, 62, 70, 76, 83, 90, 96, 104, 111, 118, 240, 273, or 287. A biologically active portion of a CRISPR repeat sequence can be a polynucleotide that comprises, for example, 8, 9, 10, 11, 12, 13, 14, 15, 16, 17, 18, 19, or 20 contiguous nucleotides of SEQ ID NO: 2, 10, 17, 24, 31, 39, 47, 55, 62, 70, 76, 83, 90, 96, 104, 111, 118, 240, 273, or 287. A biologically active portion of a tracrRNA can be a polynucleotide that comprises, for example, 8, 9, 10, 11, 12, 13, 14, 15, 16, 17, 18, 19, 20, 21, 22, 23, 24, 25, 26, 27, 28, 29,
 20 30, 35, 40, 45, 50, 55, 60, 65, 70, 75, 80 or more contiguous nucleotides of SEQ ID NO: 3, 11, 18, 25, 32, 40, 48, 56, 63, 71, 77, 84, 91, 97, 105, 112, 119, 241, 274, or 286.

In general, "variants" is intended to mean substantially similar sequences. For polynucleotides, a variant comprises a deletion and/or addition of one or more nucleotides at one or more internal sites within the native polynucleotide and/or a substitution of one or more nucleotides at one or more sites in the native
 25 polynucleotide. As used herein, a "native" or “wild type” polynucleotide or polypeptide comprises a naturally occurring nucleotide sequence or amino acid sequence, respectively. For polynucleotides, conservative variants include those sequences that, because of the degeneracy of the genetic code, encode the native amino acid sequence of the gene of interest. Naturally occurring allelic variants such as these can be identified with the use of well-known molecular biology techniques, as, for example, with polymerase
 30 chain reaction (PCR) and hybridization techniques as outlined below. Variant polynucleotides also include synthetically derived polynucleotides, such as those generated, for example, by using site-directed mutagenesis but which still encode the polypeptide or the polynucleotide of interest. Generally, variants of a particular polynucleotide disclosed herein will have at least about 40%, 45%, 50%, 55%, 60%, 65%, 70%, 75%, 80%, 85%, 90%, 91%, 92%, 93%, 94%, 95%, 96%, 97%, 98%, 99% or more sequence identity to that
 35 particular polynucleotide as determined by sequence alignment programs and parameters described elsewhere herein.

Variants of a particular polynucleotide disclosed herein (*i.e.*, the reference polynucleotide) can also be evaluated by comparison of the percent sequence identity between the polypeptide encoded by a variant polynucleotide and the polypeptide encoded by the reference polynucleotide. Percent sequence identity between any two polypeptides can be calculated using sequence alignment programs and parameters described elsewhere herein. Where any given pair of polynucleotides disclosed herein is evaluated by comparison of the percent sequence identity shared by the two polypeptides they encode, the percent sequence identity between the two encoded polypeptides is at least about 40%, 45%, 50%, 55%, 60%, 65%, 70%, 75%, 80%, 85%, 90%, 91%, 92%, 93%, 94%, 95%, 96%, 97%, 98%, 99% or more sequence identity.

In particular embodiments, the presently disclosed polynucleotides encode an RNA-guided nuclease polypeptide comprising an amino acid sequence having at least 40%, 45%, 50%, 55%, 60%, 65%, 70%, 75%, 80%, 81%, 82%, 83%, 84%, 85%, 86%, 87%, 88%, 89%, 90%, 91%, 92%, 93%, 94%, 95%, 96%, 97%, 98%, 99%, or greater identity to an amino acid sequence of SEQ ID NO: 1, 9, 16, 23, 30, 38, 46, 54, 61, 69, 75, 82, 89, 95, 103, 110, 117, 137, or 235.

A biologically active variant of an RGN polypeptide of the invention may differ by as few as about 1-15 amino acid residues, as few as about 1-10, such as about 6-10, as few as 5, as few as 4, as few as 3, as few as 2, or as few as 1 amino acid residue. In specific embodiments, the polypeptides can comprise an N-terminal or a C-terminal truncation, which can comprise at least a deletion of 10, 15, 20, 25, 30, 35, 40, 45, 50, 55, 60, 65, 70, 75, 80, 85, 90, 95, 100, 150, 200, 250, 300, 350, 400, 450, 500, 550, 600, 650, 700, 750, 800, 850, 900, 950, 1000, 1050 amino acids or more from either the N or C terminus of the polypeptide.

In certain embodiments, the presently disclosed polynucleotides comprise or encode a CRISPR repeat comprising a nucleotide sequence having at least 40%, 45%, 50%, 55%, 60%, 65%, 70%, 75%, 80%, 81%, 82%, 83%, 84%, 85%, 86%, 87%, 88%, 89%, 90%, 91%, 92%, 93%, 94%, 95%, 96%, 97%, 98%, 99%, or greater identity to the nucleotide sequence set forth as SEQ ID NO: 2, 10, 17, 24, 31, 39, 47, 55, 62, 70, 76, 83, 90, 96, 104, 111, 118, 240, 273, or 287.

The presently disclosed polynucleotides can comprise or encode a tracrRNA comprising a nucleotide sequence having at least 40%, 45%, 50%, 55%, 60%, 65%, 70%, 75%, 80%, 81%, 82%, 83%, 84%, 85%, 86%, 87%, 88%, 89%, 90%, 91%, 92%, 93%, 94%, 95%, 96%, 97%, 98%, 99%, or greater identity to the nucleotide sequence set forth as SEQ ID NO: 3, 11, 18, 25, 32, 40, 48, 56, 63, 71, 77, 84, 91, 97, 105, 112, 119, 241, 274, or 286.

Biologically active variants of a CRISPR repeat or tracrRNA of the invention may differ by as few as about 1-15 nucleotides, as few as about 1-10, such as about 6-10, as few as 5, as few as 4, as few as 3, as few as 2, or as few as 1 nucleotide. In specific embodiments, the polynucleotides can comprise a 5' or 3' truncation, which can comprise at least a deletion of 10, 15, 20, 25, 30, 35, 40, 45, 50, 55, 60, 65, 70, 75, 80 nucleotides or more from either the 5' or 3' end of the polynucleotide.

It is recognized that modifications may be made to the RGN polypeptides, CRISPR repeats, and tracrRNAs provided herein creating variant proteins and polynucleotides. Changes designed by man may be introduced through the application of site-directed mutagenesis techniques. Alternatively, native, as yet-

unknown or as yet unidentified polynucleotides and/or polypeptides structurally and/or functionally-related to the sequences disclosed herein may also be identified that fall within the scope of the present invention. Conservative amino acid substitutions may be made in nonconserved regions that do not alter the function of the RGN proteins. Alternatively, modifications may be made that improve the activity of the RGN.

5 Variant polynucleotides and proteins also encompass sequences and proteins derived from a mutagenic and recombinogenic procedure such as DNA shuffling. With such a procedure, one or more different RGN proteins disclosed herein (e.g., SEQ ID NO: 1, 9, 16, 23, 30, 38, 46, 54, 61, 69, 75, 82, 89, 95, 103, 110, 117, 137, or 235) is manipulated to create a new RGN protein possessing the desired properties. In this manner, libraries of recombinant polynucleotides are generated from a population of
10 related sequence polynucleotides comprising sequence regions that have substantial sequence identity and can be homologously recombined *in vitro* or *in vivo*. For example, using this approach, sequence motifs encoding a domain of interest may be shuffled between the RGN sequences provided herein and other known RGN genes to obtain a new gene coding for a protein with an improved property of interest, such as an increased K_m in the case of an enzyme. Strategies for such DNA shuffling are known in the art. See, for
15 example, Stemmer (1994) *Proc. Natl. Acad. Sci. USA* 91:10747-10751; Stemmer (1994) *Nature* 370:389-391; Cramer *et al.* (1997) *Nature Biotech.* 15:436-438; Moore *et al.* (1997) *J. Mol. Biol.* 272:336-347; Zhang *et al.* (1997) *Proc. Natl. Acad. Sci. USA* 94:4504-4509; Cramer *et al.* (1998) *Nature* 391:288-291; and U.S. Patent Nos. 5,605,793 and 5,837,458. A "shuffled" nucleic acid is a nucleic acid produced by a shuffling procedure such as any shuffling procedure set forth herein. Shuffled nucleic acids are produced by
20 recombining (physically or virtually) two or more nucleic acids (or character strings), for example in an artificial, and optionally recursive, fashion. Generally, one or more screening steps are used in shuffling processes to identify nucleic acids of interest; this screening step can be performed before or after any recombination step. In some (but not all) shuffling embodiments, it is desirable to perform multiple rounds of recombination prior to selection to increase the diversity of the pool to be screened. The overall process of
25 recombination and selection are optionally repeated recursively. Depending on context, shuffling can refer to an overall process of recombination and selection, or, alternately, can simply refer to the recombinational portions of the overall process.

As used herein, "sequence identity" or "identity" in the context of two polynucleotides or polypeptide sequences makes reference to the residues in the two sequences that are the same when aligned
30 for maximum correspondence over a specified comparison window. When percentage of sequence identity is used in reference to proteins it is recognized that residue positions which are not identical often differ by conservative amino acid substitutions, where amino acid residues are substituted for other amino acid residues with similar chemical properties (e.g., charge or hydrophobicity) and therefore do not change the functional properties of the molecule. When sequences differ in conservative substitutions, the percent
35 sequence identity may be adjusted upwards to correct for the conservative nature of the substitution. Sequences that differ by such conservative substitutions are said to have "sequence similarity" or "similarity". Means for making this adjustment are well known to those of skill in the art. Typically, this

involves scoring a conservative substitution as a partial rather than a full mismatch, thereby increasing the percentage sequence identity. Thus, for example, where an identical amino acid is given a score of 1 and a non-conservative substitution is given a score of zero, a conservative substitution is given a score between zero and 1. The scoring of conservative substitutions is calculated, *e.g.*, as implemented in the program
5 PC/GENE (Intelligenetics, Mountain View, California).

As used herein, "percentage of sequence identity" means the value determined by comparing two optimally aligned sequences over a comparison window, wherein the portion of the polynucleotide sequence in the comparison window may comprise additions or deletions (*i.e.*, gaps) as compared to the reference sequence (which does not comprise additions or deletions) for optimal alignment of the two sequences. The
10 percentage is calculated by determining the number of positions at which the identical nucleic acid base or amino acid residue occurs in both sequences to yield the number of matched positions, dividing the number of matched positions by the total number of positions in the window of comparison, and multiplying the result by 100 to yield the percentage of sequence identity.

Unless otherwise stated, sequence identity/similarity values provided herein refer to the value
15 obtained using GAP Version 10 using the following parameters: % identity and % similarity for a nucleotide sequence using GAP Weight of 50 and Length Weight of 3, and the nwsgapdna.cmp scoring matrix; % identity and % similarity for an amino acid sequence using GAP Weight of 8 and Length Weight of 2, and the BLOSUM62 scoring matrix; or any equivalent program thereof. By "equivalent program" is intended any sequence comparison program that, for any two sequences in question, generates an alignment having
20 identical nucleotide or amino acid residue matches and an identical percent sequence identity when compared to the corresponding alignment generated by GAP Version 10.

Two sequences are "optimally aligned" when they are aligned for similarity scoring using a defined amino acid substitution matrix (*e.g.*, BLOSUM62), gap existence penalty and gap extension penalty so as to arrive at the highest score possible for that pair of sequences. Amino acid substitution matrices and their use
25 in quantifying the similarity between two sequences are well-known in the art and described, *e.g.*, in Dayhoff et al. (1978) "A model of evolutionary change in proteins." In "Atlas of Protein Sequence and Structure," Vol. 5, Suppl. 3 (ed. M. O. Dayhoff), pp. 345-352. Natl. Biomed. Res. Found., Washington, D.C. and Henikoff et al. (1992) Proc. Natl. Acad. Sci. USA 89:10915-10919. The BLOSUM62 matrix is often used as a default scoring substitution matrix in sequence alignment protocols. The gap existence penalty is
30 imposed for the introduction of a single amino acid gap in one of the aligned sequences, and the gap extension penalty is imposed for each additional empty amino acid position inserted into an already opened gap. The alignment is defined by the amino acids positions of each sequence at which the alignment begins and ends, and optionally by the insertion of a gap or multiple gaps in one or both sequences, so as to arrive at the highest possible score. While optimal alignment and scoring can be accomplished manually, the process
35 is facilitated by the use of a computer-implemented alignment algorithm, *e.g.*, gapped BLAST 2.0, described in Altschul et al. (1997) *Nucleic Acids Res.* 25:3389-3402, and made available to the public at the National Center for Biotechnology Information Website (www.ncbi.nlm.nih.gov). Optimal alignments, including

multiple alignments, can be prepared using, *e.g.*, PSI-BLAST, available through www.ncbi.nlm.nih.gov and described by Altschul *et al.* (1997) *Nucleic Acids Res.* 25:3389-3402.

With respect to an amino acid sequence that is optimally aligned with a reference sequence, an amino acid residue "corresponds to" the position in the reference sequence with which the residue is paired in the alignment. The "position" is denoted by a number that sequentially identifies each amino acid in the reference sequence based on its position relative to the N-terminus. Owing to deletions, insertion, truncations, fusions, etc., that must be taken into account when determining an optimal alignment, in general the amino acid residue number in a test sequence as determined by simply counting from the N-terminal will not necessarily be the same as the number of its corresponding position in the reference sequence. For example, in a case where there is a deletion in an aligned test sequence, there will be no amino acid that corresponds to a position in the reference sequence at the site of deletion. Where there is an insertion in an aligned reference sequence, that insertion will not correspond to any amino acid position in the reference sequence. In the case of truncations or fusions there can be stretches of amino acids in either the reference or aligned sequence that do not correspond to any amino acid in the corresponding sequence.

V. Antibodies

Antibodies to the RGN polypeptides or ribonucleoproteins comprising the RGN polypeptides of the present invention, including those having the amino acid sequence set forth as SEQ ID NO: 1, 9, 16, 23, 30, 38, 46, 54, 61, 69, 75, 82, 89, 95, 103, 110, or 117 or active variants or fragments thereof, are also encompassed. Methods for producing antibodies are well known in the art (see, for example, Harlow and Lane (1988) *Antibodies: A Laboratory Manual*, Cold Spring Harbor Laboratory, Cold Spring Harbor, N.Y.; and U.S. Pat. No. 4,196,265). These antibodies can be used in kits for the detection and isolation of RGN polypeptides or ribonucleoproteins. Thus, this disclosure provides kits comprising antibodies that specifically bind to the polypeptides or ribonucleoproteins described herein, including, for example, polypeptides having the sequence of SEQ ID NO: 1, 9, 16, 23, 30, 38, 46, 54, 61, 69, 75, 82, 89, 95, 103, 110, or 117.

VI. Systems and Ribonucleoprotein Complexes for Binding a Target Sequence of Interest and Methods of Making the Same

The present disclosure provides a system for binding a target sequence of interest, wherein the system comprises at least one guide RNA or a nucleotide sequence encoding the same, and at least one RNA-guided nuclease or a nucleotide sequence encoding the same. The guide RNA hybridizes to the target sequence of interest and also forms a complex with the RGN polypeptide, thereby directing the RGN polypeptide to bind to the target sequence. In some of these embodiments, the RGN comprises an amino acid sequence of SEQ ID NO: 1, 9, 16, 23, 30, 38, 46, 54, 61, 69, 75, 82, 89, 95, 103, 110, 117, 137, or 235, or an active variant or fragment thereof. In various embodiments, the guide RNA comprises a CRISPR repeat sequence comprising the nucleotide sequence of SEQ ID NO: 2, 10, 17, 24, 31, 39, 47, 55, 62, 70, 76,

83, 90, 96, 104, 111, 118, 240, 273, or 287, or an active variant or fragment thereof. In particular embodiments, the guide RNA comprises a tracrRNA comprising a nucleotide sequence of SEQ ID NO: 3, 11, 18, 25, 32, 40, 48, 56, 63, 71, 77, 84, 91, 97, 105, 112, 119, 241, 274, or 286, or an active variant or fragment thereof. The guide RNA of the system can be a single guide RNA or a dual-guide RNA. In particular embodiments, the system comprises a RNA-guided nuclease that is heterologous to the guideRNA, wherein the RGN and guideRNA are not naturally complexed in nature.

The system for binding a target sequence of interest provided herein can be a ribonucleoprotein complex, which is at least one molecule of an RNA bound to at least one protein. The ribonucleoprotein complexes provided herein comprise at least one guide RNA as the RNA component and an RNA-guided nuclease as the protein component. Such ribonucleoprotein complexes can be purified from a cell or organism that naturally expresses an RGN polypeptide and has been engineered to express a particular guide RNA that is specific for a target sequence of interest. Alternatively, the ribonucleoprotein complex can be purified from a cell or organism that has been transformed with polynucleotides that encode an RGN polypeptide and a guide RNA and cultured under conditions to allow for the expression of the RGN polypeptide and guide RNA. Thus, methods are provided for making an RGN polypeptide or an RGN ribonucleoprotein complex. Such methods comprise culturing a cell comprising a nucleotide sequence encoding an RGN polypeptide, and in some embodiments a nucleotide sequence encoding a guide RNA, under conditions in which the RGN polypeptide (and in some embodiments, the guide RNA) is expressed. The RGN polypeptide or RGN ribonucleoprotein can then be purified from a lysate of the cultured cells.

Methods for purifying an RGN polypeptide or RGN ribonucleoprotein complex from a lysate of a biological sample are known in the art (*e.g.*, size exclusion and/or affinity chromatography, 2D-PAGE, HPLC, reversed-phase chromatography, immunoprecipitation). In particular methods, the RGN polypeptide is recombinantly produced and comprises a purification tag to aid in its purification, including but not limited to, glutathione-S-transferase (GST), chitin binding protein (CBP), maltose binding protein, thioredoxin (TRX), poly(NANP), tandem affinity purification (TAP) tag, myc, AcV5, AU1, AU5, E, ECS, E2, FLAG, HA, nus, Softag 1, Softag 3, Strep, SBP, Glu-Glu, HSV, KT3, S, S1, T7, V5, VSV-G, 6xHis, 10xHis, biotin carboxyl carrier protein (BCCP), and calmodulin. Generally, the tagged RGN polypeptide or RGN ribonucleoprotein complex is purified using immobilized metal affinity chromatography. It will be appreciated that other similar methods known in the art may be used, including other forms of chromatography or for example immunoprecipitation, either alone or in combination.

An "isolated" or "purified" polypeptide, or biologically active portion thereof, is substantially or essentially free from components that normally accompany or interact with the polypeptide as found in its naturally occurring environment. Thus, an isolated or purified polypeptide is substantially free of other cellular material, or culture medium when produced by recombinant techniques, or substantially free of chemical precursors or other chemicals when chemically synthesized. A protein that is substantially free of cellular material includes preparations of protein having less than about 30%, 20%, 10%, 5%, or 1% (by dry weight) of contaminating protein. When the protein of the invention or biologically active portion thereof is

recombinantly produced, optimally culture medium represents less than about 30%, 20%, 10%, 5%, or 1% (by dry weight) of chemical precursors or non-protein-of-interest chemicals.

Particular methods provided herein for binding and/or cleaving a target sequence of interest involve the use of an *in vitro* assembled RGN ribonucleoprotein complex. *In vitro* assembly of an RGN
 5 ribonucleoprotein complex can be performed using any method known in the art in which an RGN polypeptide is contacted with a guide RNA under conditions to allow for binding of the RGN polypeptide to the guide RNA. As used herein, "contact", "contacting", "contacted," refer to placing the components of a desired reaction together under conditions suitable for carrying out the desired reaction. The RGN
 10 polypeptide can be purified from a biological sample, cell lysate, or culture medium, produced via *in vitro* translation, or chemically synthesized. The guide RNA can be purified from a biological sample, cell lysate, or culture medium, transcribed *in vitro*, or chemically synthesized. The RGN polypeptide and guide RNA can be brought into contact in solution (*e.g.*, buffered saline solution) to allow for *in vitro* assembly of the RGN ribonucleoprotein complex.

VII. Methods of Binding, Cleaving, or Modifying a Target Sequence

The present disclosure provides methods for binding, cleaving, and/or modifying a target nucleotide sequence of interest. The methods include delivering a system comprising at least one guide RNA or a polynucleotide encoding the same, and at least one RGN polypeptide or a polynucleotide encoding the same to the target sequence or a cell, organelle, or embryo comprising the target sequence. In some of these
 20 embodiments, the RGN comprises the amino acid sequence of SEQ ID NO: 1, 9, 16, 23, 30, 38, 46, 54, 61, 69, 75, 82, 89, 95, 103, 110, 117, 137, or 235, or an active variant or fragment thereof. In various embodiments, the guide RNA comprises a CRISPR repeat sequence comprising the nucleotide sequence of SEQ ID NO: 2, 10, 17, 24, 31, 39, 47, 55, 62, 70, 76, 83, 90, 96, 104, 111, 118, 240, 273, or 287, or an active variant or fragment thereof. In particular embodiments, the guide RNA comprises a tracrRNA
 25 comprising the nucleotide sequence of SEQ ID NO: 3, 11, 18, 25, 32, 40, 48, 56, 63, 71, 77, 84, 91, 97, 105, 112, 119, 241, 274, or 286, or an active variant or fragment thereof. The guide RNA of the system can be a single guide RNA or a dual-guide RNA. The RGN of the system may be nuclease dead RGN, have nickase activity, or may be a fusion polypeptide. In some embodiments, the fusion polypeptide comprises a base-editing polypeptide, for example a cytidine deaminase or an adenosine deaminase. In other embodiments,
 30 the RGN fusion protein comprises a reverse transcriptase. In other embodiments, the RGN fusion protein comprises a polypeptide that recruits members of a functional nucleic acid repair complex, such as a member of the nucleotide excision repair (NER) or transcription coupled-nucleotide excision repair (TC-NER) pathway (Wei *et al.*, 2015, *PNAS USA* 112(27):E3495-504 ; Troelstra *et al.*, 1992, *Cell* 71:939-953; Marnef *et al.*, 2017, *J Mol Biol* 429(9):1277-1288), as described in U.S. Provisional Application No.
 35 62/966,203, which was filed on January 27, 2020, and is incorporated by reference in its entirety. In some embodiments, the RGN fusion protein comprises CSB (van den Boom *et al.*, 2004, *J Cell Biol* 166(1):27-36; van Gool *et al.*, 1997, *EMBO J* 16(19):5955-65; an example of which is set forth as

SEQ ID NO: 268), which is a member of the TC-NER (nucleotide excision repair) pathway and functions in the recruitment of other members. In further embodiments, the RGNABP fusion protein comprises an active domain of CSB, such as the acidic domain of CSB which comprises amino acid residues 356-394 of SEQ ID NO: 268 (Teng *et al.*, 2018, *Nat Commun* 9(1):4115).

5 In particular embodiments, the RGN and/or guide RNA is heterologous to the cell, organelle, or embryo to which the RGN and/or guide RNA (or polynucleotide(s) encoding at least one of the RGN and guide RNA) are introduced.

10 In those embodiments wherein the method comprises delivering a polynucleotide encoding a guide RNA and/or an RGN polypeptide, the cell or embryo can then be cultured under conditions in which the guide RNA and/or RGN polypeptide are expressed. In various embodiments, the method comprises contacting a target sequence with an RGN ribonucleoprotein complex. The RGN ribonucleoprotein complex may comprise an RGN that is nuclease dead or has nickase activity. In some embodiments, the RGN of the ribonucleoprotein complex is a fusion polypeptide comprising a base-editing polypeptide. In certain
15 embodiments, the method comprises introducing into a cell, organelle, or embryo comprising a target sequence an RGN ribonucleoprotein complex. The RGN ribonucleoprotein complex can be one that has been purified from a biological sample, recombinantly produced and subsequently purified, or *in vitro*-assembled as described herein. In those embodiments wherein the RGN ribonucleoprotein complex that is contacted with the target sequence or a cell organelle, or embryo has been assembled *in vitro*, the method can further comprise the *in vitro* assembly of the complex prior to contact with the target sequence, cell,
20 organelle, or embryo.

A purified or *in vitro* assembled RGN ribonucleoprotein complex can be introduced into a cell, organelle, or embryo using any method known in the art, including, but not limited to electroporation. Alternatively, an RGN polypeptide and/or polynucleotide encoding or comprising the guide RNA can be introduced into a cell, organelle, or embryo using any method known in the art (*e.g.*, electroporation).

25 Upon delivery to or contact with the target sequence or cell, organelle, or embryo comprising the target sequence, the guide RNA directs the RGN to bind to the target sequence in a sequence-specific manner. In those embodiments wherein the RGN has nuclease activity, the RGN polypeptide cleaves the target sequence of interest upon binding. The target sequence can subsequently be modified via endogenous repair mechanisms, such as non-homologous end joining, or homology-directed repair with a provided donor
30 polynucleotide.

Methods to measure binding of an RGN polypeptide to a target sequence are known in the art and include chromatin immunoprecipitation assays, gel mobility shift assays, DNA pull-down assays, reporter assays, microplate capture and detection assays. Likewise, methods to measure cleavage or modification of a target sequence are known in the art and include *in vitro* or *in vivo* cleavage assays wherein cleavage is
35 confirmed using PCR, sequencing, or gel electrophoresis, with or without the attachment of an appropriate label (*e.g.*, radioisotope, fluorescent substance) to the target sequence to facilitate detection of degradation products. Alternatively, the nicking triggered exponential amplification reaction (NTEXPAR) assay can be

used (see, *e.g.*, Zhang et al. (2016) *Chem. Sci.* 7:4951-4957). *In vivo* cleavage can be evaluated using the Surveyor assay (Guschin et al. (2010) *Methods Mol Biol* 649:247-256).

In some embodiments, the methods involve the use of a single type of RGN complexed with more than one guide RNA. The more than one guide RNA can target different regions of a single gene or can
5 target multiple genes.

In those embodiments wherein a donor polynucleotide is not provided, a double-stranded break introduced by an RGN polypeptide can be repaired by a non-homologous end-joining (NHEJ) repair process. Due to the error-prone nature of NHEJ, repair of the double-stranded break can result in a modification to the target sequence. As used herein, a “modification” in reference to a nucleic acid molecule
10 refers to a change in the nucleotide sequence of the nucleic acid molecule, which can be a deletion, insertion, or substitution of one or more nucleotides, or a combination thereof. Modification of the target sequence can result in the expression of an altered protein product or inactivation of a coding sequence.

In those embodiments wherein a donor polynucleotide is present, the donor sequence in the donor polynucleotide can be integrated into or exchanged with the target nucleotide sequence during the course of
15 repair of the introduced double-stranded break, resulting in the introduction of the exogenous donor sequence. A donor polynucleotide thus comprises a donor sequence that is desired to be introduced into a target sequence of interest. In some embodiments, the donor sequence alters the original target nucleotide sequence such that the newly integrated donor sequence will not be recognized and cleaved by the RGN. Integration of the donor sequence can be enhanced by the inclusion within the donor polynucleotide of
20 flanking sequences that have substantial sequence identity with the sequences flanking the target nucleotide sequence, allowing for a homology-directed repair process. In those embodiments wherein the RGN polypeptide introduces double-stranded staggered breaks, the donor polynucleotide can comprise a donor sequence flanked by compatible overhangs, allowing for direct ligation of the donor sequence to the cleaved target nucleotide sequence comprising overhangs by a non-homologous repair process during repair of the
25 double-stranded break.

In those embodiments wherein the method involves the use of an RGN that is a nickase (*i.e.*, is only able to cleave a single strand of a double-stranded polynucleotide), the method can comprise introducing two RGN nickases that target identical or overlapping target sequences and cleave different strands of the polynucleotide. For example, an RGN nickase that only cleaves the positive (+) strand of a double-stranded
30 polynucleotide can be introduced along with a second RGN nickase that only cleaves the negative (-) strand of a double-stranded polynucleotide.

In various embodiments, a method is provided for binding a target nucleotide sequence and detecting the target sequence, wherein the method comprises introducing into a cell, organelle, or embryo at least one guide RNA or a polynucleotide encoding the same, and at least one RGN polypeptide or a
35 polynucleotide encoding the same, expressing the guide RNA and/or RGN polypeptide (if coding sequences are introduced), wherein the RGN polypeptide is a nuclease-dead RGN and further comprises a detectable label, and the method further comprises detecting the detectable label. The detectable label may be fused to

the RGN as a fusion protein (*e.g.*, fluorescent protein) or may be a small molecule conjugated to or incorporated within the RGN polypeptide that can be detected visually or by other means.

Also provided herein are methods for modulating the expression of a target sequence or a gene of interest under the regulation of a target sequence. The methods comprise introducing into a cell, organelle, or embryo at least one guide RNA or a polynucleotide encoding the same, and at least one RGN polypeptide or a polynucleotide encoding the same, expressing the guide RNA and/or RGN polypeptide (if coding sequences are introduced), wherein the RGN polypeptide is a nuclease-dead RGN. In some of these embodiments, the nuclease-dead RGN is a fusion protein comprising an expression modulator domain (*i.e.*, epigenetic modification domain, transcriptional activation domain or a transcriptional repressor domain) as described herein.

The present disclosure also provides methods for binding and/or modifying a target nucleotide sequence of interest. The methods include delivering a system comprising at least one guide RNA or a polynucleotide encoding the same, and at least one fusion polypeptide comprises an RGN of the invention and a base-editing polypeptide, for example a cytidine deaminase or an adenosine deaminase, or a polynucleotide encoding the fusion polypeptide, to the target sequence or a cell, organelle, or embryo comprising the target sequence.

One of ordinary skill in the art will appreciate that any of the presently disclosed methods can be used to target a single target sequence or multiple target sequences. Thus, methods comprise the use of a single RGN polypeptide in combination with multiple, distinct guide RNAs, which can target multiple, distinct sequences within a single gene and/or multiple genes. Also encompassed herein are methods wherein multiple, distinct guide RNAs are introduced in combination with multiple, distinct RGN polypeptides. These guide RNAs and guide RNA/RGN polypeptide systems can target multiple, distinct sequences within a single gene and/or multiple genes.

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

In some embodiments, the kit includes instructions in one or more languages. 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 5 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 one aspect, the invention provides methods for using one or more elements of a CRISPR system. The CRISPR complex 10 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, base editing) 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 15 complexed with a guide sequence hybridized to a target sequence within the target polynucleotide.

VIII. Target polynucleotides

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 20 sampling a cell or population of cells from a human or non-human animal or plant (including microalgae) and modifying the cell or cells. Culturing may occur at any stage ex vivo. The cell or cells may even be re-introduced into the non-human animal or plant (including micro-algae).

Using natural variability, plant breeders combine most useful genes for desirable qualities, such as yield, quality, uniformity, hardiness, and resistance against pests. These desirable qualities also include 25 growth, day length preferences, temperature requirements, initiation date of floral or reproductive development, fatty acid content, insect resistance, disease resistance, nematode resistance, fungal resistance, herbicide resistance, tolerance to various environmental factors including drought, heat, wet, cold, wind, and adverse soil conditions including high salinity. The sources of these useful genes include native or foreign varieties, heirloom varieties, wild plant relatives, and induced mutations, e.g., treating plant material with 30 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 for sources of useful genes, and in varieties having desired characteristics or traits employ the present invention to induce the rise of useful genes, with more precision than previous mutagenic agents and hence accelerate and improve plant breeding programs.

35 The target polynucleotide of an RGN system 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).

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. 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 (e.g., a causal mutation). The transcribed or translated products may be known or unknown, and further may be at a normal or abnormal level. Examples of disease-associated genes and polynucleotides are available from McKusick-Nathans Institute of Genetic Medicine, Johns Hopkins University (Baltimore, Md.) and National Center for Biotechnology Information, National Library of Medicine (Bethesda, Md.), available on the World Wide Web.

Although CRISPR systems are particularly useful for their relative ease in targeting to genomic sequences of interest, there still remains an issue of what the RGN can do to address a causal mutation. One approach is to produce a fusion protein between an RGN (preferably an inactive or nickase variant of the RGN) and a base-editing enzyme or the active domain of a base editing enzyme, such as a cytidine deaminase or an adenosine deaminase base editor (U.S. Patent No. 9,840,699, herein incorporated by reference). In some embodiments, the methods comprise contacting a DNA molecule with (a) a fusion protein comprising an RGN of the invention and a base-editing polypeptide such as a deaminase; and (b) a gRNA targeting the fusion protein of (a) to a target nucleotide sequence of the DNA strand; wherein the DNA molecule is contacted with the fusion protein and the gRNA in an amount effective and under conditions suitable for the deamination of a nucleotide base. In some embodiments, the target DNA sequence comprises a sequence associated with a disease or disorder, and wherein the deamination of the nucleotide base results in a sequence that is not associated with a disease or disorder. In some embodiments, the target DNA sequence resides in an allele of a crop plant, wherein the particular allele of the trait of interest results in a plant of lesser agronomic value. The deamination of the nucleotide base results in an allele that improves the trait and increases the agronomic value of the plant.

In some embodiments, the DNA sequence comprises a T→C or A→G point mutation associated with a disease or disorder, and wherein the deamination of the mutant C or G base results in a sequence that is not associated with a disease or disorder. In some embodiments, the deamination corrects a point mutation in the sequence associated with the disease or disorder.

5 In some embodiments, the sequence associated with the disease or disorder encodes a protein, and wherein the deamination introduces a stop codon into the sequence associated with the disease or disorder, resulting in a truncation of the encoded protein. In some embodiments, the contacting is performed *in vivo* in a subject susceptible to having, having, or diagnosed with the disease or disorder. In some embodiments, the disease or disorder is a disease associated with a point mutation, or a single-base mutation, in the
10 genome. In some embodiments, the disease is a genetic disease, a cancer, a metabolic disease, or a lysosomal storage disease.

Modifying causal mutations using base-editing

An example of a genetically inherited disease which could be corrected using an approach that relies
15 on an RGN-base editor fusion protein of the invention is Hurler Syndrome. Hurler Syndrome, also known as MPS-1, is the result of a deficiency of α -L-iduronidase (IDUA) resulting in a lysosomal storage disease characterized at the molecular level by the accumulation of dermatan sulfate and heparan sulfate in lysosomes. This disease is generally an inherited genetic disorder caused by mutations in the IDUA gene encoding α -L-iduronidase. Common IDUA mutations are W402X and Q70X, both nonsense mutations
20 resulting in premature termination of translation. Such mutations are well addressed by precise genome editing (PGE) approaches, since reversion of a single nucleotide, for example by a base-editing approach, would restore the wild-type coding sequence and result in protein expression controlled by the endogenous regulatory mechanisms of the genetic locus. Additionally, since heterozygotes are known to be asymptomatic, a PGE therapy that targets one of these mutations would be useful to a large proportion of
25 patients with this disease, as only one of the mutated alleles needs to be corrected (Bunge et al. (1994) Hum. Mol. Genet. 3(6): 861-866, herein incorporated by reference).

Current treatments for Hurler Syndrome include enzyme replacement therapy and bone marrow transplants (Vellodi et al. (1997) Arch. Dis. Child. 76(2): 92-99; Peters et al. (1998) Blood 91(7): 2601-2608, herein incorporated by reference). While enzyme replacement therapy has had a dramatic effect on
30 the survival and quality of life of Hurler Syndrome patients, this approach requires costly and time-consuming weekly infusions. Additional approaches include the delivery of the IDUA gene on an expression vector or the insertion of the gene into a highly expressed locus such as that of serum albumin (U.S. Patent No. 9,956,247, herein incorporated by reference). However, these approaches do not restore the original IDUA locus to the correct coding sequence. A genome-editing strategy would have a number of
35 advantages, most notably that regulation of gene expression would be controlled by the natural mechanisms present in healthy individuals. Additionally, using base editing does not necessitate causing a double stranded DNA breaks, which could lead to large chromosomal rearrangements, cell death, or oncogenicity

by the disruption of tumor suppression mechanisms. A general strategy may be directed toward using RGN-base editor fusion proteins of the invention to target and correct certain disease-causing mutations in the human genome. It will be appreciated that similar approaches to target diseases that can be corrected by base-editing may also be pursued. It will be further appreciated that similar approaches to target disease-causing mutations in other species, particularly common household pets or livestock, can also be deployed using the RGNs of the invention. Common household pets and livestock include dogs, cats, horses, pigs, cows, sheep, chickens, donkeys, snakes, ferrets, and fish including salmon and shrimp.

Modifying causal mutations by targeted deletion

RGNs of the invention could also be useful in human therapeutic approaches where the causal mutation is more complicated. For example, some diseases such as Friedreich's Ataxia and Huntington's Disease are the result of a significant increase in repeats of a three nucleotide motif at a particular region of a gene, which affects the ability of the expressed protein to function or to be expressed. Friedreich's Ataxia (FRDA) is an autosomal recessive disease resulting in progressive degeneration of nervous tissue in the spinal cord. Reduced levels of the frataxin (FXN) protein in the mitochondria cause oxidative damages and iron deficiencies at the cellular level. The reduced FXN expression has been linked to a GAA triplet expansion within the intron 1 of the somatic and germline FXN gene. In FRDA patients, the GAA repeat frequently consists of more than 70, sometimes even more than 1000 (most commonly 600-900) triplets, whereas unaffected individuals have about 40 repeats or less (Pandolfo et al. (2012) Handbook of Clinical Neurology 103: 275-294; Campuzano et al. (1996) Science 271: 1423-1427; Pandolfo (2002) Adv. Exp. Med. Biol. 516: 99-118; all herein incorporated by reference).

The expansion of the trinucleotide repeat sequence causing Friedreich's Ataxia (FRDA) occurs in a defined genetic locus within the FXN gene, referred to as the FRDA instability region. RNA guided nucleases (RGNs) may be used for excising the instability region in FRDA patient cells. This approach requires 1) an RGN and guide RNA sequence that can be programmed to target the allele in the human genome; and 2) a delivery approach for the RGN and guide sequence. Many nucleases used for genome editing, such as the commonly used Cas9 nuclease from *S. pyogenes* (SpCas9), are too large to be packaged into adeno-associated viral (AAV) vectors, especially when considering the length of the SpCas9 gene and the guide RNA in addition to other genetic elements required for functional expression cassettes. This makes an approach using SpCas9 more difficult.

Certain RNA guided nucleases of the invention are well suited for packaging into an AAV vector along with a guide RNA. Packing two guide RNAs would likely require a second vector, but this approach still compares favorably to what would be required of a larger nuclease such as SpCas9, which may require splitting the protein sequence between two vectors. The present invention encompasses a strategy using RGNs of the invention in which a region of genomic instability is removed. Such a strategy is applicable to other diseases and disorders which have a similar genetic basis, such as Huntington's Disease. Similar strategies using RGNs of the invention may also be applicable to similar diseases and disorders in non-

human animals of agronomic or economic importance, including dogs, cats, horses, pigs, cows, sheep, chickens, donkeys, snakes, ferrets, and fish including salmon and shrimp.

Modifying causal mutations by targeted mutagenesis

5 RGNs of the invention could also be to introduce disruptive mutations that may result in a beneficial effect. Genetic defects in the genes encoding hemoglobin, particularly the beta globin chain (the HBB gene), can be responsible for a number of diseases known as hemoglobinopathies, including sickle cell anemia and thalassemias.

10 In adult humans, hemoglobin is a heterotetramer comprising two alpha (α)-like globin chains and two beta (β)-like globin chains and 4 heme groups. In adults the $\alpha_2\beta_2$ tetramer is referred to as Hemoglobin A (HbA) or adult hemoglobin. Typically, the alpha and beta globin chains are synthesized in an approximate 1:1 ratio and this ratio seems to be critical in terms of hemoglobin and red blood cell (RBC) stabilization. In a developing fetus, a different form of hemoglobin, fetal hemoglobin (HbF), is produced which has a higher binding affinity for oxygen than Hemoglobin A such that oxygen can be delivered to the baby's system via
15 the mother's blood stream. Fetal hemoglobin also contains two α globin chains, but in place of the adult β -globin chains, it has two fetal gamma (γ)-globin chains (i.e., fetal hemoglobin is $\alpha_2\gamma_2$). The regulation of the switch from production of gamma- to beta-globin is quite complex, and primarily involves a down-regulation of gamma globin transcription with a simultaneous up-regulation of beta globin transcription. At approximately 30 weeks of gestation, the synthesis of gamma globin in the fetus starts to drop while the
20 production of beta globin increases. By approximately 10 months of age, the newborn's hemoglobin is nearly all $\alpha_2\beta_2$ although some HbF persists into adulthood (approximately 1-3% of total hemoglobin). In the majority of patients with hemoglobinopathies, the genes encoding gamma globin remain present, but expression is relatively low due to normal gene repression occurring around parturition as described above.

25 Sickle cell disease is caused by a V6E mutation in the β globin gene (HBB) (a GAG to GTG at the DNA level), where the resultant hemoglobin is referred to as "hemoglobinS" or "HbS." Under lower oxygen conditions, HbS molecules aggregate and form fibrous precipitates. These aggregates cause the abnormality or 'sickling' of the RBCs, resulting in a loss of flexibility of the cells. The sickling RBCs are no longer able to squeeze into the capillary beds and can result in vaso-occlusive crisis in sickle cell patients. In addition, sickled RBCs are more fragile than normal RBCs, and tend towards hemolysis, eventually
30 leading to anemia in the patient.

Treatment and management of sickle cell patients is a life-long proposition involving antibiotic treatment, pain management and transfusions during acute episodes. One approach is the use of hydroxyurea, which exerts its effects in part by increasing the production of gamma globin. Long term side effects of chronic hydroxyurea therapy are still unknown, however, and treatment gives unwanted side
35 effects and can have variable efficacy from patient to patient. Despite an increase in the efficacy of sickle cell treatments, the life expectancy of patients is still only in the mid to late 50's and the associated morbidities of the disease have a profound impact on a patient's quality of life.

Thalassemias (alpha thalassemias and beta thalassemia) are also diseases relating to hemoglobin and typically involve a reduced expression of globin chains. This can occur through mutations in the regulatory regions of the genes or from a mutation in a globin coding sequence that results in reduced expression or reduced levels or functional globin protein. Treatment of thalassemias usually involves blood transfusions and iron chelation therapy. Bone marrow transplants are also being used for treatment of people with severe thalassemias if an appropriate donor can be identified, but this procedure can have significant risks.

One approach that has been proposed for the treatment of both SCD and beta thalassemias is to increase the expression of gamma globin so that HbF functionally replaces the aberrant adult hemoglobin. As mentioned above, treatment of SCD patients with hydroxyurea is thought to be successful in part due to its effect on increasing gamma globin expression (DeSimone (1982) Proc Nat'l Acad Sci USA 79(14):4428-31; Ley, et al., (1982) N. Engl. J. Medicine, 307: 1469-1475; Ley, et al., (1983) Blood 62: 370-380; Constantoulakis et al., (1988) Blood 72(6):1961-1967, all herein incorporated by reference). Increasing the expression of HbF involves identification of genes whose products play a role in the regulation of gamma globin expression. One such gene is BCL11A. BCL11A encodes a zinc finger protein that expressed in adult erythroid precursor cells, and down-regulation of its expression leads to an increase in gamma globin expression (Sankaran et al. (2008) Science 322: 1839, herein incorporated by reference). Use of an inhibitory RNA targeted to the BCL11A gene has been proposed (e.g., U.S. Patent Publication 2011/0182867, herein incorporated by reference) but this technology has several potential drawbacks, including that complete knock down may not be achieved, delivery of such RNAs may be problematic, and the RNAs must be present continuously, requiring multiple treatments for life.

RGNs of the invention may be used to target the BCL11A enhancer region to disrupt expression of BCL11A, thereby increasing gamma globin expression. This targeted disruption can be achieved by non-homologous end joining (NHEJ), whereby an RGN of the invention targets to a particular sequence within the BCL11A enhancer region, makes a double-stranded break, and the cell's machinery repairs the break, typically simultaneously introducing deleterious mutations. Similar to what is described for other disease targets, RGNs of the invention may have advantages over other known RGNs due to their relatively small size, which enables packaging expression cassettes for the RGN and its guide RNA into a single AAV vector for *in vivo* delivery. Similar strategies using RGNs of the invention may also be applicable to similar diseases and disorders in both humans and in non-human animals of agronomic or economic importance.

IX. Cells Comprising a Polynucleotide Genetic Modification

Provided herein are cells and organisms comprising a target sequence of interest that has been modified using a process mediated by an RGN, crRNA, and/or tracrRNA as described herein. In some of these embodiments, the RGN comprises the amino acid sequence of SEQ ID NO: 1, 9, 16, 23, 30, 38, 46, 54, 61, 69, 75, 82, 89, 95, 103, 110, 117, 137, or 235, or an active variant or fragment thereof. In various embodiments, the guide RNA comprises a CRISPR repeat sequence comprising the nucleotide sequence of SEQ ID NO: 2, 10, 17, 24, 31, 39, 47, 55, 62, 70, 76, 83, 90, 96, 104, 111, 118, 240, 273, or 287, or an

active variant or fragment thereof. In particular embodiments, the guide RNA comprises a tracrRNA comprising the nucleotide sequence of SEQ ID NO: 3, 11, 18, 25, 32, 40, 48, 56, 63, 71, 77, 84, 91, 97, 105, 112, 119, 241, 274, or 286, or an active variant or fragment thereof. The guide RNA of the system can be a single guide RNA or a dual-guide RNA.

5 The modified cells can be eukaryotic (*e.g.*, mammalian, plant, insect cell) or prokaryotic. Also provided are organelles and embryos comprising at least one nucleotide sequence that has been modified by a process utilizing an RGN, crRNA, and/or tracrRNA as described herein. The genetically modified cells, organisms, organelles, and embryos can be heterozygous or homozygous for the modified nucleotide sequence.

10 The chromosomal modification of the cell, organism, organelle, or embryo can result in altered expression (up-regulation or down-regulation), inactivation, or the expression of an altered protein product or an integrated sequence. In those instances wherein the chromosomal modification results in either the inactivation of a gene or the expression of a non-functional protein product, the genetically modified cell, organism, organelle, or embryo is referred to as a “knock out”. The knock out phenotype can be the result of
15 a deletion mutation (*i.e.*, deletion of at least one nucleotide), an insertion mutation (*i.e.*, insertion of at least one nucleotide), or a nonsense mutation (*i.e.*, substitution of at least one nucleotide such that a stop codon is introduced).

Alternatively, the chromosomal modification of a cell, organism, organelle, or embryo can produce a “knock in”, which results from the chromosomal integration of a nucleotide sequence that encodes a
20 protein. In some of these embodiments, the coding sequence is integrated into the chromosome such that the chromosomal sequence encoding the wild-type protein is inactivated, but the exogenously introduced protein is expressed.

In other embodiments, the chromosomal modification results in the production of a variant protein product. The expressed variant protein product can have at least one amino acid substitution and/or the
25 addition or deletion of at least one amino acid. The variant protein product encoded by the altered chromosomal sequence can exhibit modified characteristics or activities when compared to the wild-type protein, including but not limited to altered enzymatic activity or substrate specificity.

In yet other embodiments, the chromosomal modification can result in an altered expression pattern of a protein. As a non-limiting example, chromosomal alterations in the regulatory regions controlling the
30 expression of a protein product can result in the overexpression or downregulation of the protein product or an altered tissue or temporal expression pattern.

X. Kits and Methods for Detecting Target DNA or Single-Stranded DNA

Some RGNs (*e.g.*, APG09106.1 and APG09748, set forth as SEQ ID NOs: 54 and 137) can
35 promiscuously cleave non-targeted single-stranded DNA (ssDNA) once activated by detection of a target DNA. Thus, provided herein are compositions and methods for detecting a target DNA (double-stranded or single-stranded) in a sample. In some embodiments, the desired target may exist as RNA, such as the

genome or part of a genome of an RNA virus, such as for example a coronavirus. In some embodiments, the coronavirus may be a SARS-like coronavirus. In further embodiments, the coronavirus may be SARS-CoV-2, SARS-CoV, or a bat SARS-like coronavirus such as bat-SL-CoVZC45 (accession MG772933). In embodiments where the target exists as RNA, the target may be reverse-transcribed into a DNA molecule which can be effectively targeted by the RGN. Reverse-transcription may be followed by an amplification step, such as RT-PCR methods known in the art, which involve thermocycling, or may be by isothermal methods such as RT-LAMP (reverse transcription loop-mediated isothermal amplification) (Notomi et al., *Nucleic Acids Res* 28: E63, (2000)).

These compositions and methods involve the use of a detector ssDNA that does not hybridize with the guideRNA and is a non-target ssDNA. In some embodiments, the detector ssDNA comprises a detectable label that provides a detectable signal after cleavage of the detector ssDNA. A non-limiting example is a detector ssDNA that comprises a fluorophore/quencher pair wherein the fluorophore does not fluoresce when the detector ssDNA is whole (i.e., uncleaved) as its signal is suppressed by the presence of the quencher in close proximity. Cleavage of the detector ssDNA results in removal of the quencher and the fluorescent label can then be detected. Non-limiting examples of fluorescent labels or dyes include Cy5, fluorescein (e.g., FAM, 6 FAM, 5(6) FAM, FITC), Cy3, Alexa Fluor® dyes, and Texas Red. Non-limiting examples of quenchers include Iowa Black®FQ, Iowa Black® RQ, a Qx1 quencher, an ATT0 quencher, and a QSY dye. In some embodiments, the detector ssDNA comprises a second quencher, such as an internal quencher like ZEN™, TAO™, and Black Hole Quencher®, which can lower background and increase signal detection.

In other embodiments, the detector ssDNA comprises a detectable label that provides a detectable signal before cleavage of the detector ssDNA and cleavage of the ssDNA inhibits or prevents detection of the signal. A non-limiting example of such a scenario is a detector ssDNA that comprises a fluorescence resonance energy transfer (FRET) pair. FRET is a process by which radiationless transfer of energy occurs from an excited state of a first (donor) fluorophore to a second (acceptor) fluorophore in close proximity. The emission spectrum of the donor fluorophore overlaps with the excitation spectrum of the acceptor fluorophore. Thus, the acceptor fluorophore will fluoresce when the detector ssDNA is whole (i.e., uncleaved) and the acceptor fluorophore will no longer fluoresce when the detector ssDNA is cleaved because the donor and acceptor fluorophore will no longer be in close proximity to one another. FRET donor and acceptor fluorophores are known in the art and include, but are not limited to cyan fluorescent protein (CFP)/green fluorescent protein (GFP), Cy3/Cy5, and GFP/yellow fluorescent protein (YFP).

In some embodiments, the detector ssDNA has a length of from about 2 nucleotides to about 30 nucleotides, including but not limited to about 2, about 3, about 4, about 5, about 6, about 7, about 8, about 9, about 10, about 11, about 12, about 13, about 14, about 15, about 16, about 17, about 18, about 19, about 20, about 21, about 22, about 23, about 24, about 25 nucleotides, about 26 nucleotides, about 27 nucleotides, about 28 nucleotides, about 29 nucleotides, and about 30 nucleotides.

Methods of detecting a target DNA of a DNA molecule comprise contacting a sample with an RGN, a guide RNA capable of hybridizing with the RGN and a target DNA sequence in a DNA molecule, and a detector single-stranded DNA (ssDNA) that does not hybridize with the guide RNA, followed by measuring a detectable signal produced by cleavage of the ssDNA by the RGN, thereby detecting the target DNA sequence of the DNA molecule. In some embodiments, the method can comprise a step of amplification of the nucleic acid molecules within a sample, either before or simultaneously with contact with the RGN and guideRNA. In some of these embodiments, specific sequences to which the guide RNA will hybridize can be amplified in order to increase sensitivity of a detection method.

The sample in which a target DNA can be detected using these compositions and methods comprising a detector ssDNA include any sample comprising or believed to comprise a nucleic acid (e.g., DNA or RNA molecule). The sample can be derived from any source including a synthetic combination of purified nucleic acids or a biological sample such as respiratory swab (e.g., nasopharyngeal swab) extracts, a cell lysate, a patient sample, cells, tissues, saliva, blood, serum, plasma, urine, aspirate, biopsy samples, cerebral spinal fluid, or organism (e.g., bacteria, virus).

The contacting of the sample with the RGN, guide RNA, and detector ssDNA can include contacting *in vitro*, *ex vivo*, or *in vivo*. In some embodiments, the detector ssDNA and/or the RGN and/or guide RNA is immobilized on for example, a lateral flow device, wherein the sample contacts the immobilized detector ssDNA and/or RGN and/or guide RNA. In some embodiments, antibodies against antigen moieties on the detector ssDNA are immobilized on, for example, a lateral flow device in a manner that allows differentiation of cleaved detector ssDNA from intact detector ssDNA.

In some embodiments, the methods can further comprise determining the amount of the target DNA present in the sample. The measurement of the detectable signal in the test sample can be compared to a reference measurement (e.g., a measurement of a reference sample or series thereof comprising a known amount of target DNA).

Non-limiting examples of applications of the compositions and methods include single-nucleotide polymorphism (SNP) detection, cancer screening, detection of a bacterial infection, detection of antibiotic resistance, and detection of a viral infection.

The detectable signal produced by cleavage of the ssDNA by the RGN can be measured using any suitable method known in the art including but not limited to measuring fluorescent signal, a visual analysis of bands on a gel, a colorimetric change, and the presence or absence of an electrical signal.

The present invention provides kits for detecting a target DNA of a DNA molecule in a sample, wherein the kit comprises an RGN polypeptide, a guide RNA capable of hybridizing with the RGN and a target DNA sequence in a DNA molecule, and a detector ssDNA that does not hybridize with the guide RNA.

Also provided herein are methods of cleaving single-stranded DNAs by contacting a population of nucleic acids, wherein the population comprises a target DNA sequence of a DNA molecule and a plurality

of non-target ssDNAs with an RGN and a guide RNA capable of hybridizing with the RGN and the target DNA sequence.

The article “a” and “an” are used herein to refer to one or more than one (*i.e.*, to at least one) of the grammatical object of the article. By way of example, “a polypeptide” means one or more polypeptides.

All publications and patent applications mentioned in the specification are indicative of the level of those skilled in the art to which this disclosure pertains. All publications and patent applications are herein incorporated by reference to the same extent as if each individual publication or patent application was specifically and individually indicated to be incorporated by reference.

Although the foregoing invention has been described in some detail by way of illustration and example for purposes of clarity of understanding, it will be obvious that certain changes and modifications may be practiced within the scope of the appended embodiments.

Non-limiting embodiments include:

1. A nucleic acid molecule comprising a polynucleotide encoding an RNA-guided nuclease (RGN) polypeptide, wherein said polynucleotide comprises a nucleotide sequence encoding an RGN polypeptide comprising an amino acid sequence having at least 95% sequence identity to SEQ ID NO: 1, 9, 16, 23, 30, 38, 46, 54, 61, 69, 75, 82, 89, 95, 103, 110, or 117;

wherein said RGN polypeptide is capable of binding a target DNA sequence of a DNA molecule in an RNA-guided sequence specific manner when bound to a guide RNA (gRNA) capable of hybridizing to said target DNA sequence, and

wherein said polynucleotide encoding an RGN polypeptide is operably linked to a promoter heterologous to said polynucleotide.

2. The nucleic acid molecule of embodiment 1, wherein said RGN polypeptide is capable of cleaving said target DNA sequence upon binding.

3. The nucleic acid molecule of embodiment 2, wherein said RGN polypeptide is capable of generating a double-stranded break.

4. The nucleic acid molecule of embodiment 2, wherein said RGN polypeptide is capable of generating a single-stranded break.

5. The nucleic acid molecule of embodiment 2, wherein said RGN polypeptide is nuclease inactive.

6. The nucleic acid molecule of any one of embodiments 1-5, wherein the RGN polypeptide is operably fused to a base-editing polypeptide.

7. The nucleic acid molecule of embodiment 6, wherein the base-editing polypeptide is a deaminase.

8. The nucleic acid molecule of embodiment 7, wherein the deaminase is a cytidine deaminase or an adenosine deaminase.

9. The nucleic acid molecule of any one of embodiments 1-8, wherein the RGN polypeptide comprises one or more nuclear localization signals.

10. The nucleic acid molecule of any one of embodiments 1-9, wherein the RGN polypeptide is codon optimized for expression in a eukaryotic cell.

11. The nucleic acid molecule of any one of embodiments 1-10, wherein said target DNA sequence is located adjacent to a protospacer adjacent motif (PAM).

12. A vector comprising the nucleic acid molecule of any one of embodiments 1-11.

13. The vector of embodiment 12, further comprising at least one nucleotide sequence encoding said gRNA capable of hybridizing to said target DNA sequence.

14. The vector of embodiment 13, wherein the guide RNA comprises a CRISPR RNA comprising a CRISPR repeat sequence having at least 95% sequence identity to SEQ ID NO: 2, 10, 17, 24, 31, 39, 47, 55, 62, 70, 76, 83, 90, 96, 104, 111, or 118.

15. The vector of embodiment 13 or 14, wherein said gRNA comprises a tracrRNA.

16. The vector of embodiment 15, wherein the tracrRNA has at least 95% sequence identity to SEQ ID NO: 3, 11, 18, 25, 32, 40, 48, 56, 63, 71, 77, 84, 91, 97, 105, 112, or 119.

17. The vector of embodiment 15 or 16, where said gRNA is a single guide RNA.

18. The vector of embodiment 15 or 16, wherein said gRNA is a dual-guide RNA.

19. A cell comprising the nucleic acid molecule of any one of embodiments 1-11 or the vector of any one of embodiments 12-18.

20. A method for making an RGN polypeptide comprising culturing the cell of embodiment 18 under conditions in which the RGN polypeptide is expressed.

21. A method for making an RGN polypeptide comprising introducing into a cell a heterologous nucleic acid molecule comprising a nucleotide sequence encoding an RNA-guided nuclease (RGN) polypeptide comprising an amino acid sequence having at least 95% sequence identity to SEQ ID NO: 1, 9, 16, 23, 30, 38, 46, 54, 61, 69, 75, 82, 89, 95, 103, 110, or 117;

wherein said RGN polypeptide is capable of binding a target DNA sequence of a DNA molecule in an RNA-guided sequence specific manner when bound to a guide RNA (gRNA) capable of hybridizing to said target DNA sequence;

and culturing said cell under conditions in which the RGN polypeptide is expressed.

22. The method of embodiment 20 or 21, further comprising purifying said RGN polypeptide.

23. The method of embodiment 20 or 21, wherein said cell further expresses one or more guide RNAs that binds to said RGN polypeptide to form an RGN ribonucleoprotein complex.

24. The method of embodiment 23, further comprising purifying said RGN ribonucleoprotein complex.

25. A nucleic acid molecule comprising a polynucleotide encoding a CRISPR RNA (crRNA), wherein said crRNA comprises a spacer sequence and a CRISPR repeat sequence, wherein said CRISPR

repeat sequence comprises a nucleotide sequence having at least 95% sequence identity to SEQ ID NO: 2, 10, 17, 24, 31, 39, 47, 55, 62, 70, 76, 83, 90, 96, 104, 111, or 118;

wherein a guide RNA comprising:

a) said crRNA; and optionally,

b) a trans-activating CRISPR RNA (tracrRNA) capable of hybridizing to said CRISPR repeat sequence of said crRNA;

is capable of hybridizing to a target DNA sequence of a DNA molecule in a sequence specific manner through the spacer sequence of said crRNA when said guide RNA is bound to an RNA-guided nuclease (RGN) polypeptide, and

wherein said polynucleotide encoding a crRNA is operably linked to a promoter heterologous to said polynucleotide.

26. A vector comprising the nucleic acid molecule of embodiment 25.

27. The vector of embodiment 26, wherein said vector further comprises a polynucleotide encoding said tracrRNA.

28. The vector of embodiment 27, wherein said tracrRNA comprises a nucleotide sequence having at least 95% sequence identity to SEQ ID NO: 3, 11, 18, 25, 32, 40, 48, 56, 63, 71, 77, 84, 91, 97, 105, 112, or 119.

29. The vector of embodiment 27 or 28, wherein said polynucleotide encoding said crRNA and said polynucleotide encoding said tracrRNA are operably linked to the same promoter and are encoded as a single guide RNA.

30. The vector of embodiment 27 or 28, wherein said polynucleotide encoding said crRNA and said polynucleotide encoding said tracrRNA are operably linked to separate promoters.

31. The vector of any one of embodiments 26-30, wherein said vector further comprises a polynucleotide encoding said RGN polypeptide, wherein said RGN polypeptide comprises an amino acid sequence having at least 95% sequence identity to SEQ ID NO: 1, 9, 16, 23, 30, 38, 46, 54, 61, 69, 75, 82, 89, 95, 103, 110, or 117.

32. A nucleic acid molecule comprising a polynucleotide encoding a trans-activating CRISPR RNA (tracrRNA) comprising a nucleotide sequence having at least 95% sequence identity to SEQ ID NO: 3, 11, 18, 25, 32, 40, 48, 56, 63, 71, 77, 84, 91, 97, 105, 112, or 119;

wherein a guide RNA comprising:

a) said tracrRNA; and

b) a crRNA comprising a spacer sequence and a CRISPR repeat sequence, wherein said tracrRNA is capable of hybridizing with said CRISPR repeat sequence of said crRNA;

is capable of hybridizing to a target DNA sequence of a DNA molecule in a sequence specific manner through the spacer sequence of said crRNA when said guide RNA is bound to an RNA-guided nuclease (RGN) polypeptide, and

wherein said polynucleotide encoding a tracrRNA is operably linked to a promoter heterologous to said polynucleotide.

33. A vector comprising the nucleic acid molecule of embodiment 32.

34. The vector of embodiment 33, wherein said vector further comprises a polynucleotide encoding said crRNA.

35. The vector of embodiment 34, wherein the CRISPR repeat sequence of said crRNA comprises a nucleotide sequence having at least 95% sequence identity to SEQ ID NO: 2, 10, 17, 24, 31, 39, 47, 55, 62, 70, 76, 83, 90, 96, 104, 111, or 118.

36. The vector of embodiment 34 or 35, wherein said polynucleotide encoding said crRNA and said polynucleotide encoding said tracrRNA are operably linked to the same promoter and are encoded as a single guide RNA.

37. The vector of embodiment 34 or 35, wherein said polynucleotide encoding said crRNA and said polynucleotide encoding said tracrRNA are operably linked to separate promoters.

38. The vector of any one of embodiments 33-37, wherein said vector further comprises a polynucleotide encoding said RGN polypeptide, wherein said RGN polypeptide comprises an amino acid sequence having at least 95% sequence identity to SEQ ID NO: 1, 9, 16, 23, 30, 38, 46, 54, 61, 69, 75, 82, 89, 95, 103, 110 or 117.

39. A system for binding a target DNA sequence of a DNA molecule, said system comprising:
a) one or more guide RNAs capable of hybridizing to said target DNA sequence or one or more polynucleotides comprising nucleotide sequences encoding the one or more guide RNAs (gRNAs); and
b) an RNA-guided nuclease (RGN) polypeptide comprising an amino acid sequence having at least 95% sequence identity to SEQ ID NO: 1, 9, 16, 23, 30, 38, 46, 54, 61, 69, 75, 82, 89, 95, 103, 110, or 117; or a polynucleotide comprising a nucleotide sequence encoding the RGN polypeptide;

wherein said nucleotide sequences encoding the one or more guide RNAs and encoding the RGN polypeptide are each operably linked to a promoter heterologous to said nucleotide sequence; and

wherein the one or more guide RNAs are capable of forming a complex with the RGN polypeptide, in order to direct said RGN polypeptide to bind to said target DNA sequence of the DNA molecule.

40. The system of embodiment 39, wherein said gRNA comprises a CRISPR repeat sequence comprising a nucleotide sequence having at least 95% sequence identity to SEQ ID NO: 2, 10, 17, 24, 31, 39, 47, 55, 62, 70, 76, 83, 90, 96, 104, 111, or 118.

41. The system of embodiment 39 or 40, wherein said gRNA comprises a tracrRNA.

42. The system of embodiment 41, wherein said tracrRNA comprises a nucleotide sequence having at least 95% sequence identity to SEQ ID NO: 3, 11, 18, 25, 32, 40, 48, 56, 63, 71, 77, 84, 91, 97, 105, 112, or 119.

43. The system of embodiment 41 or 42, wherein said gRNA is a single guide RNA (sgRNA).

44. The system of embodiment 41 or 42, wherein said gRNA is a dual-guide RNA.

45. The system of any one of embodiments 39-44, wherein said target DNA sequence is located adjacent to a protospacer adjacent motif (PAM).

46. The system of any one of embodiments 39-45, wherein the target DNA sequence is within a cell.

47. The system of embodiment 46, wherein the cell is a eukaryotic cell.

48. The system of embodiment 47, wherein the eukaryotic cell is a plant cell.

49. The system of embodiment 47, wherein the eukaryotic cell is a mammalian cell.

50. The system of embodiment 47, wherein the eukaryotic cell is an insect cell.

51. The system of embodiment 46, wherein the cell is a prokaryotic cell.

52. The system of any one of embodiments 39-51, wherein when transcribed the one or more guide RNAs is capable of hybridizing to the target DNA sequence and the guide RNA is capable of forming a complex with the RGN polypeptide to direct cleavage of the target DNA sequence.

53. The system of embodiment 52, wherein said RGN polypeptide is capable of generating a double-stranded break.

54. The system of embodiment 52, wherein said RGN polypeptide is capable of generating a single-stranded break.

55. The system of embodiment 52, wherein said RGN polypeptide is nuclease inactive.

56. The system of any one of embodiments 39-55, wherein the RGN polypeptide is operably linked to a base-editing polypeptide.

57. The system of embodiment 56, wherein the base-editing polypeptide is a deaminase.

58. The system of embodiment 57, wherein the deaminase is a cytidine deaminase or an adenosine deaminase.

59. The system of any one of embodiments 39-58, wherein the RGN polypeptide comprises one or more nuclear localization signals.

60. The system of any one of embodiments 39-59, wherein the RGN polypeptide is codon optimized for expression in a eukaryotic cell.

61. The system of any one of embodiments 39-60, wherein polynucleotides comprising the nucleotide sequences encoding the one or more guide RNAs and the polynucleotide comprising the nucleotide sequence encoding an RGN polypeptide are located on one vector.

62. The system of any one of embodiments 39-61, wherein said system further comprises one or more donor polynucleotides or one or more nucleotide sequences encoding the one or more donor polynucleotides.

63. A method for binding a target DNA sequence of a DNA molecule comprising delivering a system according to any one of embodiments 39-62, to said target DNA sequence or a cell comprising the target DNA sequence.

64. The method of embodiment 63, wherein said RGN polypeptide or said guide RNA further comprises a detectable label, thereby allowing for detection of said target DNA sequence.

65. The method of embodiment 63, wherein said guide RNA or said RGN polypeptide further comprises an expression modulator, thereby modulating expression of said target DNA sequence or a gene under transcriptional control by said target DNA sequence.

66. A method for cleaving or modifying a target DNA sequence of a DNA molecule comprising delivering a system according to any one of embodiments 39-62, to said target DNA sequence or a cell comprising the DNA molecule and cleavage or modification of said target DNA sequence occurs.

67. The method of embodiment 66, wherein said modified target DNA sequence comprises insertion of heterologous DNA into the target DNA sequence.

68. The method of embodiment 66, wherein said modified target DNA sequence comprises deletion of at least one nucleotide from the target DNA sequence.

69. The method of embodiment 66, wherein said modified target DNA sequence comprises mutation of at least one nucleotide in the target DNA sequence.

70. A method for binding a target DNA sequence of a DNA molecule, said method comprising:

a) assembling an RNA-guided nuclease (RGN) ribonucleotide complex *in vitro* by combining:
i) one or more guide RNAs capable of hybridizing to the target DNA sequence; and
ii) an RGN polypeptide comprising an amino acid having at least 95% sequence identity to SEQ ID NO: 1, 9, 16, 23, 30, 38, 46, 54, 61, 69, 75, 82, 89, 95, 103, 110, or 117 under conditions suitable for formation of the RGN ribonucleotide complex; and

b) contacting said target DNA sequence or a cell comprising said target DNA sequence with the *in vitro*-assembled RGN ribonucleotide complex;

wherein the one or more guide RNAs hybridize to the target DNA sequence, thereby directing said RGN polypeptide to bind to said target DNA sequence.

71. The method of embodiment 70, wherein said RGN polypeptide or said guide RNA further comprises a detectable label, thereby allowing for detection of said target DNA sequence.

72. The method of embodiment 70, wherein said guide RNA or said RGN polypeptide further comprises an expression modulator, thereby allowing for the modulation of expression of said target DNA sequence.

73. A method for cleaving and/or modifying a target DNA sequence of a DNA molecule, comprising contacting the DNA molecule with:

a) an RNA-guided nuclease (RGN) polypeptide, wherein said RGN comprises an amino acid having at least 95% sequence identity to SEQ ID NO: 1, 9, 16, 23, 30, 38, 46, 54, 61, 69, 75, 82, 89, 95, 103, 110, or 117; and

b) one or more guide RNAs capable of targeting the RGN of (a) to the target DNA sequence; wherein the one or more guide RNAs hybridize to the target DNA sequence, thereby directing said RGN polypeptide to bind to said target DNA sequence and cleavage and/or modification of said target DNA sequence occurs.

74. The method of embodiment 73, wherein the cleavage by said RGN polypeptide generates a double-stranded break.

75. The method of embodiment 73, wherein the cleavage by said RGN polypeptide generates a single-stranded break.

5 76. The method of embodiment 73, wherein said RGN polypeptide is nuclease inactive.

77. The method of any one of embodiments 73-76, wherein said RGN polypeptide is operably linked to a base-editing polypeptide.

78. The method of embodiment 77, wherein said base-editing polypeptide comprises a deaminase.

10 79. The method of embodiment 78, wherein said deaminase is a cytidine deaminase or an adenosine deaminase.

80. The method of any one of embodiments 73-79, wherein said modified target DNA sequence comprises insertion of heterologous DNA into the target DNA sequence.

15 81. The method of any one of embodiments 73-79, wherein said modified target DNA sequence comprises deletion of at least one nucleotide from the target DNA sequence.

82. The method of any one of embodiments 73-79, wherein said modified target DNA sequence comprises mutation of at least one nucleotide in the target DNA sequence.

20 83. The method of any one of embodiments 70-82, wherein said gRNA comprises a CRISPR repeat sequence comprising a nucleotide sequence having at least 95% sequence identity to SEQ ID NO: 2, 10, 17, 24, 31, 39, 47, 55, 62, 70, 76, 83, 90, 96, 104, 111, or 118.

84. The method of any one of embodiments 70-83, wherein said gRNA comprises a tracrRNA.

85. The method of embodiment 84, wherein said tracrRNA comprises a nucleotide sequence having at least 95% sequence identity to SEQ ID NO: 3, 11, 18, 25, 32, 40, 48, 56, 63, 71, 77, 84, 91, 97, 105, 112, or 119.

25 86. The method of embodiment 84 or 85, wherein said gRNA is a single guide RNA (sgRNA).

87. The method of embodiment 84 or 85, wherein said gRNA is a dual-guide RNA.

88. The method of any one of embodiments 63-87, wherein said target DNA sequence is located adjacent to a protospacer adjacent motif (PAM).

30 89. The method of any one of embodiments 63-88, wherein the target DNA sequence is within a cell.

90. The method of embodiment 89, wherein the cell is a eukaryotic cell.

91. The method of embodiment 90, wherein the eukaryotic cell is a plant cell.

92. The method of embodiment 90, wherein the eukaryotic cell is a mammalian cell.

93. The method of embodiment 90, wherein the eukaryotic cell is an insect cell.

35 94. The method of embodiment 89, wherein the cell is a prokaryotic cell.

95. The method of any one of embodiments 89-94, further comprising culturing the cell under conditions in which the RGN polypeptide is expressed and cleaves the target DNA sequence to produce a

DNA molecule comprising a modified DNA sequence; and selecting a cell comprising said modified target DNA sequence.

96. A cell comprising a modified target DNA sequence according to the method of embodiment 95.

97. The cell of embodiment 96, wherein the cell is a eukaryotic cell.

98. The cell of embodiment 97, wherein the eukaryotic cell is a plant cell.

99. A plant comprising the cell of embodiment 98.

100. A seed comprising the cell of embodiment 98.

101. The cell of embodiment 97, wherein the eukaryotic cell is a mammalian cell.

102. The cell of embodiment 98, wherein the eukaryotic cell is an insect cell.

103. The cell of embodiment 96, wherein the cell is a prokaryotic cell.

104. A method for producing a genetically modified cell with a correction in a causal mutation for a genetically inherited disease, the method comprising introducing into the cell:

a) an RNA-guided nuclease (RGN) polypeptide, wherein the RGN polypeptide comprises an amino acid sequence having at least 95% sequence identity to SEQ ID NO: 1, 9, 16, 23, 30, 38, 46, 54, 61, 69, 75, 82, 89, 95, 103, 110, or 117; or a polynucleotide encoding said RGN polypeptide, wherein said polynucleotide encoding the RGN polypeptide is operably linked to a promoter to enable expression of the RGN polypeptide in the cell; and

b) a guide RNA (gRNA), wherein the gRNA comprises a CRISPR repeat sequence comprising a nucleotide sequence having at least 95% sequence identity to SEQ ID NO: 2, 10, 17, 24, 31, 39, 47, 55, 62, 70, 76, 83, 90, 96, 104, 111, or 118, or a polynucleotide encoding said gRNA, wherein said polynucleotide encoding the gRNA is operably linked to a promoter to enable expression of the gRNA in the cell

whereby the RGN and gRNA target to the genomic location of the causal mutation and modify the genomic sequence to remove the causal mutation.

105. The method of embodiment 104, wherein the RGN is operably linked to a base-editing polypeptide.

106. The method of embodiment 105, wherein the base-editing polypeptide is a deaminase.

107. The method of embodiment 106, wherein the deaminase is a cytidine deaminase or an adenosine deaminase.

108. The method of any one of embodiments 104-107, wherein the cell is an animal cell.

109. The method of any one of embodiments 104-107, wherein the cell is a mammalian cell.

110. The method of embodiment 108, wherein the cell is derived from a dog, cat, mouse, rat, rabbit, horse, cow, pig, or human.

111. The method of embodiment 108, wherein the genetically inherited disease is caused by a single nucleotide polymorphism.

112. The method of embodiment 111, wherein the genetically inherited disease is Hurler Syndrome.

113. The method of embodiment 112, wherein the gRNA further comprises a spacer sequence that targets a region proximal to the causal single nucleotide polymorphism.

114. A method for producing a genetically modified cell with a deletion in a disease-causing genomic region of instability, the method comprising introducing into the cell:

5 a) an RNA-guided nuclease (RGN) polypeptide, wherein the RGN polypeptide comprises an amino acid sequence having at least 95% sequence identity to SEQ ID NO: 1, 9, 16, 23, 30, 38, 46, 54, 61, 69, 75, 82, 89, 95, 103, 110, or 117; or a polynucleotide encoding said RGN polypeptide, wherein said polynucleotide encoding the RGN polypeptide is operably linked to a promoter to enable expression of the RGN polypeptide in the cell; and

10 b) a guide RNA (gRNA), wherein the gRNA comprises a CRISPR repeat sequence comprising a nucleotide sequence having at least 95% sequence identity to SEQ ID NO: 2, 10, 17, 24, 31, 39, 47, 55, 62, 70, 76, 83, 90, 96, 104, 111, or 118, or a polynucleotide encoding said gRNA, wherein said polynucleotide encoding the gRNA is operably linked to a promoter to enable expression of the gRNA in the cell, and further wherein the gRNA comprises a spacer sequence that targets the 5' flank of the genomic region of
15 instability; and

c) a second guide RNA (gRNA), wherein the gRNA comprises a CRISPR repeat sequence comprising a nucleotide sequence having at least 95% sequence identity to SEQ ID NO: 2, 10, 17, 24, 31, 39, 47, 55, 62, 70, 76, 83, 90, 96, 104, 111, or 118, or a polynucleotide encoding said gRNA, wherein said polynucleotide encoding the gRNA is operably linked to a promoter to enable expression of the second
20 gRNA in the cell, and further wherein said second gRNA comprises a spacer sequence that targets the 3' flank of the genomic region of instability;

whereby the RGN and the two gRNAs target to the genomic region of instability and at least a portion of the genomic region of instability is removed.

115. The method of embodiment 114, wherein the cell is an animal cell.

25 116. The method of embodiment 114, wherein the cell is a mammalian cell.

117. The method of embodiment 115, wherein the cell is derived from a dog, cat, mouse, rat, rabbit, horse, cow, pig, or human.

118. The method of embodiment 115, wherein the genetically inherited disease is Friedrich's Ataxia or Huntington's Disease.

30 119. The method of embodiment 118, wherein the first gRNA further comprises a spacer sequence that targets a region within or proximal to the genomic region of instability.

120. The method of embodiment 118, wherein the second gRNA further comprises a spacer sequence that targets a region within or proximal to genomic region of instability.

121. A method for producing a genetically modified mammalian hematopoietic progenitor cell
35 having decreased BCL11A mRNA and protein expression, the method comprising introducing into an isolated human hematopoietic progenitor cell:

a) an RNA-guided nuclease (RGN) polypeptide, wherein the RGN polypeptide comprises an amino acid sequence having at least 95% sequence identity to SEQ ID NO: 1, 9, 16, 23, 30, 38, 46, 54, 61, 69, 75, 82, 89, 95, 103, 110, or 117; or a polynucleotide encoding said RGN polypeptide, wherein said polynucleotide encoding the RGN polypeptide is operably linked to a promoter to enable expression of the RGN polypeptide in the cell; and

b) a guide RNA (gRNA), wherein the gRNA comprises a CRISPR repeat sequence comprising a nucleotide sequence having at least 95% sequence identity to SEQ ID NO: 2, 10, 17, 24, 31, 39, 47, 55, 62, 70, 76, 83, 90, 96, 104, 111, or 118, or a polynucleotide encoding said gRNA, wherein said polynucleotide encoding the gRNA is operably linked to a promoter to enable expression of the gRNA in the cell,

whereby the RGN and gRNA are expressed in the cell and cleave at the BCL11A enhancer region, resulting in genetic modification of the human hematopoietic progenitor cell and reducing the mRNA and/or protein expression of BCL11A.

122. The method of embodiment 121, wherein the gRNA further comprises a spacer sequence that targets a region within or proximal to the BCL11A enhancer region.

123. The method of any one of embodiments 104-122, wherein the guide RNA comprises a tracrRNA comprising a nucleotide sequence having at least 95% sequence identity to SEQ ID NO: 3, 11, 18, 25, 32, 40, 48, 56, 63, 71, 77, 84, 91, 97, 105, 112, or 119.

124. A system for binding a target DNA sequence of a DNA molecule, said system comprising:

a) one or more guide RNAs capable of hybridizing to said target DNA sequence or one or more polynucleotides comprising one or more nucleotide sequences encoding the one or more guide RNAs (gRNAs); and

b) an RNA-guided nuclease (RGN) polypeptide comprising an amino acid sequence having at least 95% sequence identity to SEQ ID NO: 1, 9, 16, 23, 30, 38, 46, 54, 61, 69, 75, 82, 89, 95, 103, 110, or 117; wherein the one or more guide RNAs are capable of hybridizing to the target DNA sequence, and wherein the one or more guide RNAs are capable of forming a complex with the RGN polypeptide, in order to direct said RGN polypeptide to bind to said target DNA sequence of the DNA molecule.

125. The system of embodiment 124, wherein said RGN polypeptide is nuclease inactive or capable of functioning as a nickase.

126. The system of embodiment 124 or 125, wherein said RGN polypeptide is operably fused to a base-editing polypeptide.

127. The system of embodiment 126, wherein the base-editing polypeptide is a deaminase.

128. The system of embodiment 127, wherein the deaminase is a cytidine deaminase or an adenosine deaminase.

129. A method of detecting a target DNA sequence of a DNA molecule in a sample, the method comprising:

a) contacting the sample with:

i) an RNA-guided nuclease (RGN) polypeptide comprising an amino acid sequence having at least 95% sequence identity to SEQ ID NO: 1, 9, 16, 23, 30, 38, 46, 54, 61, 69, 75, 82, 89, 95, 103, 110, 117, or 137, wherein said RGN polypeptide is capable of binding said target DNA sequence of a DNA molecule in an RNA-guided sequence specific manner when bound to a guide RNA capable of hybridizing to said target DNA sequence;

ii) said guide RNA; and

iii) a detector single-stranded DNA (ssDNA) that does not hybridize with the guide RNA;

and

b) measuring a detectable signal produced by cleavage of the detector ssDNA by the RGN, thereby detecting the target DNA.

130. The method of embodiment 129, wherein said sample comprises DNA molecules from a cell lysate.

131. The method of embodiment 129, wherein said sample comprises cells.

132. The method of embodiment 131, wherein said cells are eukaryotic cells.

133. The method of embodiment 129, wherein the DNA molecule comprising the target DNA sequence is produced by reverse-transcription of an RNA template molecule present in a sample comprising RNA.

134. The method of embodiment 133, wherein the RNA template molecule is an RNA virus.

135. The method of embodiment 134, wherein the RNA virus is a coronavirus.

136. The method of embodiment 135, wherein the coronavirus is a bat SARS-like coronavirus, SARS-CoV, or SARS-CoV-2.

137. The method of any one of embodiments 133-136, wherein the sample comprising RNA is derived from a sample comprising cells.

138. The method of any one of embodiments 129-137, wherein said detector ssDNA comprises a fluorophore/quencher pair.

139. The method of any one of embodiments 129-137, wherein said detector ssDNA comprises a fluorescence resonance energy transfer (FRET) pair.

140. The method of any one of embodiments 129-139, wherein said guide RNA comprises a CRISPR repeat sequence comprising a nucleotide sequence having at least 95% sequence identity to SEQ ID NO: 2, 10, 17, 24, 31, 39, 47, 55, 62, 70, 76, 83, 90, 96, 104, 111, 118, or 273.

141. The method of any one of embodiments 129-140, wherein said guide RNA comprises a tracrRNA.

142. The method of embodiment 141, wherein said tracrRNA comprises a nucleotide sequence having at least 95% sequence identity to SEQ ID NO: 3, 11, 18, 25, 32, 40, 48, 56, 63, 71, 77, 84, 91, 97, 105, 112, 119, or 274.

143. The method of embodiment 141 or 142, wherein said guide RNA is a single guide RNA.

144. The method of embodiment 141 or 142, wherein said guide RNA is a dual-guide RNA.

145. The method of any one of embodiments 129-144, wherein said method further comprises amplifying nucleic acids in the sample prior to or together with the contacting of step a).

146. The method of embodiment 145, wherein the amplification is of a DNA molecule produced by reverse transcription of an RNA molecule.

147. A kit for detecting a target DNA sequence of a DNA molecule in a sample, the kit comprising:

a) an RNA-guided nuclease (RGN) polypeptide comprising an amino acid sequence having at least 95% sequence identity to SEQ ID NO: 1, 9, 16, 23, 30, 38, 46, 54, 61, 69, 75, 82, 89, 95, 103, 110, 117, or 137, wherein said RGN polypeptide is capable of binding said target DNA sequence of a DNA molecule in an RNA-guided sequence specific manner when bound to a guide RNA capable of hybridizing to said target DNA sequence;

b) said guide RNA; and

c) a detector single-stranded DNA (ssDNA) that does not hybridize with the guide RNA.

148. The kit of embodiment 147, wherein said detector ssDNA comprises a fluorophore/quencher pair.

149. The kit of embodiment 147, wherein said detector ssDNA comprises a fluorescence resonance energy transfer (FRET) pair.

150. The kit of any one of embodiments 147-149, wherein said guide RNA comprises a CRISPR repeat sequence comprising a nucleotide sequence having at least 95% sequence identity to SEQ ID NO: 2, 10, 17, 24, 31, 39, 47, 55, 62, 70, 76, 83, 90, 96, 104, 111, 118, or 273.

151. The kit of any one of embodiments 147-150, wherein said guide RNA comprises a tracrRNA.

152. The kit of embodiment 151, wherein said tracrRNA comprises a nucleotide sequence having at least 95% sequence identity to SEQ ID NO: 3, 11, 18, 25, 32, 40, 48, 56, 63, 71, 77, 84, 91, 97, 105, 112, 119, or 274.

153. The kit of embodiment 151 or 152, wherein said guide RNA is a single guide RNA.

154. The kit of embodiment 151 or 152, wherein said guide RNA is a dual-guide RNA.

155. A method of cleaving single-stranded DNAs, the method comprising contacting a population of nucleic acids, wherein said population comprises a DNA molecule comprising a target DNA sequence and a plurality of non-target ssDNAs with:

a) an RNA-guided nuclease (RGN) polypeptide comprising an amino acid sequence having at least 95% sequence identity to SEQ ID NO: 1, 9, 16, 23, 30, 38, 46, 54, 61, 69, 75, 82, 89, 95, 103, 110, 117, or 137, wherein said RGN polypeptide is capable of binding said target DNA sequence in an RNA-guided sequence specific manner when bound to a guide RNA capable of hybridizing to said target DNA sequence;

and

b) said guide RNA;

wherein the RGN polypeptide cleaves non-target ssDNAs of said plurality.

156. The method of embodiment 155, wherein said population of nucleic acids are within a cell lysate.

157. The method of embodiment 155 or 156, wherein said guide RNA comprises a CRISPR repeat sequence comprising a nucleotide sequence having at least 95% sequence identity to SEQ ID NO: 2, 10, 17, 24, 31, 39, 47, 55, 62, 70, 76, 83, 90, 96, 104, 111, 118, or 273.

158. The method of any one of embodiments 155-157, wherein said guide RNA comprises a tracrRNA.

159. The method of embodiment 158, wherein said tracrRNA comprises a nucleotide sequence having at least 95% sequence identity to SEQ ID NO: 3, 11, 18, 25, 32, 40, 48, 56, 63, 71, 77, 84, 91, 97, 105, 112, 119, or 274.

160. The method of embodiment 158 or 159, wherein said guide RNA is a single guide RNA.

161. The method of embodiment 158 or 159, wherein said guide RNA is a dual-guide RNA.

162. A nucleic acid molecule comprising a polynucleotide encoding a CRISPR RNA (crRNA), wherein said crRNA comprises a spacer sequence and a CRISPR repeat sequence, wherein said CRISPR repeat sequence comprises a nucleotide sequence having at least 95% sequence identity to SEQ ID NO: 240; wherein a guide RNA comprising:

a) said crRNA; and optionally

b) a trans-activating CRISPR RNA (tracrRNA) capable of hybridizing to said CRISPR repeat sequence of said crRNA;

is capable of hybridizing to a target DNA sequence of a DNA molecule in a sequence specific manner through the spacer sequence of said crRNA when said guide RNA is bound to an RNA-guided nuclease (RGN) polypeptide, and

wherein said polynucleotide encoding a crRNA is operably linked to a promoter heterologous to said polynucleotide.

163. A vector comprising the nucleic acid molecule of embodiment 162.

164. The vector of embodiment 163, wherein said vector further comprises a polynucleotide encoding said tracrRNA.

165. The vector of embodiment 164, wherein said tracrRNA comprises a nucleotide sequence having at least 95% sequence identity to SEQ ID NO: 241.

166. The vector of embodiment 164 or 165, wherein said polynucleotide encoding said crRNA and said polynucleotide encoding said tracrRNA are operably linked to the same promoter and are encoded as a single guide RNA.

167. The vector of embodiment 164 or 165, wherein said polynucleotide encoding said crRNA and said polynucleotide encoding said tracrRNA are operably linked to separate promoters.

168. The vector of any one of embodiments 163-167, wherein said vector further comprises a polynucleotide encoding said RGN polypeptide, wherein said RGN polypeptide comprises an amino acid sequence having at least 95% sequence identity to SEQ ID NO: 235.

169. A nucleic acid molecule comprising a polynucleotide encoding a trans-activating CRISPR RNA (tracrRNA) comprising a nucleotide sequence having at least 95% sequence identity to SEQ ID NO: 241;

wherein a guide RNA comprising:

a) said tracrRNA; and

b) a crRNA comprising a spacer sequence and a CRISPR repeat sequence, wherein said tracrRNA is capable of hybridizing with said CRISPR repeat sequence of said crRNA;

is capable of hybridizing to a target DNA sequence of a DNA molecule in a sequence specific manner through the spacer sequence of said crRNA when said guide RNA is bound to an RNA-guided nuclease (RGN) polypeptide, and

wherein said polynucleotide encoding a tracrRNA is operably linked to a promoter heterologous to said polynucleotide.

170. A vector comprising the nucleic acid molecule of embodiment 169.

171. The vector of embodiment 170, wherein said vector further comprises a polynucleotide encoding said crRNA.

172. The vector of embodiment 171, wherein the CRISPR repeat sequence of said crRNA comprises a nucleotide sequence having at least 95% sequence identity to SEQ ID NO: 240.

173. The vector of embodiment 171 or 172, wherein said polynucleotide encoding said crRNA and said polynucleotide encoding said tracrRNA are operably linked to the same promoter and are encoded as a single guide RNA.

174. The vector of embodiment 171 or 172, wherein said polynucleotide encoding said crRNA and said polynucleotide encoding said tracrRNA are operably linked to separate promoters.

175. The vector of any one of embodiments 170-174, wherein said vector further comprises a polynucleotide encoding said RGN polypeptide, wherein said RGN polypeptide comprises an amino acid sequence having at least 95% sequence identity to SEQ ID NO: 235.

The following examples are offered by way of illustration and not by way of limitation.

EXAMPLES

Example 1. Identification of RNA-guided nucleases

Seventeen distinct CRISPR-associated RNA-guided nucleases (RGN's) were identified and are described in Table 1 below. Table 1 provides the name of each RGN, its amino acid sequence, the source from which it was derived, and processed crRNA and tracrRNA sequences (see Example 2 for methods of identification). Table 1 further provides a generic single guide RNA (sgRNA) sequence, where the poly-N indicates the location of the spacer sequence which determines the nucleic acid target sequence of the sgRNA. RGN systems APG05733.1, APG06207.1, APG01647.1, APG08032.1, APG02675.1, APG01405.1, APG06250.1, APG04293.1, and APG01308.1) had the conserved sequence in the base of the

hairpin stem of the tracrRNA of UNANNA (SEQ ID NO: 8). For APG05712.1, the sequence in the same location is CNANNG (SEQ ID NO: 37). For APG01658.1, the sequence in the same location is CNANNU (SEQ ID NO: 45). For RGN systems APG06498.1 and APG06877.1, the conserved sequence in the base of the hairpin stem of the tracrRNA is UNANNG (SEQ ID NO: 53). For APG09882.1 and APG06646.1, the sequence in the same location is UNANNC (SEQ ID NO: 68). For APG09053.1, the sequence in the same location is CNANNU (SEQ ID NO: 102).

Table 1: Summary of SEQ IDs and CRISPR associated systems

RGN ID	SEQ ID NO	Source	crRNA repeat seq (SEQ ID NO)	tracrRNA (SEQ ID NO)	sgRNA (SEQ ID NO)
APG05733.1	1	<i>Bacillus</i> sp.	2	3	4
APG06207.1	9	<i>Chryseobacterium</i> sp.	10	11	12
APG01647.1	16	<i>Sphingobacterium</i> sp.	17	18	19
APG08032.1	23	<i>Chryseobacterium</i> sp.	24	25	26
APG05712.1	30	<i>Brucella</i> sp.	31	32	33
APG01658.1	38	<i>Staphylococcus</i> sp.	39	40	41
APG06498.1	46	<i>Bacillus</i> sp.	47	48	49
APG09106.1	54	<i>Brevibacillus</i> sp.	55	56	57
APG09882.1	61	<i>Enterococcus</i> sp.	62	63	64
APG02675.1	69	<i>Sphingobacterium</i> sp.	70	71	72
APG01405.1	75	<i>Chryseobacterium</i> sp.	76	77	78
APG06250.1	82	<i>Chryseobacterium</i> sp.	83	84	85
APG06877.1	89	<i>Bacillus</i> sp.	90	91	92
APG09053.1	95	<i>Rhizobium</i> sp.	96	97	98
APG04293.1	103	<i>Myroides</i> sp.	104	105	106
APG01308.1	110	<i>Chryseobacterium</i> sp.	111	112	113
APG06646.1	117	<i>Bacillus</i> sp.	118	119	120

Example 2: Guide RNA identification and sgRNA construction

Cultures of bacteria that natively express the RNA-guided nuclease system under investigation were grown to mid-log phase (OD₆₀₀ of ~0.600), pelleted, and flash frozen. RNA was isolated from the pellets using a mirVANA miRNA Isolation Kit (Life Technologies, Carlsbad, CA), and sequencing libraries were prepared from the isolated RNA using an NEBNext Small RNA Library Prep kit (NEB, Beverly, MA). The library prep was fractionated on a 6% polyacrylamide gel to capture the RNA species less than 200nt to detect crRNAs and tracrRNAs, respectively. Deep sequencing (75 bp paired-end) was performed on a Next Seq 500 (High Output kit) by a service provider (MoGene, St. Louis, MO). Reads were quality trimmed using Cutadapt and mapped to reference genomes using Bowtie2. A custom RNAseq pipeline was written in python to detect the crRNA and tracrRNA transcripts. Processed crRNA boundaries were determined by sequence coverage of the native repeat spacer array. The anti-repeat portion of the tracrRNA was identified

using permissive BLASTn parameters. RNA sequencing depth confirmed the boundaries of the processed tracrRNA by identifying the transcript containing the anti-repeat. Manual curation of RNAs was performed using secondary structure prediction by NUPACK, an RNA folding software. sgRNA cassettes were prepared by DNA synthesis and were generally designed as follows (5'→3'): 20-30 bp spacer sequence, operably linked at its 3' end to the processed repeat portion of the crRNA, operably linked to a 4 bp noncomplementary linker (AAAG; SEQ ID NO: 123), operably linked at its 3' end to the processed tracrRNA. Other 4 bp noncomplementary linkers may also be used.

For *in vitro* assays, sgRNAs were synthesized by *in vitro* transcription of the sgRNA cassettes with a GeneArt™ Precision gRNA Synthesis Kit (ThermoFisher). Processed crRNA and tracrRNA sequences for each of the RGN polypeptides are identified and are set forth in Table 1. See below for the sgRNAs constructed for PAM libraries 1 and 2.

Example 3: Determination of PAM requirements for each RGN

PAM requirements for each RGN were determined using a PAM depletion assay essentially adapted from Kleinstiver et al. (2015) *Nature* 523:481-485 and Zetsche et al. (2015) *Cell* 163:759-771. Briefly, two plasmid libraries (L1 and L2) were generated in a pUC18 backbone (ampR), with each containing a distinct 30bp protospacer (target) sequence flanked by 8 random nucleotides (i.e., the PAM region). The target sequence and flanking PAM region of library 1 and library 2 for each RGN are set forth in Table 2.

The libraries were separately electroporated into *E. coli* BL21(DE3) cells harboring pRSF-1b expression vectors containing an RGN of the invention (codon optimized for *E. coli*) along with a cognate sgRNA containing a spacer sequence corresponding to the protospacer in L1 or L2. Sufficient library plasmid was used in the transformation reaction to obtain >10⁶ CFU. Both the RGN and sgRNA in the pRSF-1b backbone were under the control of T7 promoters. The transformation reaction was allowed to recover for 1 hr after which it was diluted into LB media containing carbenicillin and kanamycin and grown overnight. The following day the mixture was diluted into self-inducing Overnight Express™ Instant TB Medium (Millipore Sigma) to allow expression of the RGN and sgRNA, and grown for an additional 4h or 20h after which the cells were spun down and plasmid DNA was isolated with a Mini-prep kit (Qiagen, Germantown, MD). In the presence of the appropriate sgRNA, plasmids containing a PAM that is recognizable by the RGN will be cleaved resulting in their removal from the population. Plasmids containing PAMs that are not recognizable by the RGN, or that are transformed into bacteria not containing an appropriate sgRNA, will survive and replicate. The PAM and protospacer regions of uncleaved plasmids were PCR-amplified and prepared for sequencing following published protocols (16s-metagenomic library prep guide 15044223B, Illumina, San Diego, CA). Deep sequencing (75bp single end reads) was performed on a MiSeq (Illumina) by a service provider (MoGene, St. Louis, MO). Typically, 1-4M reads were obtained per amplicon. PAM regions were extracted, counted, and normalized to total reads for each sample. PAMs that lead to plasmid cleavage were identified by being underrepresented when compared to controls (i.e., when the library is transformed into *E. coli* containing the RGN but lacking an appropriate sgRNA). To

represent PAM requirements for a novel RGN, the depletion ratios (frequency in sample/frequency in control) for all sequences in the region in question were converted to enrichment values with a -log base 2 transformation. Sufficient PAMs were defined as those with enrichment values >2.3 (which corresponds to depletion ratios < ~0.2). PAMs above this threshold in both libraries were collected and used to generate web logos, which for example can be generated using a web-based service on the internet known as “weblogo”. PAM sequences were identified and reported when there was a consistent pattern in the top enriched PAMs. A consensus PAM (having an enrichment factor (EF) >2.3) for each RGN is provided in Table 2. The PAM orientation is also indicated in Table 2. As noted elsewhere in this application, APG06646.1 and APG04293.1 nucleases do not possess PAM-interacting domains. The results in Table 2 show that they also do not possess the typical PAM requirement, which is 2-5 nucleotides. APG06646.1 and APG04293.1 were shown to have single nucleotide requirements for cleavage.

Table 2: PAM or PAM-like determination

RGN ID	sgRNA L1 (SEQ ID NO)	sgRNA L2 (SEQ ID NO)	PAM (SEQ ID NO)	PAM orientation
APG05733.1	5	6	7	5'-target-PAM-3'
APG06207.1	13	14	15	5'-target-PAM-3'
APG01647.1	20	21	22	5'-target-PAM-3'
APG08032.1	27	28	29	5'-target-PAM-3'
APG05712.1	34	35	36	5'-target-PAM-3'
APG01658.1	42	43	44	5'-target-PAM-3'
APG06498.1	50	51	52	5'-target-PAM-3'
APG09106.1	58	59	60	5'-PAM-target-3'
APG09882.1	65	66	67	5'-target-PAM-3'
APG02675.1	73	74	15	5'-target-PAM-3'
APG01405.1	79	80	81	5'-target-PAM-3'
APG06250.1	86	87	88	5'-target-PAM-3'
APG06877.1	93	94	7	5'-target-PAM-3'
APG09053.1	99	100	101	5'-target-PAM-3'
APG04293.1	107	108	109	5'-target-PAM-3'
APG01308.1	114	115	116	5'-target-PAM-3'
APG06646.1	121	122	109	5'-target-PAM-3'

Example 4: Engineering the guide RNA to increase nuclease activity

4.1 RGNs APG09748 and APG09106.1

For RGNs APG09748 (set forth as SEQ ID NO: 137, and APG09748 crRNA repeat sequence, tracrRNA sequence, and generic sgRNA sequence are set forth as SEQ ID NOS: 273, 274, and 275, respectively; all sequences are described in International Appl. No. PCT/US2019/068079, which is incorporated by reference in its entirety) and APG09106.1, which have very high sequence identity and have

the same PAM, RNA folding predictions were used to determine regions in the guide RNA that can be altered to optimize nuclease activity. The stability of the crRNA:tracrRNA base pairing in the repeat:antirepeat region was increased by shortening the repeat:antirepeat region, adding G-C base pairs, and removing G-U wobble pairs. “Optimized” guide variants were tested and compared to the wild-type gRNA using the RGN APG09748 in *in vitro* cleavage assays.

To produce RGNs for RNP formation, expression plasmids containing an RGN fused to a C-terminal His6 (SEQ ID NO: 276) or His10 (SEQ ID NO: 277) tag were constructed and transformed into BL21 (DE3) strains of *E. coli*. Expression was performed using Magic Media (Thermo Fisher) supplemented with 50 µg/mL kanamycin. After lysis and clarification, the protein was purified by immobilized metal affinity chromatography and quantified using the Qubit protein quantitation kit (Thermo Fisher) or by UV-vis using a calculated extinction coefficient.

Ribonucleoprotein (RNP) was prepared by incubating the purified RGN with sgRNA at a ~2:1 ratio for 20 min at room temperature. For *in vitro* cleavage reactions, RNPs were incubated with plasmids or linear dsDNA containing the targeted protospacer flanked by a preferred PAM sequence for >30 min at room temperature. Two target nucleic acid sequences within the TRAC locus, TRAC11 (SEQ ID NO: 278) and TRAC14 (SEQ ID NO: 279), were tested. gRNAs were assayed both for targeted activity with the correct target nucleic acid sequence (for example, the gRNA has TRAC11 spacer sequence and the assayed target is TRAC11) and without the correct target nucleic acid sequence (for example, the gRNA has TRAC11 spacer sequence and the assayed target is TRAC14). Activity determined by plasmid cleavage is assessed by agarose gel electrophoresis. Results are shown in Table 3. Guide variants are listed as SEQ ID NOs: 280-283, and are provided with spacer sequences. These guide sequences use a noncomplementary nucleotide linker of AAAA (SEQ ID NO: 284). The optimized gRNA (SEQ ID NO: 285; poly-N indicates location of spacer sequence), with increased repeat:antirepeat binding, has optimized tracrRNA (SEQ ID NO: 286) and optimized crRNA (SEQ ID NO: 287) components. The optimized guide variant was able to cleave two loci where previously no cleavage was detected using the wild-type guide RNA. Through optimization of hybridization in the repeat:antirepeat region, *in vitro* cleavage of APG09748 increased from 0% cleavage to 100% cleavage for multiple targets in the TRAC locus.

Table 3: Editing efficiency of APG09748 with engineered guide variants

gRNA variant (SEQ ID NO.)	Guide Design	Assayed Target	Gel 1 - 2 µL load		Gel 2 - 1 µL load	
			% intact	% cleaved	% intact	% cleaved
280	Optimized	TRAC11	68	32	57	43
280	Optimized	TRAC14	100	0	100	0
281	Optimized	TRAC11	100	0	100	0
281	Optimized	TRAC14	70	30	69	31
282	WT	TRAC11	100	0	100	0
282	WT	TRAC14	100	0	100	0
283	WT	TRAC11	100	0	100	0

gRNA variant (SEQ ID NO.)	Guide Design	Assayed Target	Gel 1 - 2 μ L load		Gel 2 - 1 μ L load	
			% intact	% cleaved	% intact	% cleaved
283	WT	TRAC14	100	0	100	0
None		TRAC11	100	0	100	0
None		TRAC14	100	0	100	0

Additional optimized gRNA variants were designed and assayed. Further, different lengths of spacer sequence were also tested to determine how spacer length might affect cleavage efficiency. The sgRNA outside of the spacer sequence is referred to as the “backbone” in this assay. In Table 4, these are denoted as “WT” (SEQ ID NO: 288, the wild type sequence), and the three optimized sgRNAs: V1 (SEQ ID NO: 289), V2 (SEQ ID NO: 290) and V3 (SEQ ID NO: 291). All of these sequences have a poly-N to indicate the location of the spacer sequence. Guides were expressed as sgRNAs by *in vitro* transcription (IVT). Compared to the wild-type sgRNA backbone, V1 is 87.8% identical, V2 is 92.4% identical, and V3 is 85.5% identical. Synthetic tracrRNA:crRNA duplexes (“synthetic”) representing dual-guide RNAs but otherwise similar to the wild type and optimized sgRNAs recited above were also produced and tested.

For this set of assays, RGN APG09106.1 was used; otherwise, methods for *in vitro* cleavage reactions were similar to what is described above. The targeted nucleic acid sequences were Target 1 (SEQ ID NO: 292) and Target 2 (SEQ ID NO: 293). The results are shown in Table 4.

Table 4: Editing efficiency of APG09106.1 with engineered guide variants

RNA Source	Target	Spacer Length	Backbone	Spacer SEQ ID NO.	Cleavage %
Synthetic	2	18	WT	294	12.3
Synthetic	1	20	WT	295	0
Synthetic	2	20	WT	296	55.0
Synthetic	1	25	WT	297	0
Synthetic	2	25	WT	298	61.4
IVT	2	25	V1	299	1.1
IVT	2	25	V2	300	0.9
IVT	2	25	V3	301	0.7
IVT	2	20	V3	302	21.0
IVT	1	25	V3	303	2.0

4.2 RGN APG07433.1

RNA folding predictions were used to determine regions in the guide RNA for APG07433.1 (set forth as SEQ ID NO: 235 and described in U.S. Appl. Publ. No. 2019/0367949 and International Appl. Publ. No. WO 2019/236566, each of which is herein incorporated by reference in its entirety) that can be altered to optimize nuclease activity and shorten the guide RNA for packaging into viral vectors. Two areas were identified as potential locations to make alterations: the repeat:antirepeat pairing between the crRNA and tracrRNA region and the terminal hairpins in the tracrRNA. The repeat:antirepeat region was truncated to 7 (APG07433.1-7bp, SEQ ID NOs: 238 and 239), 13 (APG07433.1-13bp, SEQ ID NOs: 250 and 251), and 15

base pairs in length (APG07433.1-15bp, SEQ ID NOs: 242 and 243). Additionally, a fourth variant was tested that altered the sequence of the repeat:antirepeat region and reduced the pairing to 11 basepairs in length (APG07433.1-11bp-syn, SEQ ID NOs: 240 and 241), with an introduction of a smaller RNA bulge compared to the wildtype guide. Truncations were also made to the terminal hairpins in the tracrRNA, including a reduction of the native sequence to 40 (APG07433.1-40ntTHP, SEQ ID NOs: 254 and 255) and 42 (APG07433.1-42ntTHP, SEQ ID NOs: 244 and 245) nucleotides in length. Altered stem-loops were designed to shorten these to 35 (APG07433.1-35ntTHP-syn, SEQ ID NOs: 248 and 249) and 39 (APG07433.1-39ntTHP-syn, SEQ ID NOs: 246 and 247) nucleotides as well. One guide combined the 13-basepair shortened repeat:antirepeat region and the 42-nucleotide shortened terminal hairpin (APG07433.1-13bp42ntTH, SEQ ID NOs: 252 and 253). The effects of spacer length on cleavage was also tested. Spacer lengths of 25 (APG07433.1-native[25], SEQ ID NOs: 236 and 237; spacer sequence is set forth as SEQ ID NO: 271) and 18 (APG07433.1-native[18], SEQ ID NOs: 256 and 257; spacer sequence is set forth as SEQ ID NO: 272) nucleotides on the wild type guide RNA backbone were also tested.

The following dual guide RNAs were prepared by annealing crRNAs and tracrRNAs by preparing a solution containing 20 μ M crRNA and 10 μ M tracrRNA in annealing buffer (Synthego), then heating to 78°C for 10 min and then at 37°C for 30 min. RNPs were formed by incubation with purified APG07433.1 protein at 0.5 μ M and the dual guide RNA at 1 μ M in phosphate buffered saline for 20 minutes. The guide RNAs used in this experiment are shown in Table 5.

Table 5. Dual guide RNAs

Dual gRNA Name	crRNA SEQ ID NO	tracrRNA SEQ ID NO
APG07433.1-native[25]	236	237
APG07433.1-7bp	238	239
APG07433.1-11bp-syn	240	241
APG07433.1-15bp	242	243
APG07433.1-42ntTHP	244	245
APG07433.1-39ntTHP-syn	246	247
APG07433.1-35ntTHP-syn	248	249
APG07433.1-13bp	250	251
APG07433.1-13bp42ntTH	252	253
APG07433.1-40ntTHP	254	255
APG07433.1-native[18]	256	257
APG07433.1cr-13bp tr-35ntTHP	258	259

These RNPs were incubated with a PCR product containing the appropriate target sequence (SEQ ID NO: 260) produced by amplification using FAM and Cy3 labeled primers, to facilitate accurate and sensitive quantitation of cleavage product. The reactions contained 250 nM RNP, produced as above, and 150 nM of the FAM and Cy3 labeled PCR product in 1X Cutsmart buffer (New England Biolabs). The reaction was allowed to proceed for 15 min at 37°C and was terminated by adding RNase A to 0.1 mg/mL and EDTA to 45 mM. The quenched reaction was heated at 50°C for 30 min and 95°C for 5 minutes. The samples were then analyzed using native acrylamide gel electrophoresis on a 5% TBE gel (Bio-rad). These

were imaged on a ChemiDoc MP imager. Each of the two dyes could be used for quantitation, and each sample was performed in duplicate. The results of this experiment are shown in Table 6.

Table 6. Cleavage results

RNP	% Cleaved				
	Cy3 Channel		FAM channel		Overall average
	Rep 1	Rep 2	Rep 1	Rep 2	
Nuclease only (no guide)	0	0	0	0	0
APG07433.1-native[25]	66.41	61.16	68.81	56.85	63.3
APG07433.1-7bp	0	3.38	2.83	0	1.6
APG07433.1-11bp-syn	66.33	76.38	66.73	68	69.4
APG07433.1-15bp	50.7	65.61	54.62	59.95	57.7
APG07433.1-42ntTHP	44.81	50.59	48.84	51.77	49.0
APG07433.1-39ntTHP-syn	19.16	32.88	24.91	33.43	27.6
APG07433.1-35ntTHP-syn	26.77	38.61	32.09	40.2	34.4
APG07433.1-13bp	44.36	33.03	42.93	35.22	38.9
APG07433.1-13bp42ntTHP	35.91	36.74	33.84	39.2	36.4
APG07433.1-40ntTHP	44.52	57.14	48.34	57.84	52.0
APG07433.1-native[18]	11.1	9.76	12.93	13.24	11.8
APG07433.1cr-13bp tr-35ntTHP	10.66	12.25	12.65	13.54	12.3

This analysis demonstrates that the native backbone outperforms most of the shortened variants, and a variant containing a shortened target sequence (APG07433.1-native[18], SEQ ID NOs: 256 and 257). Of the shortened backbone sequences, that with the highest level of cleavage is the sequence named APG07433.1-11bp-syn, which comprises a 25 nt targeting sequence (spacer; SEQ ID NO: 271), SEQ ID NO: 240 for the crRNA and SEQ ID NO: 241 as the tracrRNA. This guide variant included an altered repeat:antirepeat stem loop with an engineered bulge in the stem.

Example 5: Demonstration of gene editing activity in mammalian cells

RGN expression cassettes were produced and introduced into vectors for mammalian expression. RGNs APG09748, APG09106.1, APG05712.1, APG01658.1, APG05733.1, APG06498.1, APG06646.1, APG09882.1, APG01405.1, and APG01308.1 were each codon-optimized for mammalian expression (SEQ ID NOs: 304, 305, and 411-418, respectively), and the expressed proteins were operably fused at the N-terminal end to an SV40 nuclear localization sequence (NLS; SEQ ID NO: 125) and to 3xFLAG tags (SEQ ID NO: 126), and operably fused at the C-terminal end to nucleoplasmin NLS sequences (SEQ ID NO: 127). Two copies of the NLS sequence were used, operably fused in tandem. Each expression cassette was under control of a cytomegalovirus (CMV) promoter (SEQ ID NO: 306). It is known in the art that the CMV transcription enhancer (SEQ ID NO: 307) may also be included in constructs comprising the CMV promoter. Guide RNA expression constructs encoding a single gRNA each under the control of a human RNA polymerase III U6 promoter (SEQ ID NO: 308) were produced and introduced into an expression vector. Guides targeted regions of selected genes, including RelA, AurkB, GAPDH, LINC01509, HBB, CFTR, HPRT1, TRA, EMX1, and VEGFA, as shown in Table 7. For RNA-guided nuclease APG09106.1,

specific residues were mutated to increase nuclease activity of the protein, specifically the T849 residue of APG09106 was mutated to arginine (SEQ ID NO: 309). This point mutation increased editing rates in mammalian cells.

The constructs described above were introduced into mammalian cells. One day prior to transfection, 1×10^5 HEK293T cells (Sigma) were plated in 24-well dishes in Dulbecco's modified Eagle medium (DMEM) plus 10% (vol/vol) fetal bovine serum (Gibco) and 1% Penicillin-Streptomycin (Gibco). The next day when the cells were at 50-60% confluency, 500 ng of a RGN expression plasmid plus 500 ng of a single gRNA expression plasmid were co-transfected using 1.5 μ L of Lipofectamine 3000 (Thermo Scientific) per well, following the manufacturer's instructions. After 48 hours of growth, total genomic DNA was harvested using a genomic DNA isolation kit (Machery-Nagel) according to the manufacturer's instructions.

The total genomic DNA was then analyzed to determine the rate of editing in the targeted gene. Oligonucleotides were produced to be used for PCR amplification and subsequent analysis of the amplified genomic target site (SEQ ID NOs: 310 and 311). All PCR reactions were performed using 10 μ L of 2X Master Mix Phusion High-Fidelity DNA polymerase (Thermo Scientific) in a 20 μ L reaction including 0.5 μ M of each primer. Large genomic regions encompassing each target gene were first amplified using PCR#1 primers (SEQ ID NOs: 310 and 311), using a program of: 98°C, 1 min; 30 cycles of [98°C, 10 sec; 62°C, 15 sec; 72°C, 5 min]; 72°C, 5 min; 12°C, forever.

One microliter of this PCR reaction was then further amplified using primers specific for each guide (PCR#2 primers; SEQ ID NOs: 365-370), using a program of: 98°C, 1 min; 35 cycles of [98°C, 10 sec; 67°C, 15 sec; 72°C, 30 sec]; 72°C, 5 min; 12°C, forever. Primers for PCR#2 include Nextera Read 1 and Read 2 Transposase Adapter overhang sequences for Illumina sequencing.

Following the second PCR amplification, DNA was cleaned using a PCR cleanup kit (Zymo) according to the manufacturer's instructions and eluted in water. 200-500ng of purified PCR#2 product was combined with 2 μ L of 10X NEB Buffer 2 and water in a 20 μ L reaction and annealed to form heteroduplex DNA using a program of: 95°C, 5 min; 95-85°C, cooled at a rate of 2°C / sec; 85-25°C, cooled at a rate of 0.1°C / sec.; 12°C, forever. Following annealing, 5 μ L of DNA was removed as a no enzyme control, and 1 μ L of T7 Endonuclease I (NEB) was added and the reaction incubated at 37°C for 1 hr. After incubation, 5x FlashGel loading dye (Lonza) was added and 5 μ L of each reaction and controls were analyzed by a 2.2% agarose FlashGel (Lonza) using gel electrophoresis. Following visualization of the gel, the percentage of non-homologous end joining (NHEJ) was determined using the following equation: %NHEJ events = $100 \times [1 - (1 - \text{fraction cleaved})^{1/2}]$, where (fraction cleaved) is defined as: (density of digested products)/(density of digested products + undigested parental band).

For some samples, SURVEYOR® was used to analyze the results following expression in mammalian cells. Cells were incubated at 37° C for 72 h post-transfection before genomic DNA extraction. Genomic DNA was extracted using the QuickExtract DNA Extraction Solution (Epicentre) following the manufacturer's protocol. The genomic region flanking the RGN target site was PCR amplified, and products

were purified using QiaQuick Spin Column (Qiagen) following the manufacturer's protocol. 200-500 ng total of the purified PCR products were mixed with 1 μ l 10 $\times$ Taq DNA Polymerase PCR buffer (Enzymatics) and ultrapure water to a final volume of 10 μ l, and subjected to a re-annealing process to enable heteroduplex formation: 95 $^{\circ}$ C for 10 min, 95 $^{\circ}$ C to 85 $^{\circ}$ C ramping at -2° C/s, 85 $^{\circ}$ C to 25 $^{\circ}$ C at -0.25° C/s, and 25 $^{\circ}$ C hold for 1 min.

After reannealing, products were treated with SURVEYOR[®] nuclease and SURVEYOR[®] enhancer S (Integrated DNA Technologies) following the manufacturer's recommended protocol and analyzed on 4-20% Novex TBE polyacrylamide gels (Life Technologies). Gels were stained with SYBR Gold DNA stain (Life Technologies) for 10 min and imaged with a Gel Doc gel imaging system (Bio-rad). Quantification was based on relative band intensities. Indel percentage was determined by the formula, $100 \times (1 - ((b+c)/(a+b+c))^{1/2})$, where a is the integrated intensity of the undigested PCR product, and b and c are the integrated intensities of each cleavage product.

Additionally, products from PCR#2 containing Illumina overhang sequences underwent library preparation following the Illumina 16S Metagenomic Sequencing Library protocol. Deep sequencing was performed on an Illumina Mi-Seq platform by a service provider (MOGene). Typically, 200,000 of 250 bp paired-end reads (2 x 100,000 reads) are generated per amplicon. The reads were analyzed using CRISPResso (Pinello, et al. 2016 *Nature Biotech*, 34:695-697) to calculate the rates of editing. Output alignments were hand-curated to confirm insertion and deletion sites as well as identify microhomology sites at the recombination sites. The overall rates of editing, as well as the deletion rate and insertion rate in each sample, are shown in Table 7. All experiments were performed in human cells. The "target" is the targeted sequence within the gene target. For each target sequence, the guide RNA comprised the complementary RNA spacer sequence and the appropriate sgRNA depending on the RGN used. A selected breakdown of experiments by guide RNA is shown in Tables 8.1 and 8.2.

Table 7: Overall rates of editing for gene targets

RGN	Gene Target	Guide RNA (SEQ ID NO.)	Target (SEQ ID NO.)	Overall Editing Rate	Deletion Rate	Insertion Rate
APG09106.1	AurkB	316	314	0.55%	100%	
APG09106.1	AurkB	312	315	0.60%	54%	46%
APG09106.1 T849R	AurkB	316	314	2.97%	98%	2.00%
APG09106.1 T849R	AurkB	312	315	2.36%	100%	
APG05712.1	RelA	419	482	0.36%	72.33%	27.67%
APG05712.1	AurkB	420	483	1.39%	25.94%	75.12%
APG05712.1	RelA	421	484	1.04%	32.10%	70.33%
APG05712.1	RelA	422	485	0.28%	64.13%	35.87%
APG01658.1	RelA	423	486	10.03%	70.66%	29.33%
APG01658.1	GAPDH	424	487	5.60%	76.96%	25.97%

RGN	Gene Target	Guide RNA (SEQ ID NO.)	Target (SEQ ID NO.)	Overall Editing Rate	Deletion Rate	Insertion Rate
APG01658.1	LINC01509	425	488	9.87%	75.10%	26.61%
APG01658.1	HBB	426	489	0.42%	63.86%	36.13%
APG01658.1	CFTR	427	490	6.06%	75.42%	27.86%
APG01658.1	AurkB	428	491	13.07%	88.02%	13.14%
APG05733.1	HPRT1	429	492	0.36%		100%
APG05733.1	AurkB	430	493	5.01%	86.40%	13.60%
APG05733.1	RelA	431	494	0.76%		100%
APG05733.1	HPRT1	432	495	0.17%	38.42%	61.58%
APG05733.1	RelA	433	496	0.96%	77.52%	22.48%
APG06498.1	TRA	434	497	35.35%	92.25%	8.29%
APG06498.1	TRA	435	498	21.04%	84.87%	16.34%
APG06498.1	TRA	436	499	10.16%	89.44%	10.69%
APG06498.1	TRA	437	500	13.21%	87.24%	15.00%
APG06498.1	TRA	438	501	13.56%	86.73%	13.66%
APG06498.1	TRA	439	502	9.62%	95.85%	19.90%
APG06498.1	TRA	440	503	1.51%	75.89%	24%
APG06498.1	TRA	441	504	2.63%	88.81%	11.76%
APG06498.1	TRA	442	505	4.27%	52.09%	47.90%
APG06646.1	TRA	443	497	0.33%	0%	100%
APG06646.1	VEGFA	444	506	4.52%	40.83%	63.79%
APG06646.1	AurkB	445	507	0.66%	69.38%	30.62%
APG06646.1	AurkB	446	508	6.55%	79.97%	20.37%
APG06646.1	TRA	447	509	2.47%	73.40%	40.81%
APG06646.1	TRA	448	510	0.14%	34.46%	65.55%
APG06646.1	TRA	449	511	0.61%	0%	100%
APG06646.1	AurkB	450	493	7.94%	67.95%	32.40%
APG06646.1	TRA	451	512	7.03%	86.95%	14.03%
APG06646.1	TRA	452	513	0.50%	21.80%	78.10%
APG06646.1	TRA	453	514	3.53%	86.42%	16.28%
APG06646.1	RelA	454	515	6.22%	80.39%	45.34%
APG06646.1	TRA	455	516	0.38%	0%	100%
APG06646.1	TRA	456	517	0.34%	66.76%	48.22%
APG06646.1	RelA	457	518	3.78%	75.25%	67.90%
APG06646.1	TRA	458	519	0.42%	69.61%	30.39%
APG06646.1	TRA	459	520	0.08%	100.00%	0.00%
APG06646.1	AurkB	460	521	1.09%	30.18%	69.81%
APG06646.1	AurkB	461	522	0.28%	86.60%	21.78%
APG06646.1	AurkB	462	523	10.92%	78.87%	22.05%
APG06646.1	TRA	463	504	0.08%	0%	100%
APG06646.1	TRA	464	505	2.84%	38.26%	61.47%

RGN	Gene Target	Guide RNA (SEQ ID NO.)	Target (SEQ ID NO.)	Overall Editing Rate	Deletion Rate	Insertion Rate
APG06646.1	TRA	465	524	0.43%	71.67%	28.33%
APG06646.1	TRA	466	525	0.03%	0.00%	100.00%
APG06646.1	TRA	467	526	0.08%	100%	0%
APG09882.1	RelA	468	482	0.32%	100.00%	0.00%
APG09882.1	EMX1	469	527	12.84%	82.46%	17.79%
APG09882.1	VEGFA	470	528	14.76%	92.56%	7.77%
APG09882.1	TRA	471	529	14.66%	94.19%	7.33%
APG09882.1	TRA	472	530	7.84%	95.07%	5.75%
APG09882.1	VEGFA	473	531	24.45%	88.96%	11.57%
APG09882.1	TRA	474	532	14.43%	90.24%	10.96%
APG01405.1	RelA	475	482	0.06%	0%	100%
APG01405.1	AurkB	476	533	6.81%	80.58%	20.27%
APG01405.1	AurkB	477	534	0.54%	33.83%	66.16%
APG01405.1	HPRT1	478	535	1.07%	42.14%	57.87%
APG01405.1	RelA	479	484	0.55%	2.07%	97.93%
APG01308.1	HPRT1	480	536	1.19%	80.57%	19.43%
APG01308.1	AurkB	481	537	0.90%	94.97%	6.46%

Specific insertions and deletions for respective guides are shown in Tables 8.1 and 8.2. In these tables, the target sequence is identified by bold upper case letters. The 8mer PAM regions are double underlined, with the main recognized nucleotides in bold. Insertions are identified by lowercase letters. Deletions are indicated with dashes (---). The INDEL location is calculated from the PAM proximal edge of the target sequence, with the edge being location 0. The location is positive (+) if the location is on the target side of the edge; the location is negative (-) if the location is on the PAM side of the edge.

Table 8.1: Specific insertions and deletions for Guide 831 using RGN APG09106.1

Edited target sequence	# Reads	% Reads	% of INDELS	Type	INDEL Location	Size
<u>GTCTGATTG</u> <u>CCTGTCGTTGCCCCCTCC</u> <u>CAGATCAT</u> GGAGGAGTTGGCAGA (wild type; SEQ ID NO: 316)	92294	99.40				
<u>GTCTGATTG</u> <u>CCTGTCGTTGCCCCCTCCCA</u> ----- --AGGAGTTGGCAGA (SEQ ID NO: 317)	263	0.28	54.22	Deletion	+19	8
<u>GTCTGATTG</u> <u>CCTGTCGTTGCCC</u> cctaagtgtatta agcattgtctcagagatttttGGAGGAGTTGGCAG A (SEQ ID NO: 318)	222	0.24	45.77	Insertion	+13	20

Table 8.2: Specific insertions and deletions for Guide 831 using APG09106.1 T849R

Edited target sequence	# Reads	% Reads	% of INDELs	Type	INDEL Location	Size
GTCTG ATTG CCTGTCGTTGCCCC TCC CAGATCAT GGAGGAGTTGGCAGA (wild type; SEQ ID NO: 316)	189881	97.64				
GTCTG ATTG CCTGTCGTTGCCCC----- TGGAGGAGTTGGCAGA (SEQ ID NO: 319)	602	0.309	13.129	Deletion	+14	10
GTCTG ATTG CCTGTCGTTGCCCC TCC CAGATC-- GGAGGAGTTGGCAGA (SEQ ID NO: 320)	394	0.202	8.593	Deletion	+23	2
GTCTG ATTG CCTGTCGTTGCCCC TCC CAGAT---- --AGGAGTTGGCAGA (SEQ ID NO: 321)	399	0.205	8.702	Deletion	+22	5
GTCTG ATTG CCTGTCGTTGCCCC a TC----- TG--GGAGTTGGCAGA (SEQ ID NO: 322)	379	0.194	8.266	Deletion & Mutation	+16	10
GTCTG ATTG CCTGTCGTTGCCCC T C----- TGGAGGAGTTGGCAGA (SEQ ID NO: 323)	350	0.179	7.633	Deletion	+16	8
GTCTG AT ----- TGGAGGAGTTGGCAGA (SEQ ID NO: 324)	309	0.158	6.739	Deletion	-1	26
GTCTG ATTG CCTGTCGTTGCCCC T C----- GGAGGAGTTGGCAGA (SEQ ID NO: 325)	280	0.143	6.106	Deletion	+16	9
GTCTG ATTG CCTGTCGTTGCCCC TCC ----- aGGAGGAGTTGGCAGA (SEQ ID NO: 326)	274	0.140	5.976	Deletion & Mutation	+17	7
GTCTG ATTG CCTGTCGTTGCCCC----- ---GGAGTTGGCAGA (SEQ ID NO: 327)	251	0.129	5.474	Deletion	+13	15
GTCTG ATTG CCTGTCGTTGCCCC----- ATCATGGAGGAGTTGGCAGA (SEQ ID NO: 328)	250	0.128	5.452	Deletion	+13	7
GTCTG ATTG CCTGTCGTTGCCCC T C----- CATGGAGGAGTTGGCAGA (SEQ ID NO: 329)	231	0.118	5.038	Deletion	+16	6
GTCTG ATTG CCTGTCGTTGCCCC TCCCA ----- -----GTACT (SEQ ID NO: 330)	218	0.112	4.754	Deletion	+19	30
GTCTG ATTG CCTGTCGTTGCCCC----- aATC t TGGAGGAGTTGGCAGA (SEQ ID NO: 331)	206	0.105	4.492	Deletion & Mutation	+14	5
GTCTG ATTG CCTGTCGTTGCCCC----- TgggATGGAGGAGTTGGCAGA (SEQ ID NO: 332)	162	0.083	3.533	Deletion & Mutation	+13	8
GTCTG ATTG CCTGTCGTTGCCCC T C----- -----AGTTGGCAGA (SEQ ID NO: 333)	158	0.081	3.446	Deletion	+16	14
GTCTG ATTG CCTGTCGTTGCCCC----- TCATGGAGGAGTTGGCAGA (SEQ ID NO: 334)	122	0.062	2.660	Deletion	+14	7

Example 6: Demonstration of gene editing activity in plant cells

RNA-guided nuclease activity of an RGN of the invention is demonstrated in plant cells using protocols adapted from Li, et al., 2013 (*Nat. Biotech.* 31:688-691). Briefly, a plant codon optimized version of an RGN of the invention (SEQ ID NO: 1, 9, 16, 23, 30, 38, 46, 54, 61, 69, 75, 82, 89, 95, 103, 110, or 117) operably linked to a nucleic acid sequence encoding for an N-terminal SV40 nuclear localization signal are cloned behind the strong constitutive 35S promoter in a transient transformation vector. sgRNAs

targeting one or more sites in the plant PDS gene that flank an appropriate PAM sequence are cloned behind a plant U6 promoter in a second transient expression vector. The expression vectors are introduced into *Nicotiana benthamiana* mesophyll protoplasts using PEG-mediated transformation. The transformed protoplasts are incubated in the dark for up to 36 hr. Genomic DNA is isolated from the protoplasts using a DNeasy Plant Mini Kit (Qiagen). The genomic region flanking the RGN target site is PCR amplified, and products are purified using QiaQuick Spin Column (Qiagen) following the manufacturer's protocol. 200-500 ng total of the purified PCR products are mixed with 1 µl 10× Taq DNA Polymerase PCR buffer (Enzymatics) and ultrapure water to a final volume of 10 µl, and subjected to a re-annealing process to enable heteroduplex formation: 95° C for 10 min, 95° C to 85° C ramping at -2° C/s, 85° C to 25° C at -0.25° C/s, and 25° C hold for 1 min.

After reannealing, products are treated with SURVEYOR nuclease and SURVEYOR enhancer S (Integrated DNA Technologies) following the manufacturer's recommended protocol and analyzed on 4-20% Novex TBE polyacrylamide gels (Life Technologies). Gels are stained with SYBR Gold DNA stain (Life Technologies) for 10 min and imaged with a Gel Doc gel imaging system (Bio-rad). Quantification is based on relative band intensities. Indel percentage is determined by the formula, $100 \times (1 - (1 - (b+c)/(a+b+c))^{1/2})$, where a is the integrated intensity of the undigested PCR product, and b and c are the integrated intensities of each cleavage product.

Example 7: Identification of Disease Targets

A database of clinical variants was obtained from NCBI ClinVar database, which is available through the world wide web at the NCBI ClinVar website. Pathogenic Single Nucleotide Polymorphisms (SNPs) were identified from this list. Using the genomic locus information, CRISPR targets in the region overlapping and surrounding each SNP were identified. A selection of SNPs that can be corrected using base editing in combination with an RGN system comprising APG09748 or APG09106.1 to target the causal mutation ("Casl Mut.") is listed in Table 9.1. A selection of SNPs that can be corrected using base editing in combination with an RGN system comprising APG06646.1 or APG01658.1 to target the causal mutation ("Casl Mut.") is listed in Table 9.2. In both Table 9.1 and 9.2, only one alias of each disease is listed. The "RS#" corresponds to the RS accession number through the SNP database at the NCBI website. The Chromosome Accession number provides accession reference information found through the NCBI website. Tables 9.1 and 9.2 also provide genomic target sequence information suitable for RGN systems APG09748 or APG09106.1 (in Table 9.1), or APG06646.1 or APG01658.1 (in Table 9.2) for each disease. The target sequence information also provides protospacer sequence for the production of the necessary sgRNA for the corresponding RGN of the invention.

Table 9.1: Disease Targets for APG09748 and APG09106.1

Disease	RS#	CasI Mut.	Chromosome Accession	Gene Symbol	Target (SEQ ID NO.)
Stargardt disease 1	1800728	A>G	NC_000001.10,NC_000001.11	ABCA4	313
Glycogen storage disease type 1A	1801175	C>T	NC_000017.10,NC_000017.11	G6PC	335
Severe combined immunodeficiency disease	3218716	C>T	NC_000014.8,NC_000014.9	MYH7	336
Phenylketonuria	5030858	G>A	NC_000012.11,NC_000012.12	PAH	337
Hyperphenylalaninemia	5030860	T>C	NC_000012.11,NC_000012.12	PAH	338
Alpha-1-antitrypsin deficiency	28929474	C>T	NC_000014.8,NC_000014.9	SERPINA1	339
MECP2-Related Disorders	28934906	G>A	NC_000023.10,NC_000023.11	MECP2	340
Inclusion body myopathy 2	28937594	A>G	NC_000009.11,NC_000009.12	GNE	341
Von Willebrand disease	41276738	C>T	NC_000012.11,NC_000012.12	VWF	342
Breast and/or ovarian cancer	41293455	G>A	NC_000017.10,NC_000017.11	BRCA1	343
MECP2-Related Disorders	61750240	G>A	NC_000023.10,NC_000023.11	MECP2	344
MEFV-Related Disorder	61752717	T>C	NC_000016.9,NC_000016.10	MEFV	345
Breast and/or ovarian cancer	62625307	G>A	NC_000017.10,NC_000017.11	BRCA1	346
Breast and/or ovarian cancer	62625308	G>A	NC_000017.10,NC_000017.11	BRCA1	347
Hereditary cancer-predisposing syndrome	63749795	C>T	NC_000003.11,NC_000003.12	MLH1	348
Hereditary cancer-predisposing syndrome	63749849	C>T	NC_000002.11,NC_000002.12	MSH2	349
Hereditary cancer-predisposing syndrome	63750636	C>T	NC_000002.11,NC_000002.12	MSH2	350
Carnitine palmitoyltransferase II deficiency	74315294	C>T	NC_000001.10,NC_000001.11	CPT2	351
Cystic fibrosis	74597325	C>T	NC_000007.13,NC_000007.14	CFTR	352
Deficiency of UDPglucose-hexose-1-phosphate uridylyltransferase	75391579	A>G	NC_000009.11,NC_000009.12	GALT	353
Cystic fibrosis	75527207	G>A	NC_000007.13,NC_000007.14	CFTR	354
Amyloidogenic transthyretin amyloidosis	76992529	G>A	NC_000018.9,NC_000018.10	TTR	355
Cystic fibrosis	77010898	G>A	NC_000007.13,NC_000007.14	CFTR	356

Disease	RS#	CasI Mut.	Chromosome Accession	Gene Symbol	Target (SEQ ID NO.)
Metachromatic leukodystrophy	80338815	C>T	NC_000022.10,NC_000022.11	ARSA	357
Cowden syndrome 3	80338844	C>T	NC_000011.9,NC_000011.10	SDHD	358
Smith-Lemli-Opitz syndrome	80338853	G>A	NC_000011.9,NC_000011.10	DHCR7	359
Breast and/or ovarian cancer	80356962	C>T	NC_000017.10,NC_000017.11	BRCA1	360
Breast and/or ovarian cancer	80357123	G>A	NC_000017.10,NC_000017.11	BRCA1	361
Inborn genetic diseases	80358259	A>G	NC_000018.9,NC_000018.10	NPC1	362
Breast and/or ovarian cancer	80359212	C>T	NC_000013.10,NC_000013.11	BRCA2	363
Fanconi anemia	104886457	G>A	NC_000009.11,NC_000009.12	FANCC	364
SLC26A2-Related Disorders	104893915	C>T	NC_000005.9,NC_000005.10	SLC26A2	365
Cardiomyopathy	104894368	C>T	NC_000012.11,NC_000012.12	MYL2	366
Deafness, X-linked	104894396	C>T	NC_000013.10,NC_000013.11	GJB2	367
Inborn genetic diseases	104894635	C>T	NC_000017.10,NC_000017.11	SGSH	368
Familial Mediterranean fever	104895097	C>T	NC_000016.9,NC_000016.10	MEFV	369
Familial dysautonomia	111033171	A>G	NC_000009.11,NC_000009.12	ELP1	370
Shwachman syndrome	113993993	A>G	NC_000007.13,NC_000007.14	SBDS	371
RYR1-Related Disorders	118192172	C>T	NC_000019.9,NC_000019.10	RYR1	372
Ceroid lipofuscinosis neuronal 2	119455955	G>A	NC_000011.9,NC_000011.10	TPP1	373
Medium-chain acyl-coenzyme A dehydrogenase deficiency	121434274	G>A	NC_000001.10,NC_000001.11	ACADM	374
Primary hyperoxaluria	121908529	G>A	NC_000002.11,NC_000002.12	AGXT	375
Cardiomyopathy	121908987	C>T	NC_000007.13,NC_000007.14	PRKAG2	376
Cowden syndrome	121909219	C>T	NC_000010.10,NC_000010.11	PTEN	377
Cardiomyopathy	121913628	C>T	NC_000014.8,NC_000014.9	MYH7	378
Inborn genetic diseases	121918243	G>A	NC_000001.10,NC_000001.11	MMACHC	379
PTPN11-related disorder	121918457	C>T	NC_000012.11,NC_000012.12	PTPN11	380
Juvenile myelomonocytic leukemia	121918462	C>T	NC_000012.11,NC_000012.12	PTPN11	381
Juvenile myelomonocytic leukemia	121918466	A>G	NC_000012.11,NC_000012.12	PTPN11	382

Disease	RS#	CasI Mut.	Chromosome Accession	Gene Symbol	Target (SEQ ID NO.)
Mucopolysaccharidosis type I	121965020	C>T	NC_000004.11,NC_000004.12	IDUA	383
Ceroid lipofuscinosis neuronal I	137852700	G>A	NC_000001.10,NC_000001.11	PPT1	384
CHEK2-Related Cancer Susceptibility	137853007	G>A	NC_000022.10,NC_000022.11	CHEK2	385
Colorectal cancer	137854568	C>T	NC_000005.9,NC_000005.10	APC	386
Familial hypercholesterolemia	137929307	G>A	NC_000019.9,NC_000019.10	LDLR	387
Cardio-facio-cutaneous syndrome	180177035	T>C	NC_000007.13,NC_000007.14	BRAF	388
Familial cancer of breast	180177083	G>A	NC_000016.10,NC_000016.9	PALB2	389
MYBPC3-Related Disorders	200411226	C>T	NC_000011.9,NC_000011.10	MYBPC3	390
RYR1-Related Disorders	200563280	C>T	NC_000019.9,NC_000019.10	RYR1	391
MYBPC3-Related Disorders	387907267	G>A	NC_000011.9,NC_000011.10	MYBPC3	392
Desmoid disease, hereditary	397515734	C>T	NC_000005.9,NC_000005.10	APC	393
Marfan Syndrome/Loeys-Dietz Syndrome/Familial Thoracic Aortic Aneurysms and Dissections	397515757	C>T	NC_000015.9,NC_000015.10	FBN1	394
Immunodeficiency 14	397518423	G>A	NC_000001.10,NC_000001.11	PIK3CD	395
Inborn genetic diseases	398123009	C>T	NC_000011.9,NC_000011.10	PACS1	396
B lymphoblastic leukemia lymphoma, no ICD-O subtype	529008617	G>A	NC_000001.10,NC_000001.11	MUTYH	397
Familial cancer of breast	587780021	G>A	NC_000002.11,NC_000002.12	BARD1	398
Familial hypercholesterolemia	746118995	C>T	NC_000019.9,NC_000019.10	LDLR	399
Familial hypercholesterolemia	769370816	G>A	NC_000019.10,NC_000019.9	LDLR	400

Table 9.2: Disease Targets for APG6646.1 or APG01658.1

Disease	RGN	RS#	CasI Mut.	Chromosome Accession	Gene Symbol	Target (SEQ ID NO.)
Stargardt disease 1	APG01658.1	1800553	C>T	NC_000001.10,NC_000001.11	ABCA4	538

Disease	RGN	RS#	CasI Mnt.	Chromosome Accession	Gene Symbol	Target (SEQ ID NO.)
Stargardt disease 1	APG06646.1	1800553	C>T	NC_000001.10,NC_000001.11	ABCA4	539
Hereditary cancer-predisposing syndrome	APG01658.1	5030804	A>G	NC_000003.11,NC_000003.12	VHL	540
Hereditary cancer-predisposing syndrome	APG06646.1	5030804	A>G	NC_000003.11,NC_000003.12	VHL	541
Phenylketonuria	APG01658.1	5030851	G>A	NC_000012.11,NC_000012.12	PAH	542
Phenylketonuria	APG06646.1	5030851	G>A	NC_000012.11,NC_000012.12	PAH	543
Melanoma and myeloma	APG01658.1	11547328	G>A	NC_000012.11,NC_000012.12	CDK4	544
Melanoma and myeloma	APG06646.1	11547328	G>A	NC_000012.11,NC_000012.12	CDK4	545
Familial hypercholesterolemia	APG01658.1	11547917	C>T	NC_000019.9,NC_000019.10	LDLR	546
Familial hypercholesterolemia	APG06646.1	11547917	C>T	NC_000019.9,NC_000019.10	LDLR	547
Charcot-Marie-Tooth disease	APG01658.1	61444459	G>A	NC_000001.10,NC_000001.11	LMNA	548
Charcot-Marie-Tooth disease	APG06646.1	61444459	G>A	NC_000001.10,NC_000001.11	LMNA	549
Angelman syndrome	APG01658.1	61751362	G>A	NC_000023.10,NC_000023.11	MECP2	550
Angelman syndrome	APG06646.1	61751362	G>A	NC_000023.10,NC_000023.11	MECP2	551
Phenylketonuria	APG01658.1	62514895	C>T	NC_000012.11,NC_000012.12	PAH	552
Phenylketonuria	APG06646.1	62514895	C>T	NC_000012.11,NC_000012.12	PAH	553
Phenylketonuria	APG01658.1	62516152	C>T	NC_000012.11,NC_000012.12	PAH	554
Phenylketonuria	APG06646.1	62516152	C>T	NC_000012.11,NC_000012.12	PAH	555
Lynch syndrome	APG01658.1	63749795	C>T	NC_000003.11,NC_000003.12	MLH1	556
Lynch syndrome	APG06646.1	63749795	C>T	NC_000003.11,NC_000003.12	MLH1	557
Hereditary cancer	APG01658.1	63749843	C>T	NC_000002.11,NC_000002.12	MSH6	558
Hereditary cancer	APG06646.1	63749843	C>T	NC_000002.11,NC_000002.12	MSH6	559
Hereditary cancer-predisposing syndrome	APG01658.1	63749849	C>T	NC_000002.11,NC_000002.12	MSH2	560
Hereditary cancer-predisposing syndrome	APG06646.1	63749849	C>T	NC_000002.11,NC_000002.12	MSH2	561

Disease	RGN	RS#	CasI Mnt.	Chromosome Accession	Gene Symbol	Target (SEQ ID NO.)
Hereditary cancer-predisposing syndrome	APG01658.1	63750508	C>T	NC_000002.11,NC_000002.12	MSH2	562
Hereditary cancer-predisposing syndrome	APG06646.1	63750508	C>T	NC_000002.11,NC_000002.12	MSH2	563
Hereditary cancer-predisposing syndrome	APG01658.1	63750636	C>T	NC_000002.11,NC_000002.12	MSH2	564
Hereditary cancer-predisposing syndrome	APG06646.1	63750636	C>T	NC_000002.11,NC_000002.12	MSH2	565
Hereditary cancer-predisposing syndrome	APG06646.1	63750871	G>A	NC_000007.13,NC_000007.14	PMS2	566
Lynch syndrome	APG01658.1	63751194	C>T	NC_000003.11,NC_000003.12	MLH1	567
Lynch syndrome	APG06646.1	63751194	C>T	NC_000003.11,NC_000003.12	MLH1	568
Lynch syndrome	APG01658.1	63751711	G>A	NC_000003.11,NC_000003.12	MLH1	569
Lynch syndrome	APG06646.1	63751711	G>A	NC_000003.11,NC_000003.12	MLH1	570
Cystic fibrosis	APG01658.1	75039782	C>T	NC_000007.13,NC_000007.14	CFTR	571
Cystic fibrosis	APG06646.1	75039782	C>T	NC_000007.13,NC_000007.14	CFTR	572
Deafness	APG01658.1	76434661	C>T	NC_000013.10,NC_000013.11	GJB2	573
Deafness	APG06646.1	76434661	C>T	NC_000013.10,NC_000013.11	GJB2	574
Cystic fibrosis	APG01658.1	76713772	G>A	NC_000007.13,NC_000007.14	CFTR	575
Cystic fibrosis	APG06646.1	76713772	G>A	NC_000007.13,NC_000007.14	CFTR	576
Cardiomyopathy	APG01658.1	76992529	G>A	NC_000018.9,NC_000018.10	TTR	577
Cardiomyopathy	APG06646.1	76992529	G>A	NC_000018.9,NC_000018.10	TTR	578
Cystic fibrosis	APG01658.1	77010898	G>A	NC_000007.13,NC_000007.14	CFTR	579
Cystic fibrosis	APG06646.1	77010898	G>A	NC_000007.13,NC_000007.14	CFTR	580
Cystic fibrosis	APG01658.1	80224560	G>A	NC_000007.13,NC_000007.14	CFTR	581
Cystic fibrosis	APG06646.1	80224560	G>A	NC_000007.13,NC_000007.14	CFTR	582
Cowden syndrome	APG01658.1	80338844	C>T	NC_000011.9,NC_000011.10	SDHD	583
Cowden syndrome	APG06646.1	80338844	C>T	NC_000011.9,NC_000011.10	SDHD	584

Disease	RGN	RS#	CasI Mutt.	Chromosome Accession	Gene Symbol	Target (SEQ ID NO.)
SLC26A4-Related Disorders	APG01658.1	80338849	G>A	NC_000007.13,NC_000007.14	SLC26A4	585
SLC26A4-Related Disorders	APG06646.1	80338849	G>A	NC_000007.13,NC_000007.14	SLC26A4	586
Breast-ovarian cancer	APG01658.1	80356885	C>T	NC_000017.10,NC_000017.11	BRCA1	587
Breast-ovarian cancer	APG06646.1	80356885	C>T	NC_000017.10,NC_000017.11	BRCA1	588
Breast-ovarian cancer	APG01658.1	80358557	C>T	NC_000013.10,NC_000013.11	BRCA2	589
Breast-ovarian cancer	APG06646.1	80358557	C>T	NC_000013.10,NC_000013.11	BRCA2	590
Breast-ovarian cancer	APG01658.1	80358650	G>A	NC_000013.10,NC_000013.11	BRCA2	591
Breast-ovarian cancer	APG06646.1	80358650	G>A	NC_000013.10,NC_000013.11	BRCA2	592
Breast-ovarian cancer	APG01658.1	80358659	C>T	NC_000013.10,NC_000013.11	BRCA2	593
Breast-ovarian cancer	APG06646.1	80358659	C>T	NC_000013.10,NC_000013.11	BRCA2	594
Breast-ovarian cancer	APG06646.1	80358663	C>T	NC_000013.10,NC_000013.11	BRCA2	595
Breast-ovarian cancer	APG01658.1	80358871	G>A	NC_000013.10,NC_000013.11	BRCA2	596
Breast-ovarian cancer	APG06646.1	80358871	G>A	NC_000013.10,NC_000013.11	BRCA2	597
Breast-ovarian cancer	APG01658.1	80358920	C>T	NC_000013.10,NC_000013.11	BRCA2	598
Breast-ovarian cancer	APG06646.1	80358920	C>T	NC_000013.10,NC_000013.11	BRCA2	599
Breast-ovarian cancer	APG01658.1	80358972	C>T	NC_000013.10,NC_000013.11	BRCA2	600
Breast-ovarian cancer	APG06646.1	80358972	C>T	NC_000013.10,NC_000013.11	BRCA2	601
Breast-ovarian cancer	APG01658.1	80358981	C>T	NC_000013.10,NC_000013.11	BRCA2	602
Breast-ovarian cancer	APG06646.1	80358981	C>T	NC_000013.10,NC_000013.11	BRCA2	603
Breast-ovarian cancer	APG01658.1	80359003	G>A	NC_000013.10,NC_000013.11	BRCA2	604
Breast-ovarian cancer	APG06646.1	80359003	G>A	NC_000013.10,NC_000013.11	BRCA2	605
Breast-ovarian cancer	APG01658.1	80359004	G>A	NC_000013.10,NC_000013.11	BRCA2	606
Breast-ovarian cancer	APG06646.1	80359004	G>A	NC_000013.10,NC_000013.11	BRCA2	607
Breast-ovarian cancer	APG01658.1	80359013	G>A	NC_000013.10,NC_000013.11	BRCA2	608
Breast-ovarian cancer	APG06646.1	80359013	G>A	NC_000013.10,NC_000013.11	BRCA2	609

Disease	RGN	RS#	CasI Mutt.	Chromosome Accession	Gene Symbol	Target (SEQ ID NO.)
Breast-ovarian cancer	APG01658.1	80359027	G>A	NC_000013.10,NC_000013.11	BRCA2	610
Breast-ovarian cancer	APG06646.1	80359027	G>A	NC_000013.10,NC_000013.11	BRCA2	611
Breast-ovarian cancer	APG01658.1	80359071	G>A	NC_000013.10,NC_000013.11	BRCA2	612
Breast-ovarian cancer	APG06646.1	80359071	G>A	NC_000013.10,NC_000013.11	BRCA2	613
Breast-ovarian cancer	APG01658.1	80359115	C>T	NC_000013.10,NC_000013.11	BRCA2	614
Breast-ovarian cancer	APG06646.1	80359115	C>T	NC_000013.10,NC_000013.11	BRCA2	615
Breast-ovarian cancer	APG01658.1	80359140	C>T	NC_000013.10,NC_000013.11	BRCA2	616
Breast-ovarian cancer	APG06646.1	80359140	C>T	NC_000013.10,NC_000013.11	BRCA2	617
Breast-ovarian cancer	APG06646.1	80359159	C>T	NC_000013.10,NC_000013.11	BRCA2	618
Breast-ovarian cancer	APG01658.1	80359180	C>T	NC_000013.10,NC_000013.11	BRCA2	619
Breast-ovarian cancer	APG06646.1	80359180	C>T	NC_000013.10,NC_000013.11	BRCA2	620
Breast-ovarian cancer	APG01658.1	81002846	G>A	NC_000013.10,NC_000013.11	BRCA2	621
Breast-ovarian cancer	APG06646.1	81002846	G>A	NC_000013.10,NC_000013.11	BRCA2	622
Breast-ovarian cancer	APG06646.1	81002862	A>G	NC_000013.10,NC_000013.11	BRCA2	623
Fanconi anemia	APG01658.1	104886457	G>A	NC_000009.11,NC_000009.12	FANCC	624
Fanconi anemia	APG06646.1	104886457	G>A	NC_000009.11,NC_000009.12	FANCC	625
Achondrogenesis	APG01658.1	104893915	C>T	NC_000005.9,NC_000005.10	SLC26A2	626
Achondrogenesis	APG06646.1	104893915	C>T	NC_000005.9,NC_000005.10	SLC26A2	627
RYR1-Related Disorders	APG01658.1	118192172	C>T	NC_000019.9,NC_000019.10	RYR1	628
RYR1-Related Disorders	APG06646.1	118192172	C>T	NC_000019.9,NC_000019.10	RYR1	629
CHEK2-Related Cancer Susceptibility	APG01658.1	137853007	G>A	NC_000022.10,NC_000022.11	CHEK2	630
CHEK2-Related Cancer Susceptibility	APG06646.1	137853007	G>A	NC_000022.10,NC_000022.11	CHEK2	631
Neurofibromatosis	APG06646.1	137854550	A>G	NC_000017.10,NC_000017.11	NF1	632
Neurofibromatosis	APG01658.1	137854556	G>A	NC_000017.10,NC_000017.11	NF1	633
Neurofibromatosis	APG06646.1	137854556	G>A	NC_000017.10,NC_000017.11	NF1	634

Disease	RGN	RS#	CasI Mutt.	Chromosome Accession	Gene Symbol	Target (SEQ ID NO.)
Myopathy	APG01658.1	193922390	C>T	NC_000014.8,NC_000014.9	MYH7	635
Myopathy	APG06646.1	193922390	C>T	NC_000014.8,NC_000014.9	MYH7	636
Long QT syndrome	APG01658.1	199473428	C>T	NC_000007.13,NC_000007.14	KCNH2	637
Long QT syndrome	APG06646.1	199473428	C>T	NC_000007.13,NC_000007.14	KCNH2	638
Phenylketonuria	APG01658.1	199475575	G>A	NC_000012.11,NC_000012.12	PAH	639
Phenylketonuria	APG06646.1	199475575	G>A	NC_000012.11,NC_000012.12	PAH	640
Phenylketonuria	APG01658.1	199475679	C>T	NC_000012.11,NC_000012.12	PAH	641
Phenylketonuria	APG06646.1	199475679	C>T	NC_000012.11,NC_000012.12	PAH	642
MYBPC3-Related Disorders	APG01658.1	200411226	C>T	NC_000011.9,NC_000011.10	MYBPC3	643
MYBPC3-Related Disorders	APG06646.1	200411226	C>T	NC_000011.9,NC_000011.10	MYBPC3	644
Myopathy	APG06646.1	267606908	T>C	NC_000014.8,NC_000014.9	MYH7	645
Cardiomyopathy	APG01658.1	267607003	C>T	NC_000010.10,NC_000010.11	RBM20	646
Cardiomyopathy	APG06646.1	267607003	C>T	NC_000010.10,NC_000010.11	RBM20	647
Noonan syndrome	APG01658.1	267607048	A>G	NC_000010.10,NC_000010.11	SHOC2	648
Noonan syndrome	APG06646.1	267607048	A>G	NC_000010.10,NC_000010.11	SHOC2	649
Familial hypercholesterolemia	APG01658.1	267607213	G>A	NC_000019.9,NC_000019.10	LDLR	650
Familial hypercholesterolemia	APG06646.1	267607213	G>A	NC_000019.9,NC_000019.10	LDLR	651
Lynch syndrome	APG01658.1	267607789	G>A	NC_000003.11,NC_000003.12	MLH1	652
Lynch syndrome	APG06646.1	267607789	G>A	NC_000003.11,NC_000003.12	MLH1	653
Lynch syndrome	APG01658.1	267607845	G>A	NC_000003.11,NC_000003.12	MLH1	654
Lynch syndrome	APG06646.1	267607845	G>A	NC_000003.11,NC_000003.12	MLH1	655
Hereditary cancer-predisposing syndrome	APG01658.1	267608098	A>G	NC_000002.11,NC_000002.12	MSH6	656
Hereditary cancer-predisposing syndrome	APG06646.1	267608098	A>G	NC_000002.11,NC_000002.12	MSH6	657
MYBPC3-Related Disorders	APG01658.1	387906397	A>G	NC_000011.9,NC_000011.10	MYBPC3	658

Disease	RGN	RS#	CasI Mut.	Chromosome Accession	Gene Symbol	Target (SEQ ID NO.)
MYBPC3-Related Disorders	APG06646.1	387906397	A>G	NC_000011.9,NC_000011.10	MYBPC3	659
Breast-ovarian cancer	APG01658.1	387906843	G>A	NC_000017.10,NC_000017.11	RAD51D	660
Breast-ovarian cancer	APG06646.1	387906843	G>A	NC_000017.10,NC_000017.11	RAD51D	661
MYBPC3-Related Disorders	APG01658.1	387907267	G>A	NC_000011.9,NC_000011.10	MYBPC3	662
MYBPC3-Related Disorders	APG06646.1	387907267	G>A	NC_000011.9,NC_000011.10	MYBPC3	663
Breast-ovarian cancer	APG01658.1	397507389	G>A	NC_000013.10,NC_000013.11	BRCA2	664
Breast-ovarian cancer	APG06646.1	397507389	G>A	NC_000013.10,NC_000013.11	BRCA2	665
Breast-ovarian cancer	APG01658.1	397507404	G>A	NC_000013.10,NC_000013.11	BRCA2	666
Breast-ovarian cancer	APG06646.1	397507404	G>A	NC_000013.10,NC_000013.11	BRCA2	667
Leukemia	APG01658.1	397507545	G>A	NC_000012.11,NC_000012.12	PTPN11	668
Leukemia	APG06646.1	397507545	G>A	NC_000012.11,NC_000012.12	PTPN11	669
Leukemia	APG01658.1	397507547	A>G	NC_000012.11,NC_000012.12	PTPN11	670
Leukemia	APG06646.1	397507547	A>G	NC_000012.11,NC_000012.12	PTPN11	671
Breast-ovarian cancer	APG01658.1	397507922	G>A	NC_000013.10,NC_000013.11	BRCA2	672
Breast-ovarian cancer	APG06646.1	397507922	G>A	NC_000013.10,NC_000013.11	BRCA2	673
Marfan syndrome	APG01658.1	397515757	C>T	NC_000015.9,NC_000015.10	FBN1	674
Marfan syndrome	APG06646.1	397515757	C>T	NC_000015.9,NC_000015.10	FBN1	675
MYBPC3-Related Disorders	APG01658.1	397516074	C>T	NC_000011.9,NC_000011.10	MYBPC3	676
MYBPC3-Related Disorders	APG06646.1	397516074	C>T	NC_000011.9,NC_000011.10	MYBPC3	677
Cardiomyopathy	APG01658.1	397516354	C>T	NC_000019.9,NC_000019.10	TNNI3	678
Cardiomyopathy	APG06646.1	397516354	C>T	NC_000019.9,NC_000019.10	TNNI3	679
Immunodeficiency	APG01658.1	397518423	G>A	NC_000001.10,NC_000001.11	PIK3CD	680
Immunodeficiency	APG06646.1	397518423	G>A	NC_000001.10,NC_000001.11	PIK3CD	681
B lymphoblastic leukemia lymphoma	APG01658.1	529008617	G>A	NC_000001.10,NC_000001.11	MUTYH	682
B lymphoblastic leukemia lymphoma	APG06646.1	529008617	G>A	NC_000001.10,NC_000001.11	MUTYH	683

Disease	RGN	RS#	CasI Mnt.	Chromosome Accession	Gene Symbol	Target (SEQ ID NO.)
Familial hypercholesterolemia	APG01658.1	570942190	C>T	NC_000019.9,NC_000019.10	LDLR	684
Familial hypercholesterolemia	APG06646.1	570942190	C>T	NC_000019.9,NC_000019.10	LDLR	685
Ataxia-telangiectasia syndrome	APG01658.1	587776551	G>A	NC_000011.10,NC_000011.9	ATM	686
Ataxia-telangiectasia syndrome	APG06646.1	587776551	G>A	NC_000011.10,NC_000011.9	ATM	687
Adenoid cystic carcinoma	APG01658.1	587778720	C>T	NC_000017.10,NC_000017.11	TP53	688
Adenoid cystic carcinoma	APG06646.1	587778720	C>T	NC_000017.10,NC_000017.11	TP53	689
Hereditary cancer-predisposing syndrome	APG01658.1	587779075	C>T	NC_000002.11,NC_000002.12	MSH2	690
Hereditary cancer-predisposing syndrome	APG06646.1	587779075	C>T	NC_000002.11,NC_000002.12	MSH2	691
Hereditary cancer-predisposing syndrome	APG06646.1	587779333	T>C	NC_000007.13,NC_000007.14	PMS2	692
Ataxia-telangiectasia syndrome	APG01658.1	587779866	A>G	NC_000011.10,NC_000011.9	ATM	693
Ataxia-telangiectasia syndrome	APG06646.1	587779866	A>G	NC_000011.10,NC_000011.9	ATM	694
Breast cancer	APG01658.1	587780021	G>A	NC_000002.11,NC_000002.12	BARD1	695
Breast cancer	APG06646.1	587780021	G>A	NC_000002.11,NC_000002.12	BARD1	696
Breast-ovarian cancer	APG01658.1	587781629	G>A	NC_000013.10,NC_000013.11	BRCA2	697
Breast-ovarian cancer	APG06646.1	587781629	G>A	NC_000013.10,NC_000013.11	BRCA2	698
Hereditary cancer-predisposing syndrome	APG01658.1	587782144	C>T	NC_000017.10,NC_000017.11	TP53	699
Hereditary cancer-predisposing syndrome	APG06646.1	587782144	C>T	NC_000017.10,NC_000017.11	TP53	700
Marfan syndrome	APG01658.1	727503054	A>G	NC_000015.9,NC_000015.10	FBN1	701
Marfan syndrome	APG06646.1	727503054	A>G	NC_000015.9,NC_000015.10	FBN1	702
Noonan syndrome	APG01658.1	727503110	T>C	NC_000012.11,NC_000012.12	KRAS	703
Noonan syndrome	APG06646.1	727503110	T>C	NC_000012.11,NC_000012.12	KRAS	704
Familial hypercholesterolemia	APG01658.1	746118995	C>T	NC_000019.9,NC_000019.10	LDLR	705
Familial hypercholesterolemia	APG06646.1	746118995	C>T	NC_000019.9,NC_000019.10	LDLR	706

Disease	RGN	RS#	CasI Mut.	Chromosome Accession	Gene Symbol	Target (SEQ ID NO.)
Familial hypercholesterolemia	APG01658.1	748944640	G>A	NC_000019.9,NC_000019.10	LDLR	707
Familial hypercholesterolemia	APG06646.1	748944640	G>A	NC_000019.9,NC_000019.10	LDLR	708
Familial hypercholesterolemia	APG01658.1	765696008	G>A	NC_000019.10,NC_000019.9	LDLR	709
Familial hypercholesterolemia	APG06646.1	765696008	G>A	NC_000019.10,NC_000019.9	LDLR	710
Familial hypercholesterolemia	APG01658.1	769370816	G>A	NC_000019.10,NC_000019.9	LDLR	711
Familial hypercholesterolemia	APG06646.1	769370816	G>A	NC_000019.10,NC_000019.9	LDLR	712
Familial hypercholesterolemia	APG01658.1	769737896	C>T	NC_000019.10,NC_000019.9	LDLR	713
Familial hypercholesterolemia	APG06646.1	769737896	C>T	NC_000019.10,NC_000019.9	LDLR	714
Familial hypercholesterolemia	APG01658.1	771019366	A>G	NC_000019.10,NC_000019.9	LDLR	715
Familial hypercholesterolemia	APG06646.1	771019366	A>G	NC_000019.10,NC_000019.9	LDLR	716
Paragangliomas	APG01658.1	772551056	C>T	NC_000001.11,NC_000001.10	SDHB	717
Paragangliomas	APG06646.1	772551056	C>T	NC_000001.11,NC_000001.10	SDHB	718
Hereditary cancer	APG01658.1	786201042	C>T	NC_000002.12,NC_000002.11	MSH6	719
Hereditary cancer	APG06646.1	786201042	C>T	NC_000002.12,NC_000002.11	MSH6	720
Familial hypercholesterolemia	APG01658.1	875989907	G>A	NC_000019.9,NC_000019.10	LDLR	721
Familial hypercholesterolemia	APG06646.1	875989907	G>A	NC_000019.9,NC_000019.10	LDLR	722
Familial hypercholesterolemia	APG01658.1	879254797	G>A	NC_000019.10,NC_000019.9	LDLR	723
Familial hypercholesterolemia	APG06646.1	879254797	G>A	NC_000019.10,NC_000019.9	LDLR	724
Familial hypercholesterolemia	APG01658.1	879254871	C>T	NC_000019.10,NC_000019.9	LDLR	725
Familial hypercholesterolemia	APG06646.1	879254871	C>T	NC_000019.10,NC_000019.9	LDLR	726
Familial hypercholesterolemia	APG01658.1	879255000	T>C	NC_000019.10,NC_000019.9	LDLR	727
Familial hypercholesterolemia	APG06646.1	879255000	T>C	NC_000019.10,NC_000019.9	LDLR	728

Example 8: Targeting mutations responsible for Friedreich Ataxia

The expansion of the trinucleotide repeat sequence causing Friedreich's Ataxia (FRDA) occurs in a defined genetic locus within the FXN gene, referred to as the FRDA instability region. RNA guided nucleases (RGNs) may be used for excising the instability region in FRDA patient cells. This approach requires 1) an RGN and guide RNA sequence that can be programmed to target the allele in the human genome; and 2) a delivery approach for the RGN and guide sequence. Many nucleases used for genome editing, such as the commonly used Cas9 nuclease from *S. pyogenes* (SpCas9), are too large to be packaged into adeno-associated viral (AAV) vectors, especially when considering the length of the SpCas9 gene and the guide RNA in addition to other genetic elements required for functional expression cassettes. This makes a viable approach using SpCas9 unlikely.

The compact RNA guided nucleases of the invention, such as APG09748, APG09106.1, and APG06646.1, are uniquely well suited for the excision of the FRDA instability region. Each RGN has a PAM requirement that is in the vicinity of the FRDA instability region. Additionally, each of these RGNs can be packaged into an AAV vector along with a guide RNA. Packing two guide RNAs would likely require a second vector, but this approach still compares favorably to what would be required of a larger nuclease such as SpCas9, which would require splitting the protein sequence between two vectors.

Table 10 shows the location of genomic target sequences suitable for targeting APG09748, APG09106.1, or APG06646.1 to the 5' and 3' flanks of the FRDA instability region, as well as the sequence of the sgRNAs for the genomic targets. Once at the locus, the RGN would excise the FA instability region. Excision of the region can be verified with Illumina sequencing of the locus.

Table 10: Genomic target sequences for RGN systems

Location relative to FRDA instability region	Genome target sequence for APG09748 or APG09106.1 (SEQ ID NO.)	sgRNA for APG09748 or APG09106.1 (SEQ ID NO.)	Genome target sequence for APG06646.1 (SEQ ID NO.)	sgRNA for APG06646.1 (SEQ ID NO.)
5'	401	405	729	733
5'	402	406	730	734
3'	403	407	731	735
3'	404	408	732	736

Example 9: Targeting mutations responsible for Sickle Cell Diseases

Targeting sequences within the BCL11A enhancer region (SEQ ID NO: 220) may provide a mechanism for increasing fetal hemoglobin (HbF) to either cure or alleviate the symptoms of sickle cell diseases. For example, genome wide association studies have identified a set of genetic variations at BCL11A that are associated with increased HbF levels. These variations are a collection of SNPs found in non-coding regions of BCL11A that function as a stage-specific, lineage-restricted enhancer region. Further investigation revealed that this BCL11A enhancer is required in erythroid cells for BCL11A expression

(Bauer et al, (2013) *Science* 343:253-257, incorporated by reference herein). The enhancer region was found within intron 2 of the BCL11A gene, and three areas of DNaseI hypersensitivity (often indicative of a chromatin state that is associated with regulatory potential) in intron 2 were identified. These three areas were identified as “+62”, “+58” and “+55” in accordance with the distance in kilobases from the transcription start site of BCL11A. These enhancer regions are roughly 350 (+55); 550 (+58); and 350 (+62) nucleotides in length (Bauer et al., 2013).

Example 9.1: Identifying preferred RGN systems

Here is described a potential treatment for beta-hemoglobinopathies using an RGN system that disrupts BCL11A binding to its binding site within the HBB locus, which is the gene responsible for making beta-globin in adult hemoglobin. This approach uses NHEJ which is more efficient in mammalian cells. In addition, this approach uses a nuclease of sufficiently small size that can be packaged into a single AAV vector for *in vivo* delivery.

The GATA1 enhancer motif in the human BCL11A enhancer region (SEQ ID NO: 220) is an ideal target for disruption using RNA guided nucleases (RGNs) to reduce BCL11A expression with concurrent re-expression of HbF in adult human erythrocytes (Wu et al. (2019) *Nat Med* 387:2554). Several PAM sequences compatible with APG09748 or APG09106.1 are readily apparent at the genetic locus surrounding this GATA1 site. These nucleases have a PAM sequence of 5'-DTTN-3' (SEQ ID NO: 60) and are compact in size, potentially allowing their delivery along with an appropriate guide RNA in a single AAV or adenoviral vector. In addition to its size, APG06646.1 has a minimal PAM requirement (SEQ ID NO: 109) which makes it well-suited for this approach. This delivery approach bestows multiple advantages relative to others, such as access to hematopoietic stem cells and a well-established safety profile and manufacturing techniques.

The commonly used Cas9 nuclease from *S. pyogenes* (SpyCas9) requires a PAM sequence of 5'-NGG-3', (SEQ ID NO: 101) several of which are present near the GATA1 motif. However, the size of SpyCas9 prevents packaging into a single AAV or adenoviral vector and thus forgoes the aforementioned advantages of this approach. While a dual delivery strategy may be employed, it would add significant manufacturing complexity and cost. Additionally, dual viral vector delivery significantly decreases the efficiency of gene correction, since a successful edit in a given cell requires infection with both vectors.

An expression cassette encoding a human codon optimized APG09748 (SEQ ID NO: 409), APG09106.1 (SEQ ID NO: 410), or APG06646.1 (SEQ ID NO: 415) is produced, similar to those described in Example 5. Expression cassettes which express guide RNAs for RGNs APG09748, APG09106.1, or APG06646.1 are also produced. These guide RNAs comprise: 1) a protospacer sequence that is complementary to either the non-coding or coding DNA strand within the BCL11A enhancer locus (the target sequence) and 2) an RNA sequence required for association of the guide RNA with the RGN. Because several potential PAM sequences for targeting by each RGN surround the BCL11A GATA1 enhancer motif, several potential guide RNA constructs are produced to determine the best protospacer

sequence that produces robust cleavage and NHEJ mediated disruption of the BCL11A GATA1 enhancer sequence. The target genomic sequences in Table 11 are evaluated using the sgRNA provided in Table 11.

Table 11: Target Sequences for BCL11A GATA1 enhancer locus using APG06646.1

Target genomic sequence for APG09748 or APG0916.1 (SEQ ID NO.)	sgRNA for APG09748 or APG0916.1 (SEQ ID NO.)	Target genomic sequence for APG06646.1 (SEQ ID NO.)	sgRNA for APG06646.1 (SEQ ID NO.)
221	224	737	740
222	269	738	741
223	270	739	742

5

To evaluate the efficiency with which APG09748, APG09106.1, or APG06646.1 generates insertions or deletions that disrupt the BCL11A enhancer region, human cell lines such as human embryonic kidney cells (HEK cells) are used. A DNA vector comprising an RGN expression cassette (for example, as described in Example 5) is produced. A separate vector comprising an expression cassette comprising a coding sequence for a guide RNA sequence of Table 11 is also produced. Such an expression cassette may further comprise a human RNA polymerase III U6 promoter (SEQ ID NO: 308), as described in Example 5. Alternatively, a single vector comprising expression cassettes of both the RGN and guide RNA may be used. The vector is introduced into HEK cells using standard techniques such as those described in Example 5, and the cells are cultured for 1-3 days. Following this culture period, genomic DNA is isolated and the frequency of insertions or deletions is determined by using T7 Endonuclease I digestion and/or direct DNA sequencing, as described in Example 5.

A region of DNA encompassing the target BCL11A region is amplified by PCR with primers containing Illumina Nextera XT overhang sequences. These PCR amplicons are either examined for NHEJ formation using T7 Endonuclease I digestion or undergo library preparation following the Illumina 16S Metagenomic Sequencing Library protocol or a similar Next Generation Sequencing (NGS) library preparation. Following deep sequencing, the reads generated are analyzed by CRISPResso to calculate rates of editing. Output alignments are hand-curated to confirm insertion and deletion sites. This analysis identifies the preferred RGN and the corresponding preferred guide RNA (sgRNA). The analysis may result in either APG09748, APG09106.1, or APG06646.1 being equally preferred or that one RGN is most preferred. Additionally, the analysis may determine there is more than one preferred guide RNA, or that all target genomic sequences in Table 17 are equally preferred.

Example 9.2: Assay for expression of fetal hemoglobin

In this example, APG09748, APG09106.1, or APG06646.1 generated insertions or deletions disrupting the BCL11A enhancer region are assayed for expression of fetal hemoglobin. Healthy human donor CD34⁺ hematopoietic stem cells (HSCs) are used. These HSCs are cultured and vector(s) comprising

expression cassettes comprising the coding regions of the preferred RGN and the preferred sgRNA are introduced using methods similar to those described in Example 5. Alternatively, electroporation may be used. Following electroporation, these cells are differentiated *in vitro* into erythrocytes using established protocols (for example, Giarratana et al. (2004) *Nat Biotechnology* 23:69-74, herein incorporated by reference). The expression of HbF is then measured using western blotting with an anti-human HbF antibody or quantified via High Performance Liquid Chromatography (HPLC). It is expected that successful disruption of the BCL11A enhancer locus will lead to an increase in HbF production when compared to HSCs electroporated with only the RGN but no guide.

Example 9.3: Assay for decreased sickle cell formation

In this example, APG09748, APG09106.1, or APG06646.1 generated insertions or deletions disrupting the BCL11A enhancer region are assayed for decreased sickle-cell formation. Donor CD34⁺ hematopoietic stem cells (HSCs) from patients afflicted with sickle cell disease are used. These HSCs are cultured and vector(s) comprising expression cassettes comprising the coding regions of preferred RGN and the preferred sgRNA are introduced using methods similar to those described in Example 5. Alternatively, electroporation may be used. Following electroporation, these cells are differentiated *in vitro* into erythrocytes using established protocols (Giarratana et al. (2004) *Nat Biotechnology* 23:69-74). The expression of HbF is then measured using western blotting with an anti-human HbF antibody or quantified via High Performance Liquid Chromatography (HPLC). It is expected that successful disruption of the BCL11A enhancer locus will lead to an increase in HbF production when compared to HSCs electroporated with only the RGN but no guide.

Sickle cell formation is induced in these differentiated erythrocytes by the addition of metabisulfite. The numbers of sickled vs normal erythrocytes are counted using a microscope. It is expected that the numbers of sickled cells are less in cells treated with APG09748, APG09106.1, or APG06646.1 plus sgRNAs than with cells untreated, or treated with RGNs alone.

Example 9.4: Disease treatment validation in a murine model

To evaluate the efficacy of using APG09748, APG09106.1, or APG06646.1 disruption of the BCL11A locus, suitable humanized mouse models of sickle cell anemia are used. Expression cassettes encoding for the preferred RGN and for the preferred sgRNA are packaged into AAV vectors or adenovirus vectors. In particular, adenovirus type Ad5/35 is effective at targeting HSCs. A suitable mouse model containing a humanized HBB locus with sickle cell alleles is chosen such as B6;FVB-Tg(LCR-HBA2,LCR-HBB*E26K)53Hhb/J or B6.Cg-Hbatm1Paz Hbbtm1Tow Tg(HBA-HBBs)41Paz/HhbJ. These mice are treated with granulocyte colony-stimulating factor alone or in combination with plerixafor to mobilize HSCs into circulation. AAVs or adenoviruses carrying the RGN and guide plasmid are then injected intravenously, and the mice are allowed to recover for a week. Blood obtained from these mice is tested in an *in vitro* sickling assay using metabisulfite, and the mice are followed longitudinally to monitor mortality

rates and hematopoietic function. It is expected that treatment with AAVs or adenoviruses carrying an RGN and guide RNA will reduce sickling, mortality, and improve hematopoietic function when compared to mice treated with viruses lacking both expression cassettes, or with viruses carrying the RGN expression cassette alone.

5

Example 10: Base editing activity in mammalian cells

An expression cassette that produces a cytidine deaminase-RGN fusion protein was constructed as follows. The coding sequence for RGNs (APG06646.1 described herein and APG08290.1, which was described in U.S. Appl. Publ. No. 2019/0367949 and International Appl. Publ. No. WO 2019/236566, each of which is herein incorporated by reference in its entirety) was codon optimized for mammalian expression and mutated to function as a nickase (SEQ ID NOs: 128 and 262, respectively). This coding sequence was introduced into an expression cassette which produces a fusion protein comprising an NLS at its N-terminal end (SEQ ID NO: 125), operably linked at its C-terminal end to a 3xFLAG tag (SEQ ID NO: 126), operably linked at its C-terminal end to a cytidine deaminase (SEQ ID NOs: 129-132, all of which are disclosed in International Appl. No. PCT/US2019/068079, which is herein incorporated by reference in its entirety) operably linked on its C-terminal end to an amino acid linker (SEQ ID NO: 133), operably linked on its C-terminal end to the RGN nickase, operably linked at its C-terminal end to a second NLS (SEQ ID NO: 127). These expression cassettes were each introduced into a pTwist CMV vector (Twist Bioscience) capable of driving expression of the fusion protein in mammalian cells. Separate vectors were also produced that expressed guide RNAs under control of the human U6 promoter, in mammalian cells. These guide RNAs (SEQ ID NOs: 134-136 for nAPG06646.1 and SEQ ID NOs: 263-265 for nAPG08290.1) are capable of guiding the deaminase-nRGN fusion proteins, or the RGN itself, to a targeted genomic sequence for base editing or gene editing, respectively.

500 ng of cytidine deaminase-RGN expression plasmids or standard RGN expression plasmid, and 500 ng of the guide RNA expression plasmids, were co-transfected into HEK293FT cells at 75-90% confluency in 24-well plates using Lipofectamine 2000 reagent (Life Technologies). Cells were then incubated at 37° C for 72 h. Genomic DNA was then extracted using the NucleoSpin 96 Tissue (Macherey-Nagel) following the manufacturer's protocol. The genomic region flanking the guideRNA target site was PCR amplified, and products were purified using ZR-96 DNA Clean and Concentrator (Zymo Research) following the manufacturer's protocol. The purified PCR products were then sent for Next Generation Sequencing on Illumina MiSeq (2x250). Results were analyzed for indel formation or specific cytosine mutation.

Table 12 below shows cytidine base editing and indel formation of each construct and guide combination. Interestingly, when comparing the activity of the RGN APG06646.1 with that of cytidine deaminase-nAPG06646.1 using the same guide RNA, up to 20x higher cytidine base editing than gene editing was observed. These results demonstrate that an RGN that has low nuclease activity at a particular target site can still be an efficient base editor at that site. Furthermore, since indel formation in base editing

applications is often an unwanted outcome, RGNs that have low nuclease activity at a site may be preferred for base editing applications.

Table 12: Cytidine base editing and gene editing of RGN and cytidine deaminase-nRGN

RGN or Deaminase-RGN	Guide RNA	%Total Reads with Cytidine Base Editing	% Total Reads with INDEL Formation
ARM05-nAPG06646.1	SGN000775	8.02	0.82
ARM06CTD-nAPG06646.1	SGN000775	0	0
ARM08-nAPG06646.1	SGN000775	5.15	0.46
ARM11-nAPG06646.1	SGN000775	0.66	0
APG06646.1	SGN000775	0	0.47
ARM05-nAPG06646.1	SGN000777	2.5	0.08
ARM06CTD-nAPG06646.1	SGN000777	0.53	0
ARM08-nAPG06646.1	SGN000777	8.12	0.25
ARM11-nAPG06646.1	SGN000777	3.52	1.04
APG06646.1	SGN000777	0	1.44
ARM05-nAPG06646.1	SGN000781	13.51	0.27
ARM06CTD-nAPG06646.1	SGN000781	2.69	0.13
ARM08-nAPG06646.1	SGN000781	12.97	0.12
ARM11-nAPG06646.1	SGN000781	4.7	0.14
APG06646.1	SGN000781	0	0.65
ARM05-nAPG08290.1	SGN000143	1.35	7.04
ARM06CTD-nAPG08290.1	SGN000143	1.36	3.17
ARM08-nAPG08290.1	SGN000143	2.59	10.98
ARM11-nAPG08290.1	SGN000143	4.78	5.77
APG08290.1	SGN000143	0	6.21
ARM05-nAPG08290.1	SGN000169	4.26	12.87
ARM06CTD-nAPG08290.1	SGN000169	1.63	8.29
ARM08-nAPG08290.1	SGN000169	6.99	14.27
ARM11-nAPG08290.1	SGN000169	4.99	6.39
APG08290.1	SGN000169	0	11.85
ARM05-nAPG08290.1	SGN000173	3.26	5.51
ARM06CTD-nAPG08290.1	SGN000173	0.72	0.96
ARM08-nAPG08290.1	SGN000173	6.6	6.06
ARM11-nAPG08290.1	SGN000173	2.83	4.82
APG08290.1	SGN000173	0	2.58

5

Example 11: Trans ssDNA cleavage

11.1 Determining assay conditions for trans DNA cleavage

Purified APG09748 (set forth as SEQ ID NO: 137 and described in International Appl. No. PCT/US2019/068079, which is incorporated by reference in its entirety) was incubated with single guide RNA (sgRNA) in Cutsmart buffer (New England Biolabs B7204S) at a final concentration of either 50 nM nuclease and 100 nM sgRNA or 200 nM nuclease and 400 nM sgRNA for 10 min. These RNP solutions were added to solutions of ssDNA – a target or mismatched negative control ssDNA - at a final concentration of 10 nM and reporter probes at a final concentration of 250 nM in 1.5X Cutsmart buffer (New England Biolabs B7204S). The reporter probes (TB0125 and TB0089, set forth as SEQ ID NOs: 138 and 139, respectively) contain a fluorescent dye at the 5' end (56-FAM for TB0125 and Cy5 for TB0089), a quencher at the 3' end (3IABkFQ for TB0125 and 3IAbRQSp for TB0089), and optionally an internal quencher (the internal quencher ZEN is only present on TB0125). Cleavage of the reporter probe results in dequenching of the fluorescent dye and thus an increase in fluorescence signal. To monitor fluorescence intensity, 10 µl of each reaction was incubated in a Corning low volume 384-well microplate at 30°C in a microplate reader (CLARIOstar Plus).

A number of conditions were scouted in order to determine suitable parameters for this assay. In order to determine if there are effects of quenched probe design or fluorophore characteristics, two such reporters were included as a mixture in each reaction. They were at the same concentration as each other in any given reaction. In all cases, the control or target ssDNA concentration (LE201 or LE205, set forth as SEQ ID NOs: 140 and 141, respectively) was 10 nM. The RNP names signify the nuclease and the target as indicated in Table 13 below.

Table 13. Ribonucleoprotein complexes

RNP Name	Nuclease	sgRNA	Intended Target
APG09748.1	APG09748	27sg.1 (SEQ ID NO: 144)	LE201 (SEQ ID NO: 140)
APG09748.2	APG09748	27sg.2 (SEQ ID NO: 145)	LE205 (SEQ ID NO: 141)

The results are shown in Table 14 below.

Table 14. Results of trans DNA cleavage assays

RNP Name	[RNP] (nM)	[reporters] (nM)	Target	Slope (RFU/min)	
				Cy5 channel	FAM channel
APG09748.1	25 nM	0	LE201	-0.30	-2.40
APG09748.1	25 nM	50	LE201	420.00	46.20
APG09748.1	25 nM	250	LE201	1040.40	47.40
APG09748.1	25 nM	500	LE201	1090.20	57.00
APG09748.1	25 nM	750	LE201	996.60	36.60
APG09748.1	25 nM	1000	LE201	770.40	36.00
APG09748.1	25 nM	0	LE205	0.60	-1.80
APG09748.1	25 nM	50	LE205	6.00	-1.20
APG09748.1	25 nM	250	LE205	16.80	-3.00
APG09748.1	25 nM	500	LE205	33.00	1.80
APG09748.1	25 nM	750	LE205	18.00	3.00

RNP Name	[RNP] (nM)	[reporters] (nM)	Target	Slope (RFU/min)	
				Cy5 channel	FAM channel
APG09748.1	25 nM	1000	LE205	-21.60	-51.60
APG09748.2	25 nM	0	LE201	-0.18	-1.80
APG09748.2	25 nM	50	LE201	4.20	1.20
APG09748.2	25 nM	250	LE201	42.60	3.00
APG09748.2	25 nM	500	LE201	70.80	10.20
APG09748.2	25 nM	750	LE201	67.80	10.80
APG09748.2	25 nM	1000	LE201	55.80	7.80
APG09748.2	25 nM	0	LE205	0.06	-2.40
APG09748.2	25 nM	50	LE205	321.00	64.20
APG09748.2	25 nM	250	LE205	669.60	63.00
APG09748.2	25 nM	500	LE205	706.80	52.20
APG09748.2	25 nM	750	LE205	627.00	55.20
APG09748.2	25 nM	1000	LE205	534.60	46.80
APG09748.1	100 nM	0	LE201	-0.42	-1.80
APG09748.1	100 nM	50	LE201	1063.80	155.40
APG09748.1	100 nM	250	LE201	2386.20	144.00
APG09748.1	100 nM	500	LE201	3058.80	108.60
APG09748.1	100 nM	750	LE201	2974.20	99.00
APG09748.1	100 nM	1000	LE201	3282.00	113.40
APG09748.1	100 nM	0	LE205	1.20	-0.36
APG09748.1	100 nM	50	LE205	6.60	0.18
APG09748.1	100 nM	250	LE205	27.00	1.80
APG09748.1	100 nM	500	LE205	52.80	12.60
APG09748.1	100 nM	750	LE205	57.60	9.00
APG09748.1	100 nM	1000	LE205	73.80	16.20
APG09748.2	100 nM	0	LE201	0.06	-0.48
APG09748.2	100 nM	50	LE201	25.80	9.00
APG09748.2	100 nM	250	LE201	198.00	15.00
APG09748.2	100 nM	500	LE201	265.80	16.20
APG09748.2	100 nM	750	LE201	277.20	19.20
APG09748.2	100 nM	1000	LE201	258.00	7.80
APG09748.2	100 nM	0	LE205	0.12	0.00
APG09748.2	100 nM	50	LE205	844.80	257.40
APG09748.2	100 nM	250	LE205	2091.60	270.60
APG09748.2	100 nM	500	LE205	2551.80	196.80
APG09748.2	100 nM	750	LE205	2490.60	186.00
APG09748.2	100 nM	1000	LE205	2511.00	138.00

From this experiment, it was concluded that the 100 nM concentration of RNP results generally in a higher cleavage rate of the reporter probe than the 25 nM RNP concentration. In general, reporter cleavage

rates are higher at higher concentrations of the reporter oligonucleotides up to 250 to 500 nM reporter concentration, with little benefit observed from further increase in reporter concentrations. Notably, for the TB0089 reporter (detected in the Cy5 channel), there are substantially higher levels of background activity that interfere with target differentiation, especially at reporter concentrations higher than 250 nM. Therefore, it was concluded that reporter concentrations higher than 250 nM would not be beneficial. Both probes seem suitable for observation of target recognition by the RNP.

11.2 APG09748 trans DNA cleavage and effect of purification on non-specific activity

Purified APG09748 was incubated with single guide RNA (sgRNA) in 1X Cutsmart buffer (New England Biolabs B7204S) at a final concentration of 200 nM nuclease and 400 nM sgRNA for 10 min at 37°C. These RNP solutions are then added to solutions of ssDNA – a target or mismatched negative control ssDNA - at a final concentration of 10 nM and a reporter probe (TP0003) at a final concentration of 250 nM in 1.5X Cutsmart buffer (New England Biolabs B7204S). The reporter probe contains a fluorescent dye at the 5' end and a quencher at the 3' end. Cleavage of the reporter probe results in dequenching of the fluorescent dye and thus an increase in fluorescence signal. To monitor fluorescence intensity, 10 µl of each reaction was incubated in a Corning low volume 384-well microplate at 37°C in a microplate reader (CLARIOstar Plus).

Incubation with target sequences resulted in a substantial increase in fluorescence intensity as a function of time relative to the negative control. The rate of cleavage is summarized as the slope of the linear portion of the fluorescence vs. time function as shown in Table 15.

Table 15. Results of trans DNA cleavage assay

ssDNA substrate sequence	Slope (RFU/min)
Mismatch (LE111; SEQ ID NO: 142)	549
Match (LE113; SEQ ID NO: 143)	7724

These data show differentiation of the target sequence from the negative control by the RNPs that is clearly detectable using the cleaved reporter probe.

11.3 PAM determination using a parallel plasmid DNA library

Oligonucleotides (LE00680 and LE00688 set forth as SEQ ID NOs: 266 and 267, respectively) containing a target sequence preceded by a 5 nt degenerate PAM sequence (NNNNN) were annealed and cloned into double digested pUC19 plasmid using NEBuilder HiFi DNA Assembly Master Mix (New England Biolabs). Each colony resulting from the transformation of this reaction corresponds to a clonal plasmid DNA sequence so that preparations of plasmid DNA from cultures deriving from single colonies are unique plasmid preparations sampled from the original library. Plasmid preparations were obtained from a sampling of 96 colonies. These preparations were individually subjected to Sanger sequencing to verify their PAM sequence.

Purified APG09748 was incubated with sgRNA (27sg.2) in 1X Cutsmart buffer (New England Biolabs B7204S) at a final concentration of 200 nM nuclease and 400 nM sgRNA for 20 minutes at room temperature.

These RNP solutions were added at a final concentration of 100 nM to solutions of plasmid DNA targets at a final concentration of 8.3 nM and TB0125 and TB0089 reporters (set forth as SEQ ID NOs: 138 and 139, respectively) at 250 nM and 50 nM respectively in 1.5X Cutsmart buffer (New England Biolabs B7204S). To monitor fluorescence intensity, 10 µl of each reaction was incubated in a Corning low volume 384-well microplate at 37°C in a microplate reader (CLARIOstar Plus).

The plasmid concentration in a minority of samples was either below the target for concentration normalization or the volume of the solution was insufficient to deliver the intended target amount to the reaction well. These samples were not excluded from analysis and are thus expected to contribute to error through inconsistencies between duplicates. Samples which used plasmids that, upon evaluation by Sanger sequencing, were determined to have cross contamination (multiple traces apparent in the PAM region) or alterations in the target sequence were removed from the analysis and their results are not shown below. The results of the analysis are shown in Table 16 below in descending order by slope in the FAM channel.

Table 16. PAM sequences

PAM	SEQ ID NO	Slope (RFU/min)	
		Cy5 channel	FAM channel
ATATG	147	111.52	1728.13
ATATG	147	102.42	1612.86
TATTG	148	137.15	846.12
TATTG	148	148.61	799.82
CGTTC	149	151.06	744.18
CGTTC	149	134.61	735.35
TTTTT	150	167.71	731.11
TTTTT	150	149.83	711.78
CAATC	151	120.84	675.71
AATCT	152	134.67	622.52
AATCT	152	134.1	620.2
CAATC	151	96.94	608.58
AAATT	153	152.6	607.24
AAATT	153	150.57	591.77
CATCC	154	97.34	588.7
AGATA	155	214.69	579.56
TATTC	156	124.22	570.81
ATTTT	157	134.72	551.91
AGATA	155	198.45	544.81
TATTC	156	108.57	538.18
TCTTT	158	141.66	532.87

PAM	SEQ ID NO	Slope (RFU/min)	
		Cy5 channel	FAM channel
ATTTT	157	107.9	526.75
AGATC	159	106.93	497.71
AATAA	160	78.94	484.67
TCTTT	158	117.19	484.13
GATCC	161	112.85	479.91
GATCC	161	105.04	474.94
AGATC	159	106.5	473.98
ATTCA	162	115.03	473.24
AATAA	160	77.43	465.75
AAAAT	163	78.3	458.23
TTATC	164	102.14	456.73
TATCA	165	106.45	448.78
TCACT	166	167.02	429.36
CTTAT	167	82.9	418.38
TATTG	148	116.08	417.94
TCACT	166	160.29	415.64
AAAAT	163	56.43	411.26
TGATA	168	89.02	406.14
TGATA	168	85.99	395.57
TACTA	169	90.61	391.06
ATTTT	157	113.18	382.59
TACTA	169	74.33	364.56
ATTTT	157	106.72	364.02
TATTG	148	93.95	341.36
CTTAT	167	76.12	336.8
ATATA	170	83.39	326.86
ATATA	170	72.98	318.2
TATCA	165	65.41	317.53
GACTC	171	56.56	302.2
GCTCA	172	22.1	293.68
TAACC	173	56.08	283.99
CGTGT	174	66.57	268.89
TAACC	173	56.55	265.45
TTTCG	175	59.55	262.91
CGTGT	174	50.71	262.37
TTTCG	175	61.42	261.65
CAGTA	176	51.81	240.09
GCTCA	172	19.08	228.08
CAGTA	176	51.09	220.25
CCCTT	177	17.83	136.69

PAM	SEQ ID NO	Slope (RFU/min)	
		Cy5 channel	FAM channel
CCTAT	178	40.4	125.81
TCCCT	179	39.93	124.6
AGGTT	180	40.96	117.18
AGGTT	180	40.81	114.79
TAACG	181	32.53	94.68
AACAG	182	8.19	91.72
CCTAT	178	34.51	87.57
TGAAC	183	10.22	86.38
TAAGT	184	19.35	85.12
TCCCT	179	26.67	83.02
TAACG	181	27.62	79.17
TAACG	181	28.89	78.55
TAACG	181	22.23	78.36
CTGTA	185	23.27	77.06
AGGGG	186	30.8	74.4
GCTGT	187	28.3	71.54
ATCTA	188	18.73	69.15
GCTGT	187	25.61	69.06
TTATC	164	22.8	68.13
AACAG	182	23.98	67.27
TCAGG	189	17.25	67.04
ATGTC	190	13.91	65.93
TGCTA	191	26.33	65.73
TAAAA	192	17.32	65.43
ATCTA	188	16.88	65.3
CTGTA	185	21.81	64.15
GACTC	171	24.05	64.02
ATTCA	162	25.04	63.87
TGAAC	183	24.53	63.64
CCCTT	177	21.35	61.95
ATGTC	190	12.99	60.6
ATCAG	193	22.32	59.63
ATCAG	193	18.55	56.58
CGGGG	194	27.86	56.28
TAAAA	192	17.72	56.03
TTCTC	195	25.7	55.83
GTCAT	196	23.07	55.76
GCGTG	197	17.36	55.12
GCGTG	197	22.02	55.02
GCAAT	198	15.47	54.91

PAM	SEQ ID NO	Slope (RFU/min)	
		Cy5 channel	FAM channel
TTCTC	199	21.89	54.89
GTCAT	196	17.22	54.34
GCAAT	198	17.36	53.32
AGCAT	200	22.47	52.23
GTCAA	201	14.14	52.12
AGAGC	202	19.62	51.79
AGAGC	202	15.99	50.37
GTGGG	203	22.38	50.1
CTGAA	204	21.24	49.45
CTGAT	205	12.05	49.32
ATGAA	206	17.93	49.15
ACCTT	207	19.66	49.12
ACCTT	207	22.74	49.12
GTCAA	201	13	48.21
AACCA	208	17.81	48.04
AACCA	208	15.75	47.87
GTAAA	209	16.3	47.85
TAGGA	210	18.68	47.73
CCGAA	211	20.24	47.58
AGCAT	200	22.95	47.52
CGGGG	194	20.94	47.41
GTCAT	196	15.17	47.24
CTGAA	204	20.4	46.36
GTGGG	203	19.98	46.25
TACGA	212	16.79	46.18
TGGCT	213	14.81	45.75
GTCAT	196	15.55	45.51
ATGAA	206	19.11	45.09
TAGGA	210	21.65	44.94
TTCTC	199	21.44	44.76
CCAAG	214	16.98	44.61
GTAAA	209	15.35	44.59
TGCTT	215	15.79	44.54
CTAAA	216	12.72	44.28
TGCTA	191	16	44.14
TACGA	212	16.07	44.06
TCGAT	217	22.28	43.96
TTCTC	199	15.94	42.55
CTGAT	205	16.13	42.27
TCAGG	189	11.38	41.9

PAM	SEQ ID NO	Slope (RFU/min)	
		Cy5 channel	FAM channel
CCAAG	214	11.28	41.45
AGGGG	186	16.04	41.44
TCGAT	217	18.68	40.5
CCGAA	211	15.39	39.09
CTAAA	216	10.72	30.37
GTAAC	218	4.36	9.51
TGCTT	215	-0.32	-0.7
TAAGT	184	-0.57	-1.3
CATCC	154	0.47	-1.95
TGGCT	213	-0.03	-2.2
GTAAC	219	-0.46	-2.3

Sequences with the highest slope appear to comply with the predicted PAM (DTTN set forth as SEQ ID NO: 60) determined by the plasmid depletion assay previously described in International Appl. No. PCT/US2019/068079, which is incorporated by reference in its entirety. In particular, we see a strong preference for “T” in the position two nucleotides 5’ of the target. Surprisingly, the most active PAM site observed in this assay (ATATG, SEQ ID NO: 147) did not exactly match the consensus of DTTN (SEQ ID NO: 60), suggesting some level of flexibility in this recognition site.

11.4. Trans ssDNA cleavage activity in the presence of PCR amplified DNA

PCR amplified targets were generated from genomic DNA (TRAC and VEGF amplicons set forth as SEQ ID NOs: 225 and 226, respectively) using appropriate primers. The VEGF target (set forth as SEQ ID NO: 227) was PCR amplified from HEK293T cell genomic DNA by primers with sequences LE573 and LE578 (set forth as SEQ ID NOs: 228 and 229, respectively). The TRAC target (set forth as SEQ ID NO: 230) was PCR amplified by LE257 and LE258 (set forth as SEQ ID NOs: 231 and 232, respectively).

RNPs were formed by incubation of APG09106.1 nuclease described herein and sgRNA at 0.5 μ M and 1 μ M respectively in 1X NEBuffer 2 (New England Biolabs) and incubated at room temperature for 20 minutes.

Table 17. Ribonucleoprotein complexes

RNP	Nuclease	Guide RNA	Intended target
APG09106.2	APG09106.1	27sg.2 (SEQ ID NO: 145)	LET126 amplicon Randomized PAM amplicon
APG09106.836	APG09106.1	27sg.836 (SEQ ID NO: 233)	VEGF amplicon
APG09106.838	APG09106.1	27sg.838 (SEQ ID NO: 234)	TRAC amplicon

The cleavage reaction was performed in 1.5X NEBuffer 2 with 1.5 μ M ssDNA oligonucleotide reporter with a 5’ TEX615 label and a 3’ Iowa Black FQ quencher and 100 nM of the respective PCR product. Cleavage of the reporter probe results in dequenching of the fluorescent dye and thus an increase in

fluorescence signal. To monitor fluorescence intensity, 10 μ l of each reaction was incubated in a Corning low volume 384-well microplate at 37°C in a microplate reader (CLARIOstar Plus). The results of kinetic analysis are shown in Table 18.

Table 18. Results of trans DNA cleavage assay

RNP	Target Conc. (nM)	Target	Slope (RFU/min)
28.836	30	TRAC amplicon	1204
28.838	30	TRAC amplicon	8436
28.838	30	VEGF amplicon	766
28.836	30	VEGF amplicon	3484

These results indicate specific activation of trans ssDNA cleavage activity in the presence of PCR amplified DNA from various sources. The activity is dependent on the concentration of the PCR amplified substrate.

11.5 Use of ssDNA cleavage as a diagnostic

Due to the capability of these nucleases to generate an optically detectable signal in the presence of a target DNA sequence, they promise utility for implementation into diagnostic devices for the detection of genetic disease or agents of infectious disease, such as bacteria, viruses, or fungi.

A diagnostic procedure may include isolation or amplification of nucleic acids from a sample to be tested. It may also be suitable to use some samples without performing any isolation or purification of nucleic acids, as they may be present in the sample at high enough quantities to be detectable without amplification (such as PCR) or free of materials that interfere with detection or signal production.

RNPs formed as described in the other examples could then be exposed to the sample (or processed sample as described in the preceding paragraph) along with a reporter, such as the fluorophore and quencher modified ssDNA oligonucleotides used in previous examples, or some other sort of ssDNA substrate that produces a visible or otherwise easily detectable signal when cleaved. If using fluorophore-quencher conjugated DNA oligonucleotides (as in the previously described examples), these can be detected using a fluorimeter as described in previous examples. To simplify detection, an endpoint assay can be performed instead of the kinetic assays described above, meaning that the assays can be performed for a fixed time and read out at the end of this elapsed time, relative to positive and negative controls.

These reagents may also be integrated into a lateral flow testing device which allows for the detection of a given disease-causing agent or specific nucleic acid sequence (such as a diseased allele in an individual) with very little instrumentation. In this assay, the ssDNA reporter would be conjugated to multiple molecules suitable for antibody or affinity reagent capture, such as fluorescein, biotin, and/or digoxigenin.

THAT WHICH IS CLAIMED:

1. A nucleic acid molecule comprising a polynucleotide encoding an RNA-guided nuclease (RGN) polypeptide, wherein said polynucleotide comprises a nucleotide sequence encoding an RGN polypeptide comprising an amino acid sequence having at least 95% sequence identity to SEQ ID NO: 117, wherein said RGN polypeptide is capable of binding a target DNA sequence of a DNA molecule in an RNA-guided sequence specific manner when bound to a guide RNA (gRNA) capable of hybridizing to said target DNA sequence, and

wherein said polynucleotide encoding an RGN polypeptide is operably linked to a promoter heterologous to said polynucleotide.

2. The nucleic acid molecule of claim 1, wherein said RGN polypeptide is nuclease inactive or is capable of functioning as a nickase.

3. The nucleic acid molecule of claim 1 or 2, wherein the RGN polypeptide is operably fused to a base-editing polypeptide.

4. A vector comprising the nucleic acid molecule of any one of claims 1-3.

5. The vector of claim 4, further comprising at least one nucleotide sequence encoding said gRNA capable of hybridizing to said target DNA sequence, and wherein the guide RNA comprises a CRISPR RNA comprising a CRISPR repeat sequence having at least 95% sequence identity to SEQ ID NO: 118.

6. The vector of claim 5, wherein said gRNA comprises a tracrRNA having at least 95% sequence identity to SEQ ID NO: 119.

7. A cell comprising the nucleic acid molecule of any one of claims 1-3 or the vector of any one of claims 4-6.

8. A system for binding a target DNA sequence of a DNA molecule, said system comprising:
a) one or more guide RNAs capable of hybridizing to said target DNA sequence or one or more polynucleotides comprising nucleotide sequences encoding the one or more guide RNAs (gRNAs); and
b) an RNA-guided nuclease (RGN) polypeptide comprising an amino acid sequence having at least 95% sequence identity to SEQ ID NO: 117;

or a polynucleotide comprising a nucleotide sequence encoding the RGN polypeptide;

wherein said nucleotide sequences encoding the one or more guide RNAs and encoding the RGN polypeptide are each operably linked to a promoter heterologous to said nucleotide sequence; and

wherein the one or more guide RNAs are capable of forming a complex with the RGN polypeptide, in order to direct said RGN polypeptide to bind to said target DNA sequence of the DNA molecule.

9. The system of claim 8, wherein the target DNA sequence is within a cell.

10. The system of claim 8 or 9, wherein said RGN polypeptide is nuclease inactive or is capable of functioning as a nickase, and wherein the RGN polypeptide is operably linked to a base-editing polypeptide.

11. The system of claim 8 or 9, wherein said system further comprises one or more donor polynucleotides or one or more nucleotide sequences encoding the one or more donor polynucleotides.

12. An *in vitro* or *ex vivo* method for binding a target DNA sequence of a DNA molecule comprising delivering a system according to any one of claims 8-11, to said target DNA sequence or a cell comprising the target DNA sequence.

13. An *in vitro* or *ex vivo* method for cleaving or modifying a target DNA sequence of a DNA molecule comprising delivering a system according to any one of claims 8-11, to said target DNA sequence or a cell comprising the DNA molecule and cleavage or modification of said target DNA sequence occurs.

14. A method for binding a target DNA sequence of a DNA molecule, said method comprising:
a) assembling an RNA-guided nuclease (RGN) ribonucleotide complex *in vitro* by combining:
i) one or more guide RNAs capable of hybridizing to the target DNA sequence; and
ii) an RGN polypeptide comprising an amino acid sequence having at least 95% sequence identity to SEQ ID NO: 117 under conditions suitable for formation of the RGN ribonucleotide complex; and

b) contacting said target DNA sequence or a cell comprising said target DNA sequence with the *in vitro*-assembled RGN ribonucleotide complex;

wherein the one or more guide RNAs hybridize to the target DNA sequence, thereby directing said RGN polypeptide to bind to said target DNA sequence.

15. An *in vitro* or *ex vivo* method for cleaving and/or modifying a target DNA sequence of a DNA molecule, comprising contacting the DNA molecule with:

a) an RNA-guided nuclease (RGN) polypeptide, wherein said RGN comprises an amino acid sequence having at least 95% sequence identity to SEQ ID NO: 117; and

b) one or more guide RNAs capable of targeting the RGN of (a) to the target DNA sequence;

wherein the one or more guide RNAs hybridize to the target DNA sequence, thereby directing said RGN polypeptide to bind to said target DNA sequence and cleavage and/or modification of said target DNA sequence occurs.

16. The *in vitro* or *ex vivo* method of claim 15, wherein said modified target DNA sequence comprises deletion or mutation of at least one nucleotide in the target DNA sequence.

17. The *in vitro* or *ex vivo* method of claim 15 or 16, wherein said RGN polypeptide is nuclease inactive or functions as a nickase, and wherein the RGN polypeptide is operably linked to a base-editing polypeptide.

18. The *in vitro* or *ex vivo* method of claim 15, wherein said modified target DNA sequence comprises insertion of heterologous DNA into the target DNA sequence.

19. The *in vitro* or *ex vivo* method of any one of claims 15-18, wherein the target DNA sequence is within a cell.

20. The *in vitro* or *ex vivo* method of claim 19, further comprising culturing the cell under conditions in which the RGN polypeptide is expressed and cleaves the target DNA sequence to produce a DNA molecule comprising a modified DNA sequence; and selecting a cell comprising said modified target DNA sequence.

21. A cell comprising a modified target DNA sequence according to the method of claim 20.

22. A system for binding a target DNA sequence of a DNA molecule, said system comprising:
a) one or more guide RNAs capable of hybridizing to said target DNA sequence or one or more polynucleotides comprising one or more nucleotide sequences encoding the one or more guide RNAs (gRNAs); and

b) an RNA-guided nuclease (RGN) polypeptide comprising an amino acid sequence having at least 95% sequence identity to SEQ ID NO: 117,

wherein the one or more guide RNAs are capable of hybridizing to the target DNA sequence, and
wherein the one or more guide RNAs are capable of forming a complex with the RGN polypeptide, in order to direct said RGN polypeptide to bind to said target DNA sequence of the DNA molecule.