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site_type	site_start	site_end	site_ strand	relative_ start	site_sequence	genome wide hits with 1 mismatches	genome wide hits with 2 mismatches	genome wide hits with 3 mismatches
(N20)NGG	46414444	46414445	1	50	TGACATCAATTATTATACATCGG (SEQ ID NO: 1)	1	4	28
(N20)NGG	46414510	46414511	1	116	CCTGCCTCCGCTCTACTCACTGG (SEQ ID NO: 2)	2	2	25
(N20)NGG	46414523	46414524	1	129	TACTCACTGGTGTTCATCTTTGG (SEQ ID NO: 3)	2	3	25
(N20)NGG	46414531	46414532	1	137	GGTGTTCATCTTTGGTTTTGTGG (SEQ ID NO: 4)	2	2	34
(N20)NGG	46414532	46414533	1	138	GTGTTCATCTTTGGTTTTGTGGG (SEQ ID NO: 5)	2	3	64
(N20)NGG	46414543	46414544	1	149	TGGTTTTGTGCCCAACATCCTGG (SEQ ID NO: 6)	2	4	32
(N20)NGG	46414572	46414573	1	178	TCATCCTGATAAACTGCAAAAGG (SEQ ID NO: 7)	1	1	35
(N20)NGG	46414609	46414610	1	215	TGACATCTACCTGCTCAACCTGG (SEQ ID NO: 8)	2	5	37
(N20)NGG	46414650	46414651	1	256	TCCTTCTTACTGTCCCCTTCTGG (SEQ ID NO: 9)	1	2	35
(N20)NGG	46414651	46414652	1	257	CCTTCTTACTGTCCCCTTCTGGG (SEQ ID NO: 10)	2	3	44
(N20)NGG	46414674	46414675	1	280	CTCACTATGCTGCCGCCCAGTGG (SEQ ID NO: 11)	1	2	16
(N20)NGG	46414675	46414676	1	281	TCACTATGCTGCCGCCCAGTGGG (SEQ ID NO: 12)	1	1	7
(N20)NGG	46414682	46414683	1	288	GCTGCCGCCCAGTGGGACTTTGG (SEQ ID NO: 13)	1	2	10
(N20)NGG	46414709	46414710	1	315	ACAATGTGTCAACTCTTGACAGG (SEQ ID NO: 14)	1	1	6
(N20)NGG	46414710	46414711	1	316	CAATGTGTCAACTCTTGACAGGG (SEQ ID NO: 15)	1	2	11

FIG. 1

(57) Abstract: Disclosed herein are methods, compositions, and kits for high efficiency, site-specific genomic editing of cells.

WO 2014/165825 A2

THERAPEUTIC USES OF GENOME EDITING WITH CRISPR/Cas SYSTEMS

RELATED APPLICATIONS

[0001] This application claims the benefit of U.S. Provisional Application Serial No. 61/808,594, filed April 4, 2013, the teachings of which are incorporated herein by reference in their entirety.

GOVERNMENT SUPPORT

[0002] This invention was made with government support under R01-HL118744, R00-HL098364 and R01-DK095384 awarded by the National Institutes of Health. The government has certain rights in the invention.

BACKGROUND OF THE INVENTION

[0003] Clustered regularly interspaced short palindromic repeats (CRISPR)/CRISPR-associated (Cas) systems are a new class of genome-editing tools that target desired genomic sites in mammalian cells. Recently published type II CRISPR/Cas systems use Cas9 nuclease that is targeted to a genomic site by complexing with a synthetic guide RNA that hybridizes to a 20-nucleotide DNA sequence and immediately preceding an NGG motif recognized by Cas9 (thus, a $(N)_{20}$NGG target DNA sequence). This results in a double-strand break three nucleotides upstream of the NGG motif. The double strand break instigates either non-homologous end-joining, which is error-prone and conducive to frameshift

mutations that knock out gene alleles, or homology-directed repair, which can be exploited with the use of an exogenously introduced double-strand or single-strand DNA repair template to knock in or correct a mutation in the genome. Thus, CRISPR/Cas systems could be useful tools for therapeutic applications, but unfortunately prior published reports have demonstrated an efficiency of allele targeting of only 2%-4% in human stem cells (Mali *et al.*, *Science 339*:823-826 (2013)).

SUMMARY OF THE INVENTION

[0004] Work described herein demonstrates methods of allele targeting using CRISPR/Cas systems resulting in mutant cells with efficiencies of up to 80%. In particular, work described herein surprisingly and unexpectedly demonstrates that a multiple guide strategy (e.g., using two or more ribonucleic acids which guide Cas protein to and hybridize to a target polynucleotide sequence) efficiently and effectively deletes target polynucleotide sequences (e.g., B2M, HPRT, CCR5 and/or CXCR4) in primary somatic cells (e.g., human blood cells, e.g., CD34+ and T cells), in contrast to a single guide strategy which has been demonstrated by the inventors to efficiently delete target polynucleotide sequences in cell lines (e.g., 293T) but not in primary somatic cells. These vastly improved methods permit CRISPR/Cas systems to be utilized effectively for the first time for therapeutic purposes. Methods of delivery of CRISPR/Cas systems to human stem cells are provided. In addition, methods of specifically identifying useful RNA guide sequences are provided, along with particular guide sequences useful in targeting specific genes (e.g., B2M, HPRT, CCR5 and/or CXCR4). Moreover, methods of treatment (e.g., methods of treating HIV infection) utilizing the compositions and methods disclosed herein are provided.

[0005] In some aspects, the present invention provides a method for altering a target polynucleotide sequence in a cell comprising contacting the polynucleotide sequence with a clustered regularly interspaced short palindromic repeats-associated (Cas) protein and from one to two ribonucleic acids, wherein the ribonucleic acids direct Cas protein to and hybridize to a target motif of the target polynucleotide sequence, wherein the target polynucleotide sequence is cleaved, and wherein the

efficiency of alteration of cells that express Cas protein is from about 50% to about 80%.

[0006] In some aspects, the present invention provides a method for treating or preventing a disorder associated with expression of a polynucleotide sequence in a subject, the method comprising (a) altering a target polynucleotide sequence in a cell *ex vivo* by contacting the polynucleotide sequence with a clustered regularly interspaced short palindromic repeats-associated (Cas) protein and from one to two ribonucleic acids, wherein the ribonucleic acids direct Cas protein to and hybridize to a target motif of the target polynucleotide sequence, wherein the target polynucleotide sequence is cleaved, and wherein the efficiency of alteration of cells that express Cas protein is from about 50% to about 80%, and (b) introducing the cell into the subject, thereby treating or preventing a disorder associated with expression of the polynucleotide sequence.

[0007] In some aspects, the present invention provides a method for simultaneously altering multiple target polynucleotide sequences in a cell comprising contacting the polynucleotide sequences with a clustered regularly interspaced short palindromic repeats-associated (Cas) protein and multiple ribonucleic acids, wherein the ribonucleic acids direct Cas protein to and hybridize to target motifs of the target polynucleotide sequences, wherein the target polynucleotide sequences are cleaved, and wherein the efficiency of alteration of cells that express Cas protein is from about 50% to about 80%.

[0008] In some aspects, the present invention provides a method for treating or preventing a disorder associated with expression of polynucleotide sequences in a subject, the method comprising (a) altering target polynucleotide sequences in a cell *ex vivo* by contacting the polynucleotide sequences with a clustered regularly interspaced short palindromic repeats-associated (Cas) protein and multiple ribonucleic acids, wherein the ribonucleic acids direct Cas protein to and hybridize to target motifs of the target polynucleotide sequences, wherein the target polynucleotide sequences are cleaved, and wherein the efficiency of alteration of cells that express Cas protein is from about 50% to about 80%, and (b) introducing the cell into the subject, thereby treating or preventing a disorder associated with expression of the polynucleotide sequences.

[0009] In some embodiments, the Cas protein is *Streptococcus pyogenes* Cas9 protein or a functional portion thereof. In some embodiments, the functional portion comprises a combination of operably linked Cas9 protein functional domains selected from the group consisting of a DNA binding domain, at least one RNA binding domain, a helicase domain, and an endonuclease domain. In some embodiments, the functional domains form a complex.

[0010] In some embodiments, the Cas protein is Cas9 protein from any bacterial species or functional portion thereof. In some embodiments, the functional portion comprises a combination of operably linked Cas9 protein functional domains selected from the group consisting of a DNA binding domain, at least one RNA binding domain, a helicase domain, and an endonuclease domain. In some embodiments, the functional domains form a complex.

[0011] In some embodiments, the Cas protein is complexed with the one to two ribonucleic acids. In some embodiments, the Cas protein is complexed with the multiple ribonucleic acids.

[0012] In some embodiments, the target motif is a 20-nucleotide DNA sequence. In some embodiments, each target motif is a 20-nucleotide DNA sequence. In some embodiments, the target motif is a 20-nucleotide DNA sequence beginning with G and immediately precedes an NGG motif recognized by the Cas protein. In some embodiments, each target motif is a 20-nucleotide DNA sequence beginning with G and immediately precedes an NGG motif recognized by the Cas protein. In some embodiments, the target motif is a 20-nucleotide DNA sequence and immediately precedes an NGG motif recognized by the Cas protein. In some embodiments, each target motif is a 20-nucleotide DNA sequence and immediately precedes an NGG motif recognized by the Cas protein. In some embodiments, the target motif is $G(N)_{19}NGG$. In some embodiments, each target motif is $G(N)_{19}NGG$. In some embodiments, the target motif is $(N)_{20}NGG$. In some embodiments, each target motif is $(N)_{20}NGG$.

[0013] In some embodiments, the target polynucleotide sequence is cleaved such that a double-strand break results. In some embodiments, each target polynucleotide sequence is cleaved such that a double-strand break results. In some embodiments, the target polynucleotide sequence is cleaved such that a single-strand

break results. In some embodiments, each target polynucleotide sequence is cleaved such that a single-strand break results.

[0014] In some embodiments, the alteration is an indel. In some embodiments, the alteration results in reduced expression of the target polynucleotide sequence. In some embodiments, the alteration results in reduced expression of the target polynucleotide sequences. In some embodiments, the alteration results in a knock out of the target polynucleotide sequence.

[0015] In some embodiments, the alteration results in a knock out of the target polynucleotide sequences. In some embodiments, the alteration results in correction of the target polynucleotide sequence from an undesired sequence to a desired sequence. In some embodiments, the alteration results in correction of the target polynucleotide sequences from undesired sequences to desired sequences. In some embodiments, the alteration is a homozygous alteration. In some embodiments, each alteration is a homozygous alteration.

[0016] In some embodiments, subsequent to cleavage of the target polynucleotide sequence, homology-directed repair occurs. In some embodiments, homology-directed repair is performed using an exogenously introduced DNA repair template. In some embodiments, the exogenously introduced DNA repair template is single-stranded. In some embodiments, the exogenously introduced DNA repair template is double-stranded.

[0017] In some embodiments, subsequent to cleavage of the target polynucleotide sequences, homology-directed repair occurs. In some embodiments, homology-directed repair is performed using an exogenously introduced DNA repair template. In some embodiments, the exogenously introduced DNA repair template is single-stranded. In some embodiments, the exogenously introduced DNA repair template is double-stranded.

[0018] In some embodiments, the cell is a peripheral blood cell. In some embodiments, the cell is a stem cell or a pluripotent cell. In some embodiments, the cell is a hematopoietic stem cell. In some embodiments, the cell is a $CD34^+$ cell. In some embodiments, the cell is a $CD34^+$ mobilized peripheral blood cell. In some embodiments, the cell is a $CD34^+$ cord blood cell. In some embodiments, the cell is a

$CD34^+$ bone marrow cell. In some embodiments, the cell is a $CD34^+CD38$-Lineage-$CD90^+CD45RA^-$ cell. In some embodiments, the cell is a hepatocyte.

[0019] In some embodiments, the target polynucleotide sequence is CCR5. In some embodiments, at least one of the one to two ribonucleic acids comprises a sequence selected from the group consisting of the ribonucleic acid sequences of Fig. 1. In some embodiments, at least one of the one to two ribonucleic acids comprises a sequence with a single nucleotide mismatch to a sequence selected from the group consisting of the ribonucleic acid sequences of Fig. 1.

[0020] In some embodiments, the target polynucleotide sequence is CXCR4. In some embodiments, at least one of the one to two ribonucleic acids comprises a sequence selected from the group consisting of the ribonucleic acid sequences of Fig. 2. In some embodiments, at least one of the one to two ribonucleic acids comprises a sequence with a single nucleotide mismatch to a sequence selected from the group consisting of the ribonucleic acid sequences of Fig. 2.

[0021] In some embodiments, the target polynucleotide sequences comprise multiple different portions of CCR5. In some embodiments, each of the multiple ribonucleic acids comprises a different sequence selected from the group consisting of the ribonucleic acid sequences of Fig. 1. In some embodiments, each of the multiple ribonucleic acids comprises a sequence with a single nucleotide mismatch to a different sequence selected from the group consisting of the ribonucleic acid sequences of Fig. 1.

[0022] In some embodiments, the target polynucleotide sequences comprise multiple different portions of CXCR4. In some embodiments, each of the multiple ribonucleic acids comprises a different sequence selected from the group consisting of the ribonucleic acid sequences of Fig. 2. In some embodiments, each of the multiple ribonucleic acids comprises a sequence with a single nucleotide mismatch to a different sequence selected from the group consisting of the ribonucleic acid sequences of Fig. 2.

[0023] In some embodiments, the target polynucleotide sequences comprise at least a portion of CCR5 and at least a portion of CXCR4. In some embodiments, each of the multiple ribonucleic acids comprises a different sequence selected from the group consisting of the ribonucleic acid sequences of Fig. 1 and the ribonucleic acid

sequences of Fig. 2. In some embodiments, each of the multiple ribonucleic acids comprises a sequence with a single nucleotide mismatch to a different sequence selected from the group consisting of the ribonucleic acid sequences of Fig. 1 and the ribonucleic acid sequences of Fig 2.

[0024] In some embodiments, the disorder is a genetic disorder. In some embodiments, the disorder is a monogenic disorder. In some embodiments, the disorder is human immunodeficiency virus (HIV) infection. In some embodiments, the disorder is acquired immunodeficiency syndrome (AIDS).

[0025] In some embodiments, the one to two ribonucleic acids are designed to hybridize to a target motif immediately adjacent to a deoxyribonucleic acid motif recognized by the Cas protein. In some embodiments, each of the one to two ribonucleic acids are designed to hybridize to target motifs immediately adjacent to deoxyribonucleic acid motifs recognized by the Cas protein which flank a mutant allele located between the target motifs. In some embodiments, the multiple ribonucleic acids are designed to hybridize to target motifs immediately adjacent to deoxyribonucleic acid motifs recognized by the Cas protein. In some embodiments, the multiple ribonucleic acids are designed to hybridize to target motifs immediately adjacent to deoxyribonucleic acid motifs recognized by the Cas protein which flank mutant alleles located between the target motifs. In some embodiments, the one to two ribonucleic acids are selected to minimize hybridization with nucleic acid sequences other than the target polynucleotide sequence. In some embodiments, the multiple ribonucleic acids are selected to minimize hybridization with nucleic acid sequences other than the target polynucleotide sequence.

[0026] In some embodiments, the target motif is selected such that it contains at least two mismatches when compared with all other genomic nucleotide sequences in the cell. In some embodiments, each target motif is selected such that it contains at least two mismatches when compared with all other genomic nucleotide sequences in the cell. In some embodiments, the target motif is selected such that it contains at least one mismatch when compared with all other genomic nucleotide sequences in the cell.

[0027] In some embodiments, the target motif is selected such that it contains at least one mismatch when compared with all other genomic nucleotide sequences in

the cell. In some embodiments, the one to two ribonucleic acids hybridize to a target motif that it contains at least two mismatches when compared with all other genomic nucleotide sequences in the cell.

[0028] In some embodiments, each of the multiple ribonucleic acids hybridize to target motifs that contain at least two mismatches when compared with all other genomic nucleotide sequences in the cell. In some embodiments, the one to two ribonucleic acids hybridize to a target motif that contains at least one mismatch when compared with all other genomic nucleotide sequences in the cell. In some embodiments, each of the multiple ribonucleic acids hybridize to target motifs that contain at least one mismatch when compared with all other genomic nucleotide sequences in the cell.

[0029] In some embodiments, the efficiency of alteration at each loci is from about 50% to about 80%. In some embodiments, the efficiency of alteration is at least about 5%. In some embodiments, the efficiency of alteration is at least about 10%. In some embodiments, the efficiency of alteration is from about 50% to about 80%.

[0030] In some embodiments, the Cas protein is encoded by a modified nucleic acid. In some embodiments, the modified nucleic acid comprises a ribonucleic acid containing at least one modified nucleotide selected from the group consisting of pseudouridine, 5-methylcytodine, 2-thio-uridine, 5-methyluridine-5'-triphosphate, 4-thiouridine-5'-triphosphate, 5,6-dihydrouridine-5'-triphosphate, and 5-azauridine-5'-triphosphate. In some embodiments, at least one of the ribonucleic acids is a modified ribonucleic acid comprising one to two modified nucleotides selected from the group consisting of pseudouridine, 5-methylcytodine, 2-thio-uridine, 5-methyluridine-5'-triphosphate, 4-thiouridine-5'-triphosphate, 5,6-dihydrouridine-5'-triphosphate, and 5-azauridine-5'-triphosphate.

[0031] In some embodiments, any of the Cas protein or the ribonucleic acids are expressed from a plasmid.

[0032] In some embodiments, any of the Cas protein or the ribonucleic acids are expressed using a promoter optimized for increased expression in stem cells. In some embodiments, the promoter is selected from the group consisting of a Cytomegalovirus (CMV) early enhancer element and a chicken beta-actin promoter, a

chicken beta-actin promoter, an elongation factor-1 alpha promoter, and a ubiquitin promoter.

[0033] In some embodiments, the method further comprises selecting cells that express the Cas protein. In some embodiments, selecting cells comprises FACS. In some embodiments, FACs is used to select cells which co-express Cas and a fluorescent protein selected from the group consisting of green fluorescent protein and red fluorescent protein.

[0034] In some aspects, the present invention provides a method for altering a target polynucleotide sequence in a cell comprising contacting the polynucleotide sequence in a cell selected from the group consisting of a human pluripotent cell, a primary human cell, and a non-transformed human cell, with a clustered regularly interspaced short palindromic repeats-associated (Cas) protein and from one to two ribonucleic acids, wherein the ribonucleic acids direct Cas protein to and hybridize to a target motif of the target polynucleotide sequence, wherein the target polynucleotide sequence is cleaved, and wherein the efficiency of alteration of cells that express Cas protein is from about 8% to about 80%.

[0035] In some aspects, the present invention provides a method for treating or preventing a disorder associated with expression of a polynucleotide sequence in a subject, the method comprising (a) altering a target polynucleotide sequence in a cell *ex vivo* by contacting the polynucleotide sequence in a cell selected from the group consisting of a human pluripotent cell, a primary human cell, and a non-transformed human cell, with a clustered regularly interspaced short palindromic repeats-associated (Cas) protein and from one to two ribonucleic acids, wherein the ribonucleic acids direct Cas protein to and hybridize to a target motif of the target polynucleotide sequence, wherein the target polynucleotide sequence is cleaved, and wherein the efficiency of alteration is from about 8% to about 80%, and (b) introducing the cell into the subject, thereby treating or preventing a disorder associated with expression of the polynucleotide sequence.

[0036] In some aspects, the present invention provides a method for simultaneously altering multiple target polynucleotide sequences in a cell comprising contacting the polynucleotide sequences in a cell selected from the group consisting of a human pluripotent cell, a primary human cell, and a non-transformed human cell,

with a clustered regularly interspaced short palindromic repeats-associated (Cas) protein and multiple ribonucleic acids, wherein the ribonucleic acids direct Cas protein to and hybridize to target motifs of the target polynucleotide sequences, wherein the target polynucleotide sequences are cleaved, and wherein the efficiency of alteration of cells that express Cas protein is from about 8% to about 80%.

[0037] In some aspects, the present invention provides a method for treating or preventing a disorder associated with expression of polynucleotide sequences in a subject, the method comprising (a) altering target polynucleotide sequences in a cell *ex vivo* by contacting the polynucleotide sequences in a cell selected from the group consisting of a human pluripotent cell, a primary human cell, and a non-transformed human cell, with a clustered regularly interspaced short palindromic repeats-associated (Cas) protein and multiple ribonucleic acids, wherein the ribonucleic acids direct Cas protein to and hybridize to target motifs of the target polynucleotide sequences, wherein the target polynucleotide sequences are cleaved, and wherein the efficiency of alteration of cells that express Cas protein is from about 8% to about 80%, and (b) introducing the cell into the subject, thereby treating or preventing a disorder associated with expression of the polynucleotide sequences.

[0038] In some aspects, the present invention provides a composition comprising at least one ribonucleic acid having a sequence selected from the group consisting of the ribonucleic acid sequences of Fig 1.

[0039] In some aspects, the present invention provides a composition comprising at least one ribonucleic acid comprising a sequence with a single nucleotide mismatch to a sequence selected from the group consisting of the ribonucleic acid sequences of Fig 1.

[0040] In some aspects, the present invention provides a composition comprising at least one ribonucleic acid having a sequence selected from the group consisting of the ribonucleic acid sequences of Fig 2.

[0041] In some aspects, the present invention provides a composition comprising at least one ribonucleic acid comprising a sequence with a single nucleotide mismatch to a sequence selected from the group consisting of the ribonucleic acid sequences of Fig 2.

[0042] In some aspects, the present invention provides a composition comprising at least one ribonucleic acid having a sequence selected from the group consisting of the ribonucleic acid sequences of Fig. 1, the ribonucleic acid sequences of Fig. 2, a sequence with a single nucleotide mismatch to a ribonucleic acid sequence of Fig. 1, and a sequence with a single nucleotide mismatch to a ribonucleic acid sequences of Fig. 2.

[0043] In some embodiments, the composition further comprises a nucleic acid sequence encoding a Cas protein. In some embodiments, the composition further comprises a nucleic acid sequence encoding a Cas9 protein or a functional portion thereof. In some embodiments, the nucleic acid comprises a modified ribonucleic acid comprising at least one modified nucleotide selected from the group consisting of pseudouridine, 5-methylcytodine, 2-thio-uridine, 5-methyluridine-5'-triphosphate, 4-thiouridine-5'-triphosphate, 5,6-dihydrouridine-5'-triphosphate, and 5-azauridine-5'-triphosphate.

[0044] In some aspects, the present invention provides a composition comprising a chimeric nucleic acid comprising a ribonucleic acid encoding a Cas protein and at least one additional ribonucleic acid having a sequence selected from the group consisting of the ribonucleic acid sequences of Fig. 1.

[0045] In some aspects, the present invention provides a composition comprising a chimeric nucleic acid comprising a ribonucleic acid encoding a Cas protein and at least one additional ribonucleic acid having a sequence selected from the group consisting of the ribonucleic acid sequences of Fig. 2.

[0046] In some aspects, the present invention provides a composition comprising a chimeric nucleic acid comprising a ribonucleic acid encoding a Cas protein and at least one additional ribonucleic acid sequence comprising a sequence with a single nucleotide mismatch to a sequence selected from the group consisting of the ribonucleic acid sequences of Fig 1.

[0047] In some aspects, the present invention provides a composition comprising a chimeric nucleic acid comprising a ribonucleic acid encoding a Cas protein and at least one additional ribonucleic acid sequence comprising a sequence with a single nucleotide mismatch to a sequence selected from the group consisting of the ribonucleic acid sequences of Fig 2.

[0048] In some aspects, the present invention provides a composition comprising a chimeric nucleic acid comprising a ribonucleic acid encoding a Cas protein and at least one additional ribonucleic acid having a sequence selected from the group consisting of the ribonucleic acid sequences of Fig. 1, the ribonucleic acid sequences of Fig. 2, a sequence with a single nucleotide mismatch to a ribonucleic acid sequence of Fig. 1, and a sequence with a single nucleotide mismatch to a ribonucleic acid sequences of Fig. 2.

[0049] In some embodiments, the composition further comprises a nucleic acid sequence encoding a fluorescent protein selected from the group consisting of green fluorescent protein and red fluorescent protein.

[0050] In some embodiments, the composition further comprises a promoter operably linked to the chimeric nucleic acid. In some embodiments, the promoter is optimized for increased expression in human stem cells. In some embodiments, the promoter is selected from the group consisting of a Cytomegalovirus (CMV) early enhancer element and a chicken beta-actin promoter, a chicken beta-actin promoter, an elongation factor-1 alpha promoter, and a ubiquitin promoter.

[0051] In some embodiments, the Cas protein comprises a Cas9 protein or a functional portion thereof.

[0052] In some aspects, the present invention provides a kit for altering a target polynucleotide sequence in a cell comprising a Cas9 protein or a nucleic acid encoding the Cas9 protein, and at least one ribonucleic acid sequence selected from the group consisting of the ribonucleic acid sequences of Fig. 1, the ribonucleic acid sequences of Fig. 2, a sequence with a single nucleotide mismatch to a ribonucleic acid sequence of Fig. 1, and a sequence with a single nucleotide mismatch to a ribonucleic acid sequences of Fig. 2. In some embodiments, the kit further comprises one or more cell lines, cultures, or populations selected from the group consisting of human pluripotent cells, primary human cells, and non-transformed cells. In some embodiments, the kit further comprises a DNA repair template.

[0053] In some embodiments, the cell comprises a primary cell. In some embodiments, the cell comprises a primary somatic cell. In some embodiments, the cell comprises an autologous primary somatic cell. In some embodiments, the cell comprises an allogeneic primary somatic cell. In some embodiments, the target

polynucleotide sequence is B2M. In some embodiments, at least one of the one to two ribonucleic acids comprises a sequence optimized to target the B2M gene. In some embodiments, at least one of the one to two ribonucleic acids comprises a sequence with a single nucleotide mismatch to a sequence optimized to target the B2M gene. In some embodiments, the target polynucleotide sequences comprises multiple different portions of B2M. In some embodiments, each of the multiple ribonucleic acids comprises a different sequence optimized to target the B2M gene. In some embodiments, each of the multiple ribonucleic acids comprises a sequence with a single nucleotide mismatch to a different sequence optimized to target the B2M gene. In some embodiments, the one to two ribonucleic acids comprise two guide ribonucleic acid sequences.

[0054] In some embodiments, the one to two ribonucleic acids comprise two guide ribonucleic acid sequences. In some embodiments, the target polynucleotide sequence comprises CCR5. In some embodiments, the cell comprises a primary CD34+ hematopoietic progenitor cell. In some embodiments, the two guide ribonucleic acid sequences comprise any combination of two guide ribonucleic acid sequences which are complementary to a different sequence selected from the group consisting of SEQ ID NOs: 1-139. In some embodiments, the two guide ribonucleic acid sequences comprise any combination of two guide ribonucleic acid sequences which are complementary to offset sequences selected from the group consisting of SEQ ID NOs: 1-139. In some embodiments, the two guide ribonucleic acid sequences comprise any combination of two guide ribonucleic acid sequences which hybridize to and target Cas protein to offset target sites in CCR5 selected from the group consisting of SEQ ID NOs: 1-139. In some embodiments, the two guide ribonucleic acid sequences comprise any combination of two guide ribonucleic acid sequences from SEQ ID NOs: 298-303. In some embodiments, the two guide ribonucleic acid sequences comprise a pair of guide ribonucleic acids selected from the group consisting of SEQ ID NOs: 299 and 303, SEQ ID NOs: 298 and 300, SEQ ID NOs: 299 and 300, SEQ ID NOs: 298 and 303, SEQ ID NOs: 299 and 301, SEQ ID NOs: 298 and 299, SEQ ID NOs: 301 and 303, SEQ ID NOs: 298 and 302, and SEQ ID NOs: 298 and 301. In some embodiments, the two guide ribonucleic acid sequences comprise any combination of two guide ribonucleic acid sequences which are

complementary to a different sequence selected from the group consisting of SEQ ID NOs: 304-333. In some embodiments, the two guide ribonucleic acid sequences comprise any combination of two guide ribonucleic acid sequences which are complementary to offset sequences selected from the group consisting of SEQ ID NOs: 304-333. In some embodiments, the two guide ribonucleic acid sequences comprise any combination of two guide ribonucleic acid sequences which hybridize to and target Cas protein to offset target sites in CCR5 selected from the group consisting of SEQ ID NOs: 304-333. In some embodiments, the target polynucleotide sequence comprises CXCR4. In some embodiments, the cell comprises a primary CD34+ hematopoietic progenitor cell. In some embodiments, the two guide ribonucleic acid sequences comprise any combination of two guide ribonucleic acid sequences which are complementary to a different sequence selected from the group consisting of SEQ ID NOs: 140-297. In some embodiments, the two guide ribonucleic acid sequences comprise any combination of two guide ribonucleic acid sequences which are complementary to offset sequences selected from the group consisting of SEQ ID NOs: 140-297. In some embodiments, the two guide ribonucleic acid sequences comprise any combination of two guide ribonucleic acid sequences which hybridize to and target Cas protein to offset target sites in CXCR4 selected from the group consisting of SEQ ID NOs: 140-297. In some embodiments, the target polynucleotide sequence comprises B2M. In some embodiments, the cell comprises a primary cell. In some embodiments, the two guide ribonucleic acid sequences comprise any combination of two guide ribonucleic acid sequences which are complementary to different sequences in the B2M gene. In some embodiments, the two guide ribonucleic acid sequences comprise any combination of two guide ribonucleic acid sequences which are complementary to offset sequences in the B2M gene. In some embodiments, the two guide ribonucleic acid sequences comprise any combination of two guide ribonucleic acid sequences which hybridize to and target Cas protein to offset target sites in B2M.

[0055] In some aspects, the invention provides a method for altering a target polynucleotide sequence in a primary cell comprising contacting the polynucleotide sequence with a clustered regularly interspaced short palindromic repeats-associated (Cas) protein and at least two ribonucleic acids, wherein the at least two ribonucleic

acids comprise guide ribonucleic acids which direct Cas protein to and hybridize to a target motif of the target polynucleotide sequence, wherein the target polynucleotide sequence is cleaved. In some embodiments, the efficiency of alteration of cells that express Cas protein is from about 50% to about 80%.

[0056] In some aspects, the invention provides a method for altering a target polynucleotide sequence in a primary cell comprising contacting the polynucleotide sequence with a clustered regularly interspaced short palindromic repeats-associated (Cas) protein and at least two ribonucleic acids, wherein the at least two ribonucleic acids comprise guide ribonucleic acids which direct Cas protein to and hybridize to a target motif of the target polynucleotide sequence, wherein the target polynucleotide sequence is cleaved, and wherein the efficiency of alteration of cells that express Cas protein is from about 50% to about 80%.

[0057] In some aspects, the invention provides a method for treating or preventing a disorder associated with expression of a polynucleotide sequence in a subject, the method comprising (a) altering a target polynucleotide sequence in a primary cell *ex vivo* by contacting the polynucleotide sequence with a clustered regularly interspaced short palindromic repeats-associated (Cas) protein and at least two ribonucleic acids, wherein the at least two ribonucleic acids comprise guide ribonucleic acids which direct Cas protein to and hybridize to a target motif of the target polynucleotide sequence, wherein the target polynucleotide sequence is cleaved, and (b) introducing the cell into the subject, thereby treating or preventing a disorder associated with expression of the polynucleotide sequence. In some embodiments, the efficiency of alteration of cells that express Cas protein is from about 50% to about 80%.

[0058] In some aspects, the invention provides a method for treating or preventing a disorder associated with expression of a polynucleotide sequence in a subject, the method comprising (a) altering a target polynucleotide sequence in a primary cell *ex vivo* by contacting the polynucleotide sequence with a clustered regularly interspaced short palindromic repeats-associated (Cas) protein and at least two ribonucleic acids, wherein the at least two ribonucleic acids comprise guide ribonucleic acids which direct Cas protein to and hybridize to a target motif of the target polynucleotide sequence, wherein the target polynucleotide sequence is

cleaved, and wherein the efficiency of alteration of cells that express Cas protein is from about 50% to about 80%, and (b) introducing the cell into the subject, thereby treating or preventing a disorder associated with expression of the polynucleotide sequence.

[0059] In some aspects, the invention provides, a method for simultaneously altering multiple target polynucleotide sequences in a primary cell comprising contacting the polynucleotide sequences with a clustered regularly interspaced short palindromic repeats-associated (Cas) protein and multiple ribonucleic acids, wherein the ribonucleic acids comprise guide ribonucleic acids which direct Cas protein to and hybridize to target motifs of the target polynucleotide sequences, wherein the target polynucleotide sequences are cleaved. In some embodiments, the efficiency of alteration of cells that express Cas protein is from about 50% to about 80%.

[0060] In some aspects, the invention provides, a method for simultaneously altering multiple target polynucleotide sequences in a primary cell comprising contacting the polynucleotide sequences with a clustered regularly interspaced short palindromic repeats-associated (Cas) protein and multiple ribonucleic acids, wherein the ribonucleic acids comprise guide ribonucleic acids which direct Cas protein to and hybridize to target motifs of the target polynucleotide sequences, wherein the target polynucleotide sequences are cleaved, and wherein the efficiency of alteration of cells that express Cas protein is from about 50% to about 80%.

[0061] In some aspects, the disclosure provides a method for treating or preventing a disorder associated with expression of polynucleotide sequences in a subject, the method comprising (a) altering target polynucleotide sequences in a primary cell *ex vivo* by contacting the polynucleotide sequences with a clustered regularly interspaced short palindromic repeats-associated (Cas) protein and multiple ribonucleic acids, wherein the ribonucleic acids comprise guide ribonucleic acids which direct Cas protein to and hybridize to target motifs of the target polynucleotide sequences, wherein the target polynucleotide sequences are cleaved, and (b) introducing the cell into the subject, thereby treating or preventing a disorder associated with expression of the polynucleotide sequences. In some embodiments, the efficiency of alteration of cells that express Cas protein is from about 50% to about 80%.

[0062] In some aspects, the disclosure provides a method for treating or preventing a disorder associated with expression of polynucleotide sequences in a subject, the method comprising (a) altering target polynucleotide sequences in a primary cell *ex vivo* by contacting the polynucleotide sequences with a clustered regularly interspaced short palindromic repeats-associated (Cas) protein and multiple ribonucleic acids, wherein the ribonucleic acids comprise guide ribonucleic acids which direct Cas protein to and hybridize to target motifs of the target polynucleotide sequences, wherein the target polynucleotide sequences are cleaved, and wherein the efficiency of alteration of cells that express Cas protein is from about 50% to about 80%, and (b) introducing the cell into the subject, thereby treating or preventing a disorder associated with expression of the polynucleotide sequences.

[0063] In some embodiments, the Cas protein is *Streptococcus pyogenes* Cas9 protein or a functional portion thereof. In some embodiments, the functional portion comprises a combination of operably linked Cas9 protein functional domains selected from the group consisting of a DNA binding domain, at least one RNA binding domain, a helicase domain, and an endonuclease domain. In some embodiments, the functional domains form a complex. In some embodiments, the Cas protein is Cas9 protein from any bacterial species or functional portion thereof. In some embodiments, the functional portion comprises a combination of operably linked Cas9 protein functional domains selected from the group consisting of a DNA binding domain, at least one RNA binding domain, a helicase domain, and an endonuclease domain. In some embodiments, the functional domains form a complex. In some embodiments, the Cas protein is complexed with the one to two ribonucleic acids. In some embodiments, the Cas protein is complexed with the multiple ribonucleic acids.

[0064] In some embodiments, the target motif is a 20-nucleotide DNA sequence. In some embodiments, each target motif is a 20-nucleotide DNA sequence. In some embodiments, the target motif is a 20-nucleotide DNA sequence beginning with G and immediately precedes an NGG motif recognized by the Cas protein. In some embodiments, each target motif is a 20-nucleotide DNA sequence beginning with G and immediately precedes an NGG motif recognized by the Cas protein. In some embodiments, the target motif is a 20-nucleotide DNA sequence and immediately precedes an NGG motif recognized by the Cas protein. In some

embodiments, each target motif is a 20-nucleotide DNA sequence and immediately precedes an NGG motif recognized by the Cas protein. In some embodiments, the target motif is $G(N)_{19}NGG$. In some embodiments,each target motif is $G(N)_{19}NGG$. In some embodiments, the target motif is $(N)_{20}NGG$. In some embodiments, each target motif is $(N)_{20}NGG$. In some embodiments, the target motif comprises a sequence selected from the group consisting of SEQ ID NOs: 1-297 or 304-333. In some embodiments, the target motif comprises a sequence selected from the group consisting of SEQ ID NOs: 1-297 or 304-333. In some embodiments, the target polynucleotide sequence is cleaved such that a double-strand break results. In some embodiments, each target polynucleotide sequence is cleaved such that a double-strand break results. In some embodiments, the target polynucleotide sequence is cleaved such that a single-strand break results. In some embodiments, each target polynucleotide sequence is cleaved such that a single-strand break results. In some embodiments, the alteration is an indel. In some embodiments, the alteration results in reduced expression of the target polynucleotide sequence. In some embodiments, the alteration results in reduced expression of the target polynucleotide sequences. In some embodiments, the alteration results in a knock out of the target polynucleotide sequence. In some embodiments, the alteration results in a knock out of the target polynucleotide sequences. In some embodiments, the alteration results in correction of the target polynucleotide sequence from an undesired sequence to a desired sequence. In some embodiments, the alteration results in correction of the target polynucleotide sequences from undesired sequences to desired sequences. In some embodiments, the alteration is a homozygous alteration.

[0065] In some embodiments, each alteration is a homozygous alteration. In some embodiments, subsequent to cleavage of the target polynucleotide sequence, homology-directed repair occurs. In some embodiments, homology-directed repair is performed using an exogenously introduced DNA repair template. In some embodiments, the exogenously introduced DNA repair template is single-stranded. In some embodiments, the exogenously introduced DNA repair template is double-stranded. In some embodiments, subsequent to cleavage of the target polynucleotide sequences, homology-directed repair occurs. In some embodiments, homology-directed repair is performed using an exogenously introduced DNA repair template. In

some embodiments, the exogenously introduced DNA repair template is single-stranded. In some embodiments, the exogenously introduced DNA repair template is double-stranded. In some embodiments, the cell is a peripheral blood cell. In some embodiments, the cell is a stem cell or a pluripotent cell. In some embodiments, the cell is a hematopoietic stem cell. In some embodiments, the cell is a $CD34^+$ cell. In some embodiments, the cell is a $CD34^+$ mobilized peripheral blood cell. In some embodiments, the cell is a $CD34^+$ cord blood cell. In some embodiments, the cell is a $CD34^+$ bone marrow cell. In some embodiments, the cell is a $CD34^+CD38$-Lineage-$CD90^+CD45RA^-$ cell. In some embodiments, the cell is a hepatocyte. In some embodiments, the cell is a primary cell. In some embodiments, the target polynucleotide sequence is CCR5.

[0066] In some embodiments, the two ribonucleic acids comprise a different sequence selected from the group consisting of SEQ ID NOs: 298-303. In some embodiments, the two guide ribonucleic acid sequences comprise a pair of guide ribonucleic acids selected from the group consisting of SEQ ID NOs: 299 and 303, SEQ ID NOs: 298 and 300, SEQ ID NOs: 299 and 300, SEQ ID NOs: 298 and 303, SEQ ID NOs: 299 and 301, SEQ ID NOs: 298 and 299, SEQ ID NOs: 301 and 303, SEQ ID NOs: 298 and 302, and SEQ ID NOs: 298 and 301. In some embodiments, the two ribonucleic acids comprise sequences which are complementary to and/or hybridize to different sequences selected from the group consisting of SEQ ID NOs: 1-139 and 304-333. In some embodiments, the two ribonucleic acids comprise sequences which are complementary to and/or hybridize to different sequences with a single nucleotide mismatch to a sequence selected from the group consisting of SEQ ID NOs: 1-139 and 304-333. In some embodiments, the two ribonucleic acids comprise sequences which are complementary to and/or hybridizes to offset sequences selected from the group consisting of SEQ ID NOs: 1-139 and 304-333. In some embodiments, the two ribonucleic acids comprise sequences which are complementary to and/or hybridize to offsets sequences with a single nucleotide mismatch to a sequence selected from the group consisting of SEQ ID NOs: 1-139 and 304-333. In some embodiments, the target polynucleotide sequence is CXCR4. In some embodiments, the two ribonucleic acids comprise sequences which are complementary to and/or hybridize to different sequences selected from the group

consisting of SEQ ID NO: 140-297. In some embodiments, the two ribonucleic acids comprise sequences which are complementary to and/or hybridize to different sequences with a single nucleotide mismatch to a sequence selected from the group consisting of SEQ ID NO: 140-297. In some embodiments, the two ribonucleic acids comprise sequences which are complementary to and/or hybridize to offset sequences selected from the group consisting of SEQ ID NO: 140-297. In some embodiments, the two ribonucleic acids comprise sequences which are complementary to and/or hybridize to offset sequences with a single nucleotide mismatch to a sequence selected from the group consisting of SEQ ID NO: 140-297. In some embodiments, the target polynucleotide sequences comprise multiple different portions of CCR5. In some embodiments, each of the multiple ribonucleic acids comprises a sequence which is complementary to and/or hybridizes to a different sequence selected from the group consisting of SEQ ID NOs: 1-139 and 304-333. In some embodiments, each of the multiple ribonucleic acids comprises a sequence which is complementary to and/or hybridizes to an offset sequence selected from the group consisting of SEQ ID NOs: 1-139 and 304-333. In some embodiments, each of the multiple ribonucleic acids comprises a sequence which is complementary to and/or hybridizes to a sequence with a single nucleotide mismatch to a different sequence selected from the group consisting of SEQ ID NOs: 1-139 and 304-333. In some embodiments, each of the multiple ribonucleic acids comprises a sequence which is complementary to and/or hybridizes to a sequence with a single nucleotide mismatch to an offset sequence selected from the group consisting of SEQ ID NOs: 1-139 and 304-333.

[0067] In some embodiments, the target polynucleotide sequences comprise multiple different portions of CXCR4. In some embodiments, each of the multiple ribonucleic acids comprises a sequence which is complementary to and/or hybridizes to a different sequence selected from the group consisting of SEQ ID NOs: 140-297 and 304-333. In some embodiments, each of the multiple ribonucleic acids comprises a sequence which is complementary to and/or hybridizes to an offset sequence selected from the group consisting of SEQ ID NOs: 140-297 and 304-333. In some embodiments, each of the multiple ribonucleic acids comprises a sequence which is complementary to and/or hybridizes to a sequence with a single nucleotide mismatch to a different sequence selected from the group consisting of SEQ ID NOs: 140-297

and 304-333. In some embodiments, each of the multiple ribonucleic acids comprises a sequence which is complementary to and/or hybridizes to a sequence with a single nucleotide mismatch to an offset sequence selected from the group consisting of SEQ ID NOs: 140-297 and 304-333.

[0068] In some embodiments, the target polynucleotide sequences comprise at least a portion of CCR5 and at least a portion of CXCR4. In some embodiments, each of the multiple ribonucleic acids comprises a sequence which is complementary to and/or hybridizes to a different sequence selected from the group consisting of SEQ ID NOs: 1-297 and 304-333. In some embodiments, each of the multiple ribonucleic acids comprises a sequence which is complementary to and/or hybridizes to an offset sequence selected from the group consisting of SEQ ID NOs: 1-297 and 304-333. In some embodiments, each of the multiple ribonucleic acids comprises a sequence which is complementary to and/or hybridizes to a sequence with a single nucleotide mismatch to a different sequence selected from the group consisting of SEQ ID NOs: 1-297 and 304-333. In some embodiments, each of the multiple ribonucleic acids comprises a sequence which is complementary to and/or hybridizes to a sequence with a single nucleotide mismatch to an offset sequence selected from the group consisting of SEQ ID NOs: 1-297 and 304-333. In some embodiments, the multiple ribonucleic acids comprise at least two ribonucleic acid sequences which are complementary to and/or hybridize to offset sequences selected from the group consisting of SEQ ID NOs: 1-139 and 304-333, and at least two ribonucleic acid sequences which are complementary to and/or hybridize to offset sequences selected from the group consisting of SEQ ID NOs: 140-297. In some embodiments, the multiple ribonucleic acids comprises at least two ribonucleic acid sequences which are complementary to and/or hybridize to different sequences with a single nucleotide mismatch to an offset sequence selected from the group consisting of SEQ ID NOs: 1-139 and 304-333, and at least two ribonucleic acid sequences which are complementary to and/or hybridize to different sequences with a single nucleotide mismatch to an offset sequence selected from the group consisting of SEQ ID NOs: 140-297.

[0069] In some embodiments, the disorder is a genetic disorder. In some embodiments, the disorder is a monogenic disorder. In some embodiments, the

disorder is human immunodeficiency virus (HIV) infection. In some embodiments, the disorder is acquired immunodeficiency syndrome (AIDS). In some embodiments, the two ribonucleic acids are designed to hybridize to a target motif immediately adjacent to a deoxyribonucleic acid motif recognized by the Cas protein. In some embodiments, each of the two ribonucleic acids are designed to hybridize to target motifs immediately adjacent to deoxyribonucleic acid motifs recognized by the Cas protein which flank a mutant allele located between the target motifs. In some embodiments, the multiple ribonucleic acids are designed to hybridize to target motifs immediately adjacent to deoxyribonucleic acid motifs recognized by the Cas protein. In some embodiments, the multiple ribonucleic acids are designed to hybridize to target motifs immediately adjacent to deoxyribonucleic acid motifs recognized by the Cas protein which flank mutant alleles located between the target motifs. In some embodiments, the two ribonucleic acids are selected to minimize hybridization with nucleic acid sequences other than the target polynucleotide sequence. In some embodiments, the multiple ribonucleic acids are selected to minimize hybridization with nucleic acid sequences other than the target polynucleotide sequence. In some embodiments, the target motif is selected such that it contains at least two mismatches when compared with all other genomic nucleotide sequences in the cell. In some embodiments, each target motif is selected such that it contains at least two mismatches when compared with all other genomic nucleotide sequences in the cell. In some embodiments, the target motif is selected such that it contains at least one mismatch when compared with all other genomic nucleotide sequences in the cell. In some embodiments, the target motif is selected such that it contains at least one mismatch when compared with all other genomic nucleotide sequences in the cell. In some embodiments, the two ribonucleic acids hybridize to a target motif that contains at least two mismatches when compared with all other genomic nucleotide sequences in the cell. In some embodiments, each of the multiple ribonucleic acids hybridize to target motifs that contain at least two mismatches when compared with all other genomic nucleotide sequences in the cell.

[0070] In some embodiments, the two ribonucleic acids hybridize to a target motif that contains at least one mismatch when compared with all other genomic nucleotide sequences in the cell. In some embodiments, each of the multiple

ribonucleic acids hybridize to target motifs that contain at least one mismatch when compared with all other genomic nucleotide sequences in the cell. In some embodiments, the efficiency of alteration at each loci is from about 50% to about 80%.

[0071] In some embodiments, the Cas protein is encoded by a modified nucleic acid. In some embodiments, the modified nucleic acid comprises a ribonucleic acid containing at least one modified nucleotide selected from the group consisting of pseudouridine, 5-methylcytodine, 2-thio-uridine, 5-methyluridine-5'-triphosphate, 4-thiouridine-5'-triphosphate, 5,6-dihydrouridine-5'-triphosphate, and 5-azauridine-5'-triphosphate. In some embodiments, at least one of the two ribonucleic acids is a modified ribonucleic acid comprising one to two modified nucleotides selected from the group consisting of pseudouridine, 5-methylcytodine, 2-thio-uridine, 5-methyluridine-5'-triphosphate, 4-thiouridine-5'-triphosphate, 5,6-dihydrouridine-5'-triphosphate, and 5-azauridine-5'-triphosphate. In some embodiments, the two ribonucleic acids comprise modified ribonucleic acids comprising one to two modified nucleotides selected from the group consisting of pseudouridine, 5-methylcytodine, 2-thio-uridine, 5-methyluridine-5'-triphosphate, 4-thiouridine-5'-triphosphate, 5,6-dihydrouridine-5'-triphosphate, and 5-azauridine-5'-triphosphate.

[0072] In some embodiments, any of the Cas protein or the ribonucleic acids are expressed from a plasmid. In some embodiments, any of the Cas protein or the ribonucleic acids are expressed using a promoter optimized for increased expression in stem cells. In some embodiments, the promoter is selected from the group consisting of a Cytomegalovirus (CMV) early enhancer element and a chicken beta-actin promoter, a chicken beta-actin promoter, an elongation factor-1 alpha promoter, and a ubiquitin promoter. In some embodiments, the method comprises selecting cells that express the Cas protein. In some embodiments, selecting cells comprises FACS. In some embodiments, FACs is used to select cells which co-express Cas and a fluorescent protein.

[0073] In some aspects, the invention provides a method for altering a target polynucleotide sequence in a cell comprising contacting the polynucleotide sequence in a cell selected from the group consisting of a human pluripotent cell, a primary human cell, and a non-transformed human cell, with a clustered regularly interspaced

short palindromic repeats-associated (Cas) protein and two ribonucleic acids, wherein the ribonucleic acids direct Cas protein to and hybridize to a target motif of the target polynucleotide sequence, wherein the target polynucleotide sequence is cleaved. In some embodiments, the efficiency of alteration of cells that express Cas protein is from about 8% to about 80%.

[0074] In some aspects, the invention provides a method for altering a target polynucleotide sequence in a cell comprising contacting the polynucleotide sequence in a cell selected from the group consisting of a human pluripotent cell, a primary human cell, and a non-transformed human cell, with a clustered regularly interspaced short palindromic repeats-associated (Cas) protein and two ribonucleic acids, wherein the ribonucleic acids direct Cas protein to and hybridize to a target motif of the target polynucleotide sequence, wherein the target polynucleotide sequence is cleaved, and wherein the efficiency of alteration of cells that express Cas protein is from about 8% to about 80%.

[0075] In some aspects, the invention provides a method for treating or preventing a disorder associated with expression of a polynucleotide sequence in a subject, the method comprising (a) altering a target polynucleotide sequence in a cell *ex vivo* by contacting the polynucleotide sequence in a cell selected from the group consisting of a human pluripotent cell, a primary human cell, and a non-transformed human cell, with a clustered regularly interspaced short palindromic repeats-associated (Cas) protein and two ribonucleic acids, wherein the ribonucleic acids direct Cas protein to and hybridize to a target motif of the target polynucleotide sequence, wherein the target polynucleotide sequence is cleaved, and (b) introducing the cell into the subject, thereby treating or preventing a disorder associated with expression of the polynucleotide sequence. In some embodiments, the efficiency of alteration is from about 8% to about 80%.

[0076] In some aspects, the invention provides a method for treating or preventing a disorder associated with expression of a polynucleotide sequence in a subject, the method comprising (a) altering a target polynucleotide sequence in a cell *ex vivo* by contacting the polynucleotide sequence in a cell selected from the group consisting of a human pluripotent cell, a primary human cell, and a non-transformed human cell, with a clustered regularly interspaced short palindromic repeats-

associated (Cas) protein and two ribonucleic acids, wherein the ribonucleic acids direct Cas protein to and hybridize to a target motif of the target polynucleotide sequence, wherein the target polynucleotide sequence is cleaved, and wherein the efficiency of alteration is from about 8% to about 80%, and (b) introducing the cell into the subject, thereby treating or preventing a disorder associated with expression of the polynucleotide sequence.

[0077] In some aspects, the invention provides a method for simultaneously altering multiple target polynucleotide sequences in a cell comprising contacting the polynucleotide sequences in a cell selected from the group consisting of a human pluripotent cell, a primary human cell, and a non-transformed human cell, with a clustered regularly interspaced short palindromic repeats-associated (Cas) protein and multiple ribonucleic acids, wherein the ribonucleic acids direct Cas protein to and hybridize to target motifs of the target polynucleotide sequences, wherein the target polynucleotide sequences are cleaved. In some embodiments, the efficiency of alteration of cells that express Cas protein is from about 8% to about 80%.

[0078] In some aspects, the invention provides a method for simultaneously altering multiple target polynucleotide sequences in a cell comprising contacting the polynucleotide sequences in a cell selected from the group consisting of a human pluripotent cell, a primary human cell, and a non-transformed human cell, with a clustered regularly interspaced short palindromic repeats-associated (Cas) protein and multiple ribonucleic acids, wherein the ribonucleic acids direct Cas protein to and hybridize to target motifs of the target polynucleotide sequences, wherein the target polynucleotide sequences are cleaved, and wherein the efficiency of alteration of cells that express Cas protein is from about 8% to about 80%.

[0079] In some aspects, the invention provides a method for treating or preventing a disorder associated with expression of polynucleotide sequences in a subject, the method comprising (a) altering target polynucleotide sequences in a cell *ex vivo* by contacting the polynucleotide sequences in a cell selected from the group consisting of a human pluripotent cell, a primary human cell, and a non-transformed human cell, with a clustered regularly interspaced short palindromic repeats-associated (Cas) protein and multiple ribonucleic acids, wherein the ribonucleic acids direct Cas protein to and hybridize to target motifs of the target polynucleotide

sequences, wherein the target polynucleotide sequences are cleaved, and (b) introducing the cell into the subject, thereby treating or preventing a disorder associated with expression of the polynucleotide sequences. In some embodiments, the efficiency of alteration of cells that express Cas protein is from about 8% to about 80%.

[0080] In some aspects, the invention provides a method for treating or preventing a disorder associated with expression of polynucleotide sequences in a subject, the method comprising (a) altering target polynucleotide sequences in a cell *ex vivo* by contacting the polynucleotide sequences in a cell selected from the group consisting of a human pluripotent cell, a primary human cell, and a non-transformed human cell, with a clustered regularly interspaced short palindromic repeats-associated (Cas) protein and multiple ribonucleic acids, wherein the ribonucleic acids direct Cas protein to and hybridize to target motifs of the target polynucleotide sequences, wherein the target polynucleotide sequences are cleaved, and wherein the efficiency of alteration of cells that express Cas protein is from about 8% to about 80%, and (b) introducing the cell into the subject, thereby treating or preventing a disorder associated with expression of the polynucleotide sequences.

[0081] In some aspects, the disclosure provides a composition comprising at least two ribonucleic acids each comprising a different sequence selected from the group consisting of SEQ ID NOs: 298-303.

[0082] In some aspects, the disclosure provides a composition comprising at least two ribonucleic acids each comprising a sequence which is complementary to and/or hybridizes to a different sequence selected from the group consisting of SEQ ID NOs: 1-139 and 304-333.

[0083] In some aspects, the disclosure provides a composition comprising at least two ribonucleic acids each comprising a sequence which is complementary to and/or hybridizes to a sequence with a single nucleotide mismatch to a sequence selected from the group consisting of SEQ ID NOs: 1-139 and 304-333.

[0084] In some aspects, the disclosure provides a composition comprising at least two ribonucleic acids each comprising a sequence which is complementary to and/or hybridizes to a different sequence selected from the group consisting of SEQ ID NOs: 140-297.

[0085] In some aspects, the disclosure provides a composition comprising at least two ribonucleic acids each comprising a sequence which is complementary to and/or hybridizes to a sequence with a single nucleotide mismatch to a sequence selected from the group consisting of SEQ ID NOs: 140-297.

[0086] In some embodiments, at least one of the two ribonucleic acids is a modified ribonucleic acid comprising one to two modified nucleotides selected from the group consisting of pseudouridine, 5-methylcytodine, 2-thio-uridine, 5-methyluridine-5'-triphosphate, 4-thiouridine-5'-triphosphate, 5,6-dihydrouridine-5'-triphosphate, and 5-azauridine-5'-triphosphate.

[0087] In some embodiments, the two ribonucleic acids comprise modified ribonucleic acids comprising one to two modified nucleotides selected from the group consisting of pseudouridine, 5-methylcytodine, 2-thio-uridine, 5-methyluridine-5'-triphosphate, 4-thiouridine-5'-triphosphate, 5,6-dihydrouridine-5'-triphosphate, and 5-azauridine-5'-triphosphate. In some embodiments, the composition includes a nucleic acid sequence encoding a Cas protein. In some embodiments, the composition includes a nucleic acid sequence encoding a Cas9 protein or a functional portion thereof. In some embodiments, the nucleic acid comprises a modified ribonucleic acid comprising at least one modified nucleotide selected from the group consisting of pseudouridine, 5-methylcytodine, 2-thio-uridine, 5-methyluridine-5'-triphosphate, 4-thiouridine-5'-triphosphate, 5,6-dihydrouridine-5'-triphosphate, and 5-azauridine-5'-triphosphate.

[0088] In some aspects, the invention provides a composition comprising a chimeric nucleic acid comprising a ribonucleic acid encoding a Cas protein and at least two additional ribonucleic acids each having a sequence selected from the group consisting of the ribonucleic acid sequences of SEQ ID NOs: 298-303.

[0089] In some aspects, the invention provides a chimeric nucleic acid comprising a ribonucleic acid encoding a Cas protein and at least two additional ribonucleic acids each having a sequence which is complementary to and/or hybridizes to a different sequence selected from the group consisting of SEQ ID NOs: 1-139 and 304-333.

[0090] In some aspects, the invention provides a composition comprising a chimeric nucleic acid comprising a ribonucleic acid encoding a Cas protein and at

least two additional ribonucleic acids each having a sequence which is complementary to and/or hybridizes to a different sequence selected from the group consisting of SEQ ID NOs: 140-297.

[0091] In some aspects, the invention provides a composition comprising a chimeric nucleic acid comprising a ribonucleic acid encoding a Cas protein and at least two additional ribonucleic acid sequences each comprising a sequence which is complementary to and/or hybridizes to a sequence with a single nucleotide mismatch to a sequence selected from the group consisting of SEQ ID NOs: 1-139 and 304-333.

[0092] In some aspects, the invention provides a composition comprising a chimeric nucleic acid comprising a ribonucleic acid encoding a Cas protein and at least two additional ribonucleic acid sequences each comprising a sequence which is complementary to and/or hybridizes to a sequence with a single nucleotide mismatch to a sequence selected from the group consisting of SEQ ID NOs: 140-297.

[0093] In some embodiments, the composition includes a nucleic acid sequence encoding a detectable marker. In some embodiments, the composition includes a nucleic acid sequence encoding a fluorescent protein. In some embodiments, the composition includes a promoter operably linked to the chimeric nucleic acid. In some embodiments, the promoter is optimized for increased expression in human stem cells. In some embodiments, the promoter is selected from the group consisting of a Cytomegalovirus (CMV) early enhancer element and a chicken beta-actin promoter, a chicken beta-actin promoter, an elongation factor-1 alpha promoter, and a ubiquitin promoter. In some embodiments, the chimeric nucleic acid comprises at least one modified nucleotide selected from the group consisting of pseudouridine, 5-methylcytodine, 2-thio-uridine, 5-methyluridine-5'-triphosphate, 4-thiouridine-5'-triphosphate, 5,6-dihydrouridine-5'-triphosphate, and 5-azauridine-5'-triphosphate. In some embodiments, the Cas protein comprises a Cas9 protein or a functional portion thereof.

[0094] In some aspects, the invention provides a kit for altering a target polynucleotide sequence in a cell comprising a Cas9 protein or a nucleic acid encoding the Cas9 protein, and at least two ribonucleic acids each comprising a different sequence selected from the group consisting of the ribonucleic acid sequences of SEQ ID NOs: 298-303.

[0095] In some aspects, the invention provides a kit for altering a target polynucleotide sequence in a cell comprising a Cas9 protein or a nucleic acid encoding the Cas9 protein, and at least two ribonucleic acids each comprising a sequence which is complementary to and/or hybridizes to a different sequence selected from the group consisting of SEQ ID NOs: 1-139 and 304-333.

[0096] In some aspects, the invention provides a kit for altering a target polynucleotide sequence in a cell comprising a Cas9 protein or a nucleic acid encoding the Cas9 protein, and at least two ribonucleic acids each comprising a sequence which is complementary to and/or hybridizes to a different sequence selected from the group consisting of SEQ ID NOs: 140-297.

[0097] In some embodiments, the kit includes one or more cell lines, cultures, or populations selected from the group consisting of human pluripotent cells, primary human cells, and non-transformed cells. In some aspects, the kit includes a DNA repair template.

[0098] In some aspects, the invention provides a method of administering cells to a subject in need of such cells, the method comprising: (a) contacting a cell or population of cells *ex vivo* with a Cas protein and two ribonucleic acids which direct Cas protein to and hybridize to a target polynucleotide sequence encoding B2M in the cell or population of cells, wherein the target polynucleotide sequence is cleaved; and (b) administering the resulting cells from (a) to a subject in need of such cells.

[0099] In some aspects, the invention provides a method of administering cells to a subject in need of such cells, the method comprising: (a) contacting a cell or population of cells *ex vivo* with (i) a Cas protein, (ii) at least two ribonucleic acids which direct Cas protein to and hybridize to a target polynucleotide sequence encoding B2M in the cell or population of cells, and (iii) at least two additional ribonucleic acids which direct Cas protein to and hybridize to a target polynucleotide sequence in the cell or population of cells, wherein the target polynucleotide sequences are cleaved; and (b) administering the resulting cell or cells from (a) to a subject in need of such cells.

[0100] In some embodiments, cleavage of the target polynucleotide sequence encoding B2M in the cell or population of cells reduces the likelihood that the resulting cell or cells will trigger a host immune response when the cells are

administered to the subject. In some aspects, the target polynucleotide sequence comprises CCR5. In some embodiments, the at least two ribonucleic acids comprise two different sequences selected from the group consisting of SEQ ID NOs: 298-303. In some embodiments, the at least two ribonucleic acids each comprise sequences which are complementary to and/or hybridize to a different sequence selected from the group consisting of SEQ ID NOs: 1-139 and 304-333. In some embodiments, the at least two ribonucleic acids each comprise sequences which are complementary to and/or hybridize to sequences comprising at least one nucleotide mismatch to different sequences selected from the group consisting of SEQ ID NOs: 1-139 and 304-333. In some embodiments, the target polynucleotide sequence comprises CXCR4. In some embodiments, the at least two ribonucleic acids each comprise sequences which are complementary to and/or hybridize to a different sequence selected from the group consisting of SEQ ID NOs: 140-297. In some embodiments, the at least two ribonucleic acids each comprise sequences which are complementary to and/or hybridize to sequences comprising at least one nucleotide mismatch to different sequences selected from the group consisting of SEQ ID NOs: 140-297. In some embodiments, the cell or population of cells comprises primary cells. In some embodiments, the subject in need of administration of cells is suffering from a disorder. In some embodiments, the disorder comprises a genetic disorder. In some embodiments, the disorder comprises an infection. In some embodiments, the disorder comprises HIV or AIDs. In some embodiments, the disorder comprises cancer.

[0101] In some aspects, the invention provides a method of reducing the likelihood that cells administered to a subject will trigger a host immune response in the subject, the method comprising: (a) contacting a cell or population of cells *ex vivo* with a Cas protein and two ribonucleic acids which direct Cas protein to and hybridize to a target polynucleotide sequence encoding B2M in the cell or population of cells, wherein the target polynucleotide sequence encoding B2M is cleaved, thereby reducing the likelihood that cells administered to the subject will trigger a host immune response in the subject; and (b) administering the resulting cells from (a) to a subject in need of such cells.

[0102] In some aspects, the invention provides a method of reducing the likelihood that cells administered to a subject will trigger a host immune response in the subject, the method comprising: (a) contacting a cell or population of cells *ex vivo* with (i) a Cas protein, (ii) at least two ribonucleic acids which direct Cas protein to and hybridize to a target polynucleotide sequence encoding B2M in the cell or population of cells, wherein the target polynucleotide sequence encoding B2M in the cell or population of cells is cleaved, thereby reducing the likelihood that the cell or population of cells will trigger a host immune response in the subject, and (iii) at least two additional ribonucleic acids which direct Cas protein to and hybridize to a target polynucleotide sequence in the cell or population of cells, wherein the target polynucleotide sequence is cleaved; and (b) administering the resulting cell or cells from (a) to a subject in need of such cells.

[0103] In some embodiments, the target polynucleotide sequence comprises CCR5. In some embodiments, the at least two ribonucleic acids comprise two different sequences selected from the group consisting of SEQ ID NOs: 298-303. In some embodiments, the at least two ribonucleic acids each comprise sequences which are complementary to and/or hybridize to a different sequence selected from the group consisting of SEQ ID NOs: 1-139 and 304-333. In some embodiments, the at least two ribonucleic acids each comprise sequences which are complementary to and/or hybridize to sequences comprising at least one nucleotide mismatch to different sequences selected from the group consisting of SEQ ID NOs: 1-139 and 304-333. In some embodiments, the target polynucleotide sequence comprises CXCR4. In some embodiments, the at least two ribonucleic acids each comprise sequences which are complementary to and/or hybridize to a different sequence selected from the group consisting of SEQ ID NOs: 140-297. In some embodiments, the at least two ribonucleic acids each comprise sequences which are complementary to and/or hybridize to sequences comprising at least one nucleotide mismatch to different sequences selected from the group consisting of SEQ ID NOs: 140-297. In some embodiments, the cell or population of cells comprises primary cells. In some embodiments, the subject in need of administration of cells is suffering from a disorder. In some embodiments, the disorder comprises a genetic disorder. In some embodiments, the disorder comprises an infection. In some embodiments, the

disorder comprises HIV or AIDs. In some embodiments, the disorder comprises cancer.

BRIEF DESCRIPTION OF THE DRAWINGS

[0104] The patent or application file contains at least one drawing executed in color. Copies of this patent or patent application publication with color drawings will be provided by the Office upon request and payment of the necessary fee.

[0105] Fig. 1 shows exemplary guide RNA sequences useful when the target polynucleotide sequence is human CCR5.

[0106] Fig. 2 shows exemplary guide RNA sequences useful when the target polynucleotide sequence is human CXCR4.

[0107] Fig. 3 shows an exemplary amino acid sequence of a Cas protein. Yellow highlights indicate Ruv-C-like domain. Underlining indicates HNH nuclease domain.

[0108] Figs. 4A, 4B, 4C, 4D and 4E demonstrate that a single guide strategy achieves high efficiency genome editing in cell lines, but not in clinically relevant primary somatic cells. Fig. 4A is a table showing CRISPR-targeting sites in the CCR5 locus (single guides), which were found by scanning the human chemokine receptor CCR5 gene for optimized guide RNA sequences using a CRISPR design program (available on the world wide web at http://CRISPR.mit.edu) (left panel). A total of 11 guide RNAs having a score greater than 50 was tested for editing efficiency in a K562 cell line. Fig. 4A (right panel) shows the editing efficiency of 7 of selected guides (% indels) was measured by a CEL surveyor assay. Fig. 4B shows a comparative analysis of genome-editing efficiency in cell lines 293T, K562 (left two panels) and primary human CD34+ HSPCs (right two panels) illustrating inefficient genome editing efficiency in primary CD34+ cells. Cells were transfected with Cas9 (lane 1) together with guide RNA (lane 2) or expression plasmids (lane 3). Fig. 4C is a schematic illustrating CRISPR-targeting sites in the CCR5 locus (single guides). Fig. 4D shows the results of targeting the B2M locus with single guide RNAs in 293T cells. Fig. 4E shows the results of flow cytometry analysis using a single guide strategy targeting B2M in 293T cell, which demonstrate that B2M CRISPRs ablate B2M surface expression with high efficiency.

[0109] Figs. 5A, 5B and 5C demonstrate that a double guide strategy achieves genome editing with high efficacy in clinically relevant cells. Fig. 5A shows that as compared to single guide (A or B), 2-guide combination (A+B) showed robust editing efficiency in targeting CCR5 in K562 cell line. Fig. 5B shows various guide combinations and spacing between each guide pair with orientation (upper panel). The PCR results (bottom left panel) and CEL assay (bottom right) show robust genome editing for tested guide pairs. Fig. 5C shows the results of PCR analysis indicating that with 2-guide combination wild-type Cas9 effectively deleted the DNA sequence between the two guides, in contrast to Nickase (D10A) which did not effectively delete the DNA sequence between the two guides. Fig. 5D is a schematic showing double B2M CRISPR combinations.

[0110] Figs. 6A and 6B demonstrate effective genome-editing in human CD34+ HSPC using a two-guide approach. Fig. 6A is a representative gel picture showing efficient clonal deletion frequency using two guides. Clonal deletion efficiency was determined by PCR carried on individual colony grown on methyl cellulose. Fig. 6B is a Table showing data obtained from two independent clonal deletion experiments, which suggests efficacious genome-editing in primary human CD34+ cells using a two-guide approach.

[0111] Figs. 7A, 7B and 7C demonstrate that in contrast to primary cells, the double guide strategy does not improve B2M editing efficiency in 293T cells. Fig. 7A shows the gating strategy for flow cytometry analysis of 293T cells electroporated with 1 μg Cas9 plus either 0.5 μg gRNA or 0.25 μg + 0.25 μg gRNA targeting B2M 72 hours post-transfection in a 6-well format. Fig. 7B shows the results of a SURVEYOR assay with B2M CRISPR gRNAs in 293T cells (72 h). Fig. 7C shows that the double guide strategy does not improve B2M cutting efficiency in 293T cells, in contrast to the double guide strategy which significantly improves B2M cutting efficiency in primary cells (Fig. 5).

[0112] Figs. 8A, 8B, 8C and 8D demonstrate ablation of B2M surface expression in somatic cells (e.g., primary CD4+ T cells) using a double guide strategy. Fig. 8A shows the results of a flow cytometry analysis demonstrating B2M knock-out efficiency in CD4+ T cells (total live cells). Fig. 8B shows the results of a flow cytometry analysis demonstrating B2M knock-out efficiency in CD4+ T cells

(gated on GFP+ cells). Fig. 8C shows a Table quantifying the results of a flow cytometry analysis demonstrating B2M knock-out efficiency in CD4+ T cells. Fig. 8D shows the results of a flow cytometry analysis of cells gated on live/7AAD neg/GFP+ cells, demonstrating that the double guide strategy results in ablation of B2M surface expression.

DETAILED DESCRIPTION OF THE INVENTION

[0113] Work described herein demonstrates methods of allele targeting using CRISPR/Cas systems resulting in mutant cells with efficiencies of up to 80%. In particular, work described herein surprisingly and unexpectedly demonstrates that a multiple guide strategy (e.g., using two or more ribonucleic acids which guide Cas protein to and hybridize to a target polynucleotide sequence) efficiently and effectively deletes target polynucleotide sequences (e.g., B2M, HPRT, CCR5 and/or CXCR4) in primary somatic cells (e.g., human blood cells, e.g., CD34+ and T cells), in contrast to a single guide strategy which has been demonstrated by the inventors to efficiently delete target polynucleotide sequences in cell lines (e.g., 293T) but not in primary somatic cells. These vastly improved methods permit CRISPR/Cas systems to be utilized effectively for the first time for therapeutic purposes. Methods of delivery of CRISPR/Cas systems to human stem cells are provided. In addition, methods of specifically identifying useful RNA guide sequences are provided, along with particular guide sequences useful in targeting specific genes (e.g., B2M, HPRT, CCR5 and/or CXCR4). Moreover, methods of treatment (e.g., methods of treating HIV infection) utilizing the compositions and methods disclosed herein are provided. Moreover, methods of administering cells (e.g., methods of administering a cell that has a reduced likelihood of triggering a host immune response) utilizing the compositions and methods disclosed herein are provided.

[0114] In one aspect, the present invention provides a method for altering a target polynucleotide sequence in a cell.

[0115] An exemplary method for altering a target polynucleotide sequence in a cell comprises contacting the polynucleotide sequence with a clustered regularly interspaced short palindromic repeats-associated (Cas) protein and from one to two ribonucleic acids, wherein the ribonucleic acids direct Cas protein to and hybridize to

a target motif of the target polynucleotide sequence, wherein the target polynucleotide sequence is cleaved, and wherein the efficiency of alteration of cells that express Cas protein is from about 50% to about 80%.

[0116] As used herein, the term "contacting" (i.e., contacting a polynucleotide sequence with a clustered regularly interspaced short palindromic repeats-associated (Cas) protein and/or ribonucleic acids) is intended to include incubating the Cas protein and/or the ribonucleic acids in the cell together *in vitro* (e.g., adding the Cas protein or nucleic acid encoding the Cas protein to cells in culture) or contacting a cell *ex vivo*. The step of contacting a target polynucleotide sequence with a Cas protein and/or ribonucleic acids as disclosed herein can be conducted in any suitable manner. For example, the cells may be treated in adherent culture, or in suspension culture. It is understood that the cells contacted with a Cas protein and/or ribonucleic acids as disclosed herein can also be simultaneously or subsequently contacted with another agent, such as a growth factor or other differentiation agent or environments to stabilize the cells, or to differentiate the cells further.

[0117] In another aspect, the present invention provides a method for treating or preventing a disorder associated with expression of a polynucleotide sequence in a subject.

[0118] The terms "treat", "treating", "treatment", etc., as applied to an isolated cell, include subjecting the cell to any kind of process or condition or performing any kind of manipulation or procedure on the cell. As applied to a subject, the terms refer to providing a cell in which a target polynucleotide sequence has been altered *ex vivo* according to the methods described herein to an individual. The individual is usually ill or injured, or at increased risk of becoming ill relative to an average member of the population and in need of such attention, care, or management.

[0119] As used herein, the term "treating" and "treatment" refers to administering to a subject an effective amount of cells with target polynucleotide sequences altered ex *vivo* according to the methods described herein so that the subject has a reduction in at least one symptom of the disease or an improvement in the disease, for example, beneficial or desired clinical results. For purposes of this invention, beneficial or desired clinical results include, but are not limited to, alleviation of one or more symptoms, diminishment of extent of disease, stabilized

(i.e., not worsening) state of disease, delay or slowing of disease progression, amelioration or palliation of the disease state, and remission (whether partial or total) , whether detectable or undetectable. Treating can refer to prolonging survival as compared to expected survival if not receiving treatment. Thus, one of skill in the art realizes that a treatment may improve the disease condition, but may not be a complete cure for the disease. As used herein, the term "treatment" includes prophylaxis. Alternatively, treatment is “effective” if the progression of a disease is reduced or halted. "Treatment" can also mean prolonging survival as compared to expected survival if not receiving treatment. Those in need of treatment include those already diagnosed with a disorder associated with expression of a polynucleotide sequence, as well as those likely to develop such a disorder due to genetic susceptibility or other factors.

[0120] By “treatment,” “prevention” or “amelioration” of a disease or disorder is meant delaying or preventing the onset of such a disease or disorder, reversing, alleviating, ameliorating, inhibiting, slowing down or stopping the progression, aggravation or deterioration the progression or severity of a condition associated with such a disease or disorder. In one embodiment, the symptoms of a disease or disorder are alleviated by at least 5%, at least 10%, at least 20%, at least 30%, at least 40%, or at least 50%.

[0121] An exemplary method for treating or preventing a disorder associated with expression of a polynucleotide sequence in a subject comprises (a) altering a target polynucleotide sequence in a cell *ex vivo* by contacting the polynucleotide sequence with a clustered regularly interspaced short palindromic repeats-associated (Cas) protein and from one to two ribonucleic acids, wherein the ribonucleic acids direct Cas protein to and hybridize to a target motif of the target polynucleotide sequence, wherein the target polynucleotide sequence is cleaved, and wherein the efficiency of alteration of cells that express Cas protein is from about 50% to about 80%, and (b) introducing the cell into the subject, thereby treating or preventing a disorder associated with expression of the polynucleotide sequence.

[0122] The present invention contemplates altering target polynucleotide sequences in any manner which is available to the skilled artisan utilizing a CRISPR/Cas system of the present invention. Any CRISPR/Cas system that is

capable of altering a target polynucleotide sequence in a cell can be used. Such CRISPR-Cas systems can employ a variety of Cas proteins (Haft *et* al. *PLoS Comput Biol.* 2005;1(6)e60). The molecular machinery of such Cas proteins that allows the CRISPR/Cas system to alter target polynucleotide sequences in cells include RNA binding proteins, endo- and exo-nucleases, helicases, and polymerases. In some embodiments, the CRISPR/Cas system is a CRISPR type I system. In some embodiments, the CRISPR/Cas system is a CRISPR type II system.

[0123] The CRISPR/Cas systems of the present invention can be used to alter a target polynucleotide sequence in a cell. The present invention contemplates altering target polynucleotide sequences in a cell for any purpose. In some embodiments, the target polynucleotide sequence in a cell is altered to produce a mutant cell. As used herein, a "mutant cell" refers to a cell with a resulting genotype that differs from its original genotype. In some instances, a "mutant cell" exhibits a mutant phenotype, for example when a normally functioning gene is altered using the CRISPR/Cas systems of the present invention. In other instances, a "mutant cell" exhibits a wild-type phenotype, for example when a CRISPR/Cas system of the present invention is used to correct a mutant genotype. In some embodiments, the target polynucleotide sequence in a cell is altered to correct or repair a genetic mutation (e.g., to restore a normal phenotype to the cell). In some embodiments, the target polynucleotide sequence in a cell is altered to induce a genetic mutation (e.g., to disrupt the function of a gene or genomic element).

[0124] In some embodiments, the alteration is an indel. As used herein, "indel" refers to a mutation resulting from an insertion, deletion, or a combination thereof. As will be appreciated by those skilled in the art, an indel in a coding region of a genomic sequence will result in a frameshift mutation, unless the length of the indel is a multiple of three. In some embodiments, the alteration is a point mutation. As used herein, "point mutation" refers to a substitution that replaces one of the nucleotides. A CRISPR/Cas system of the present invention can be used to induce an indel of any length or a point mutation in a target polynucleotide sequence.

[0125] In some embodiments, the alteration results in a knock out of the target polynucleotide sequence or a portion thereof. Knocking out a target polynucleotide sequence or a portion thereof using a CRISPR/Cas system of the present invention

can be useful for a variety of applications. For example, knocking out a target polynucleotide sequence in a cell can be performed *in vitro* for research purposes. For *ex vivo* purposes, knocking out a target polynucleotide sequence in a cell can be useful for treating or preventing a disorder associated with expression of the target polynucleotide sequence (e.g., by knocking out a mutant allele in a cell *ex vivo* and introducing those cells comprising the knocked out mutant allele into a subject).

[0126] As used herein, "knock out" includes deleting all or a portion of the target polynucleotide sequence in a way that interferes with the function of the target polynucleotide sequence. For example, a knock out can be achieved by altering a target polynucleotide sequence by inducing an indel in the target polynucleotide sequence in a functional domain of the target polynucleotide sequence (e.g., a DNA binding domain). Those skilled in the art will readily appreciate how to use the CRISPR/Cas systems of the present invention to knock out a target polynucleotide sequence or a portion thereof based upon the details described herein.

[0127] In some embodiments, the alteration results in reduced expression of the target polynucleotide sequence. The terms "decrease," "reduced," "reduction," and "decrease" are all used herein generally to mean a decrease by a statistically significant amount. However, for avoidance of doubt, decrease," "reduced," "reduction," "decrease" means a decrease by at least 10% as compared to a reference level, for example a decrease by at least about 20%, or at least about 30%, or at least about 40%, or at least about 50%, or at least about 60%, or at least about 70%, or at least about 80%, or at least about 90% or up to and including a 100% decrease (i.e. absent level as compared to a reference sample), or any decrease between 10-100% as compared to a reference level.

[0128] The terms "increased" ,"increase" or "enhance" or "activate" are all used herein to generally mean an increase by a statically significant amount; for the avoidance of any doubt, the terms "increased", "increase" or "enhance" or "activate" means an increase of at least 10% as compared to a reference level, for example an increase of at least about 20%, or at least about 30%, or at least about 40%, or at least about 50%, or at least about 60%, or at least about 70%, or at least about 80%, or at least about 90% or up to and including a 100% increase or any increase between 10-100% as compared to a reference level, or at least about a 2-fold, or at least about a 3-

fold, or at least about a 4-fold, or at least about a 5-fold or at least about a 10-fold increase, or any increase between 2-fold and 10-fold or greater as compared to a reference level.

[0129] The term “statistically significant" or “significantly" refers to statistical significance and generally means a two standard deviation (2SD) below normal, or lower, concentration of the marker. The term refers to statistical evidence that there is a difference. It is defined as the probability of making a decision to reject the null hypothesis when the null hypothesis is actually true. The decision is often made using the p-value.

[0130] In some embodiments, the alteration is a homozygous alteration. In some embodiments, the alteration is a heterozygous alteration.

[0131] In some embodiments, the alteration results in correction of the target polynucleotide sequence from an undesired sequence to a desired sequence. The CRISPR/Cas systems of the present invention can be used to correct any type of mutation or error in a target polynucleotide sequence. For example, the CRISPR/Cas systems of the present invention can be used to insert a nucleotide sequence that is missing from a target polynucleotide sequence due to a deletion. The CRISPR/Cas systems of the present invention can also be used to delete or excise a nucleotide sequence from a target polynucleotide sequence due to an insertion mutation. In some instances, the CRISPR/Cas systems of the present invention can be used to replace an incorrect nucleotide sequence with a correct nucleotide sequence (e.g., to restore function to a target polynucleotide sequence that is impaired due to a loss of function mutation, i.e., a SNP).

[0132] The CRISPR/Cas systems of the present invention can alter target polynucleotides with surprisingly high efficiency as compared to conventional CRISPR/Cas systems. In certain embodiments, the efficiency of alteration is at least about 5%. In certain embodiments, the efficiency of alteration is at least about 10%. In certain embodiments, the efficiency of alteration is from about 10% to about 80%. In certain embodiments, the efficiency of alteration is from about 30% to about 80%. In certain embodiments, the efficiency of alteration is from about 50% to about 80%. In some embodiments, the efficiency of alteration is greater than or equal to about 80%.

[0133] The CRISPR/Cas systems of the present invention can be used to alter any target polynucleotide sequence in a cell. Those skilled in the art will readily appreciate that desirable target polynucleotide sequences to be altered in any particular cell may correspond to any genomic sequence for which expression of the genomic sequence is associated with a disorder or otherwise facilitates entry of a pathogen into the cell. For example, a desirable target polynucleotide sequence to alter in a cell may be a polynucleotide sequence corresponding to a genomic sequence which contains a disease associated single polynucleotide polymorphism. In such example, the CRISPR/Cas systems of the present invention can be used to correct the disease associated SNP in a cell by replacing it with a wild-type allele. As another example, a polynucleotide sequence of a target gene which is responsible for entry or proliferation of a pathogen into a cell may be a suitable target for deletion or insertion to disrupt the function of the target gene to prevent the pathogen from entering the cell or proliferating inside the cell.

[0134] In some embodiments, the target polynucleotide sequence is a genomic sequence. In some embodiments, the target polynucleotide sequence is a human genomic sequence. In some embodiments, the target polynucleotide sequence is a mammalian genomic sequence. In some embodiments, the target polynucleotide sequence is a vertebrate genomic sequence.

[0135] In some embodiments, a target polynucleotide sequence is a pathogenic genomic sequence. Exemplary pathogenic genomic sequences include, but are not limited to a viral genomic sequence, a bacterial genomic sequence, a fungal genomic sequence, a toxin genomic sequence, or a parasitic genomic sequence. In such embodiments, the CRISPR/Cas systems of the present invention can be used to disrupt the function of a pathogen (e.g., to treat or prevent an infection by the pathogen) by cleaving a genomic sequence of the pathogen (e.g., a genomic sequence that is critical for entry into a cell, or responsible for multiplication, growth or survival once the pathogen is inside a cell).

[0136] In some embodiments, the target polynucleotide sequence is beta-2-microglobulin (B2M; Gene ID: 567). The B2M polynucleotide sequence encodes a serum protein associated with the heavy chain of the major histocompatibility complex (MHC) class I molecules which are expressed on the surface of virtually all

nucleated cells. B2M protein comprises a beta-pleated sheet structure that has been found to form amyloid fibrils in certain pathological conditions. The B2M gene has 4 exons which span approximately 8 kb. B2M has been observed in the serum of normal individuals and in elevated amounts in urine from patients having Wilson disease, cadmium poisoning, and various conditions leading to renal tubular dysfunction. Other pathological conditions known to be associated with the B2M include, without limitation, a homozygous mutation (e.g., ala11pro) in the B2M gene has been reported in individuals having familial hypercatabolic hypoproteinemia, a heterozygous mutation (e.g., asp76asn) in the B2M gene has been reported in individuals having familial visceral amyloidosis

[0137] In some embodiments, the target polynucleotide sequence is a variant of B2M. In some embodiments, the target polynucleotide sequence is a homolog of B2M. In some embodiments, the target polynucleotide sequence is an ortholog of B2M.

[0138] In some embodiments, the target polynucleotide sequence is hypoxanthine phosphoribosyltransferase 1 (HPRT1; Gene ID: 3251).

[0139] In some embodiments, the target polynucleotide sequence is CCR5 (Gene ID: 1234, also known as CC-CKR-5, CCCKR5, CCR-5, CD195, CKR-5, CKR5, CMKBR5, and IDDM22). In some embodiments, the target polynucleotide sequence is a variant of CCR5. In some embodiments, the target polynucleotide sequence is a homolog of CCR5. In some embodiments, the target polynucleotide sequence is an ortholog of CCR5.

[0140] In some embodiments, the target polynucleotide sequence is CXCR4 (Gene ID: 7852, also known as FB22; HM89; LAP3; LCR1; NPYR; WHIM; CD184; LESTR; NPY3R; NPYRL; HSY3RR; NPYY3R; and D2S201E). In some embodiments, the target polynucleotide sequence is a variant of CXCR4. In some embodiments, the target polynucleotide sequence is a homolog of CXCR4. In some embodiments, the target polynucleotide sequence is an ortholog of CXCR4.It should be appreciated that the CRISPR/Cas systems of the present invention can cleave target polynucleotide sequences in a variety of ways. In some embodiments, the target polynucleotide sequence is cleaved such that a double-strand break results. In some

embodiments, the target polynucleotide sequence is cleaved such that a single-strand break results.

[0141] The methods of the present invention can be used to alter any target polynucleotide sequence in a cell, as long as the target polynucleotide sequence in the cell contains a suitable target motif that allows at least one ribonucleic acid of the CRISPR/Cas system to direct the Cas protein to and hybridize to the target motif. Those skilled in the art will appreciate that the target motif for targeting a particular polynucleotide depends on the CRISPR/Cas system being used, and the sequence of the polynucleotide to be targeted.

[0142] In some embodiments, the target motif is at least 20 bp in length. In some embodiments, the target motif is a 20-nucleotide DNA sequence. In some embodiments, the target motif is a 20-nucleotide DNA sequence beginning with G and immediately precedes an NGG motif recognized by the Cas protein. In some embodiments, the target motif is $G(N)_{19}NGG$. In some embodiments, the target motif is a 20-nucleotide DNA sequence and immediately precedes an NGG motif recognized by the Cas protein. In some embodiments, the target motif is $(N)_{20}NGG$.

[0143] The target motifs of the present invention can be selected to minimize off-target effects of the CRISPR/Cas systems of the present invention. In some embodiments, the target motif is selected such that it contains at least two mismatches when compared with all other genomic nucleotide sequences in the cell. In some embodiments, the target motif is selected such that it contains at least one mismatch when compared with all other genomic nucleotide sequences in the cell. Those skilled in the art will appreciate that a variety of techniques can be used to select suitable target motifs for minimizing off-target effects (e.g., bioinformatics analyses).

[0144] In some embodiments, the target motif comprises a DNA sequence selected from the group consisting of SEQ ID NOs: 1-139. In some embodiments, the target motif comprises a DNA sequence comprising at least one nucleotide mismatch compared to a DNA sequence selected from the group consisting of SEQ ID NOs: 1-139. In some embodiments, the target motif comprises a DNA sequence comprising at least two nucleotide mismatches compared to a DNA sequence selected from the group consisting of SEQ ID NOs: 1-139. In some embodiments, the target motif comprises a DNA sequence selected from the group consisting of SEQ ID NOs: 140-

297. In some embodiments, the target motif comprises a DNA sequence comprising at least one nucleotide mismatch compared to a DNA sequence selected from the group consisting of SEQ ID NOs: 140-297. In some embodiments, the target motif comprises a DNA sequence comprising at least two nucleotide mismatches compared to a DNA sequence selected from the group consisting of SEQ ID NOs: 140-297. In some embodiments, the target motif comprises a DNA sequence selected from the group consisting of SEQ ID NOs: 304-333. In some embodiments, the target motif comprises a DNA sequence comprising at least one nucleotide mismatch compared to a DNA sequence selected from the group consisting of SEQ ID NOs: 304-333. In some embodiments, the target motif comprises a DNA sequence comprising at least two nucleotide mismatches compared to a DNA sequence selected from the group consisting of SEQ ID NOs: 304-333. In some embodiments, the CRISPR/Cas systems of the present invention utilize homology-directed repair to correct target polynucleotide sequences. In some embodiments, subsequent to cleavage of the target polynucleotide sequence, homology-directed repair occurs. In some embodiments, homology-directed repair is performed using an exogenously introduced DNA repair template. The exogenously introduced DNA repair template can be single-stranded or double-stranded. The DNA repair template can be of any length. Those skilled in the art will appreciate that the length of any particular DNA repair template will depend on the target polynucleotide sequence that is to be corrected. The DNA repair template can be designed to repair or replace any target polynucleotide sequence, particularly target polynucleotide sequences comprising disease associated polymorphisms (e.g., SNPs). For example, homology-directed repair of a mutant allele comprising such SNPs can be achieved with a CRISPR/Cas system by selecting two target motifs which flank the mutant allele, and an designing a DNA repair template to match the wild-type allele.

[0145] In some embodiments, a CRISPR/Cas system of the present invention includes a Cas protein and at least one to two one ribonucleic acids that are capable of directing the Cas protein to and hybridizing to a target motif of a target polynucleotide sequence.

[0146] As used herein, “protein” and “polypeptide” are used interchangeably to refer to a series of amino acid residues joined by peptide bonds (i.e., a polymer of

amino acids) and include modified amino acids (e.g., phosphorylated, glycated, glycosolated, etc.) and amino acid analogs. Exemplary polypeptides or proteins include gene products, naturally occurring proteins, homologs, paralogs, fragments and other equivalents, variants, and analogs of the above.

[0147] In some embodiments, a Cas protein comprises one or more amino acid substitutions or modifications. In some embodiments, the one or more amino acid substitutions comprises a conservative amino acid substitution. In some instances, substitutions and/or modifications can prevent or reduce proteolytic degradation and/or extend the half-life of the polypeptide in a cell. In some embodiments, the Cas protein can comprise a peptide bond replacement (e.g., urea, thiourea, carbamate, sulfonyl urea, etc.). In some embodiments, the Cas protein can comprise a naturally occurring amino acid. In some embodiments, the Cas protein can comprise an alternative amino acid (e.g., D-amino acids, beta-amino acids, homocysteine, phosphoserine, etc.). In some embodiments, a Cas protein can comprise a modification to include a moiety (e.g., PEGylation, glycosylation, lipidation, acetylation, end-capping, etc.).

[0148] In some embodiments, a Cas protein comprises a core Cas protein. Exemplary Cas core proteins include, but are not limited to Cas1, Cas2, Cas3, Cas4, Cas5, Cas6, Cas7, Cas8 and Cas9. In some embodiments, a Cas protein comprises a Cas protein of an *E. coli* subtype (also known as CASS2). Exemplary Cas proteins of the *E. Coli* subtype include, but are not limited to Cse1, Cse2, Cse3, Cse4, and Cas5e. In some embodiments, a Cas protein comprises a Cas protein of the Ypest subtype (also known as CASS3). Exemplary Cas proteins of the Ypest subtype include, but are not limited to Csy1, Csy2, Csy3, and Csy4. In some embodiments, a Cas protein comprises a Cas protein of the Nmeni subtype (also known as CASS4). Exemplary Cas proteins of the Nmeni subtype include, but are not limited to Csn1 and Csn2. In some embodiments, a Cas protein comprises a Cas protein of the Dvulg subtype (also known as CASS1). Exemplary Cas proteins of the Dvulg subtype include Csd1, Csd2, and Cas5d. In some embodiments, a Cas protein comprises a Cas protein of the Tneap subtype (also known as CASS7). Exemplary Cas proteins of the Tneap subtype include, but are not limited to, Cst1, Cst2, Cas5t. In some embodiments, a Cas protein comprises a Cas protein of the Hmari subtype. Exemplary Cas proteins of

the Hmari subtype include, but are not limited to Csh1, Csh2, and Cas5h. In some embodiments, a Cas protein comprises a Cas protein of the Apern subtype (also known as CASS5). Exemplary Cas proteins of the Apern subtype include, but are not limited to Csa1, Csa2, Csa3, Csa4, Csa5, and Cas5a. In some embodiments, a Cas protein comprises a Cas protein of the Mtube subtype (also known as CASS6). Exemplary Cas proteins of the Mtube subtype include, but are not limited to Csm1, Csm2, Csm3, Csm4, and Csm5. In some embodiments, a Cas protein comprises a RAMP module Cas protein. Exemplary RAMP module Cas proteins include, but are not limited to, Cmr1, Cmr2, Cmr3, Cmr4, Cmr5, and Cmr6.

[0149] In some embodiments, the Cas protein is a *Streptococcus pyogenes* Cas9 protein or a functional portion thereof. In some embodiments, the Cas protein is Cas9 protein from any bacterial species or functional portion thereof. Cas9 protein is a member of the type II CRISPR systems which typically include a trans-coded small RNA (tracrRNA), endogenous ribonuclease 3 (rnc) and a Cas protein. Cas 9 protein (also known as CRISPR-associated endonuclease Cas9/Csn1) is a polypeptide comprising 1368 amino acids. An exemplary amino acid sequence of a Cas9 protein (SEQ ID NO: 298) is shown in Figure 3. Cas 9 contains 2 enconuclease domains, including an RuvC-like domain (residues 7-22, 759-766 and 982-989) which cleaves target DNA that is noncomplementary to crRNA, and an HNH nuclease domain (residues 810-872) which cleave target DNA complementary to crRNA. In Figure 3, the RuvC-like domain is highlighted in yellow and the HNH nuclease domain is underlined.

[0150] As used herein, "functional portion" refers to a portion of a peptide which retains its ability to complex with at least one ribonucleic acid (e.g., guide RNA (gRNA)) and cleave a target polynucleotide sequence. In some embodiments, the functional portion comprises a combination of operably linked Cas9 protein functional domains selected from the group consisting of a DNA binding domain, at least one RNA binding domain, a helicase domain, and an endonuclease domain. In some embodiments, the functional domains form a complex.

[0151] In some embodiments, a functional portion of the Cas9 protein comprises a functional portion of a RuvC-like domain. In some embodiments, a

functional portion of the Cas9 protein comprises a functional portion of the HNH nuclease domain.

[0152] It should be appreciated that the present invention contemplates various of ways of contacting a target polynucleotide sequence with a Cas protein (e.g., Cas9). In some embodiments, exogenous Cas protein can be introduced into the cell in polypeptide form. In certain embodiments, Cas proteins can be conjugated to or fused to a cell-penetrating polypeptide or cell-penetrating peptide. As used herein, "cell-penetrating polypeptide" and "cell-penetrating peptide" refers to a polypeptide or peptide, respectively, which facilitates the uptake of molecule into a cell. The cell-penetrating polypeptides can contain a detectable label.

[0153] In certain embodiments, Cas proteins can be conjugated to or fused to a charged protein (e.g., that carries a positive, negative or overall neutral electric charge). Such linkage may be covalent. In some embodiments, the Cas protein can be fused to a superpositively charged GFP to significantly increase the ability of the Cas protein to penetrate a cell (Cronican *et al. ACS Chem Biol.*2010;5(8):747-52).

[0154] In certain embodiments, the Cas protein can be fused to a protein transduction domain (PTD) to facilitate its entry into a cell. Exemplary PTDs include Tat, oligoarginine, and penetratin.

[0155] In some embodiments, the Cas9 protein comprises a Cas9 polypeptide fused to a cell-penetrating peptide. In some embodiments, the Cas9 protein comprises a Cas9 polypeptide fused to a PTD. In some embodiments, the Cas9 protein comprises a Cas9 polypeptide fused to a tat domain. In some embodiments, the Cas9 protein comprises a Cas9 polypeptide fused to an oligoarginine domain. In some embodiments, the Cas9 protein comprises a Cas9 polypeptide fused to a penetratin domain. In some embodiments, the Cas9 protein comprises a Cas9 polypeptide fused to a superpositively charged GFP.

[0156] In some embodiments, the Cas protein can be introduced into a cell containing the target polynucleotide sequence in the form of a nucleic acid encoding the Cas protein (e.g., Cas9). The process of introducing the nucleic acids into cells can be achieved by any suitable technique. Suitable techniques include calcium phosphate or lipid-mediated transfection, electroporation, and transduction or infection using a viral vector. In some embodiments, the nucleic acid comprises

DNA. In some embodiments, the nucleic acid comprises a modified DNA, as described herein. In some embodiments, the nucleic acid comprises mRNA. In some embodiments, the nucleic acid comprises a modified mRNA, as described herein (e.g., a synthetic, modified mRNA).

[0157] In some embodiments, the Cas protein is complexed with the one to two ribonucleic acids. In some embodiments, the Cas protein is complexed with two ribonucleic acids. In some embodiments, the Cas protein is encoded by a modified nucleic acid, as described herein (e.g., a synthetic, modified mRNA).

[0158] The methods of the present invention contemplate the use of any ribonucleic acid that is capable of directing a Cas protein to and hybridizing to a target motif of a target polynucleotide sequence. In some embodiments, at least one of the ribonucleic acids comprises tracrRNA. In some embodiments, at least one of the ribonucleic acids comprises CRISPR RNA (crRNA). In some embodiments, at least one of the ribonucleic acids comprises a guide RNA that directs the Cas protein to and hybridizes to a target motif of the target polynucleotide sequence in a cell. In some embodiments, both of the one to two ribonucleic acids comprise a guide RNA that directs the Cas protein to and hybridizes to a target motif of the target polynucleotide sequence in a cell.The ribonucleic acids of the present invention can be selected to hybridize to a variety of different target motifs, depending on the particular CRISPR/Cas system employed, and the sequence of the target polynucleotide, as will be appreciated by those skilled in the art. The one to two ribonucleic acids can also be selected to minimize hybridization with nucleic acid sequences other than the target polynucleotide sequence. In some embodiments, the one to two ribonucleic acids hybridize to a target motif that contains at least two mismatches when compared with all other genomic nucleotide sequences in the cell. In some embodiments, the one to two ribonucleic acids hybridize to a target motif that contains at least one mismatch when compared with all other genomic nucleotide sequences in the cell. In some embodiments, the one to two ribonucleic acids are designed to hybridize to a target motif immediately adjacent to a deoxyribonucleic acid motif recognized by the Cas protein. In some embodiments, each of the one to two ribonucleic acids are designed to hybridize to target motifs immediately adjacent

to deoxyribonucleic acid motifs recognized by the Cas protein which flank a mutant allele located between the target motifs.

[0159] In some embodiments, at least one of the one to two ribonucleic acids comprises a sequence selected from the group consisting of the ribonucleic acid sequences of Fig. 1. In some embodiments, at least one of the one to two ribonucleic acids comprises a sequence with a single nucleotide mismatch to a sequence selected from the group consisting of the ribonucleic acid sequences of Fig. 1.

[0160] In some embodiments, at least one of the one to two ribonucleic acids comprises a sequence selected from the group consisting of the ribonucleic acid sequences of Fig. 2. In some embodiments, at least one of the one to two ribonucleic acids comprises a sequence with a single nucleotide mismatch to a sequence selected from the group consisting of the ribonucleic acid sequences of Fig. 2.

[0161] In some embodiments, each of the one to two ribonucleic acids comprises guide RNAs that directs the Cas protein to and hybridizes to a target motif of the target polynucleotide sequence in a cell. In some embodiments, the two guide RNA sequences comprise any combination of two guide ribonucleic acid sequences selected from the group consisting of SEQ ID NOs: 1-139. In some embodiments, the two guide RNA sequences comprise any combination of two guide ribonucleic acid sequences comprising RNA sequences which are complementary to two different sequences selected from the group consisting of SEQ ID NOs: 1-139. In some embodiments, the two guide RNA sequences comprise any combination of two guide ribonucleic acid sequences comprising RNA sequences which are complementary to two different offset sequences selected from the group consisting of SEQ ID NOs: 1-139. In some embodiments, the two guide RNA sequences comprise any combination of two guide ribonucleic acid sequences comprising RNA sequences which are complementary to two different sequences comprising at least one nucleotide mismatch compared to a sequence selected from the group consisting of SEQ ID NOs: 1-139. In some embodiments, the two guide RNA sequences comprise any combination of two guide ribonucleic acid sequences comprising RNA sequences which are complementary to two different offset sequences comprising at least one nucleotide mismatch compared to a sequence selected from the group consisting of SEQ ID NOs: 1-139. In some embodiments, the two guide RNA sequences comprise

any combination of two guide ribonucleic acid sequences comprising RNA sequences which hybridize to two different sequences selected from the group consisting of SEQ ID NOs: 1-139. In some embodiments, the two guide RNA sequences comprise any combination of two guide ribonucleic acid sequences comprising RNA sequences which hybridize to two different offset sequences selected from the group consisting of SEQ ID NOs: 1-139. In some embodiments, the two guide RNA sequences comprise any combination of two guide ribonucleic acid sequences comprising RNA sequences which hybridize to two different sequences comprising at least one nucleotide mismatch compared to a sequence selected from the group consisting of SEQ ID NOs: 1-139. In some embodiments, the two guide RNA sequences comprise any combination of two guide ribonucleic acid sequences comprising RNA sequences which hybridize to two different offset sequences comprising at least one nucleotide mismatch compared to a sequence selected from the group consisting of SEQ ID NOs: 1-139.

[0162] In some embodiments, the two guide RNA sequences comprise any combination of two guide ribonucleic acid sequences selected from the group consisting of SEQ ID NOs: 140-297. In some embodiments, the two guide RNA sequences comprise any combination of two guide ribonucleic acid sequences comprising RNA sequences which are complementary to two different sequences selected from the group consisting of SEQ ID NOs: 140-297. In some embodiments, the two guide RNA sequences comprise any combination of two guide ribonucleic acid sequences comprising RNA sequences which are complementary to two different offset sequences selected from the group consisting of SEQ ID NOs: 140-297. In some embodiments, the two guide RNA sequences comprise any combination of two guide ribonucleic acid sequences comprising RNA sequences which are complementary to two different sequences comprising at least one nucleotide mismatch compared to a sequence selected from the group consisting of SEQ ID NOs: 140-297. In some embodiments, the two guide RNA sequences comprise any combination of two guide ribonucleic acid sequences comprising RNA sequences which are complementary to two different offset sequences comprising at least one nucleotide mismatch compared to a sequence selected from the group consisting of SEQ ID NOs: 140-297. In some embodiments, the two guide RNA sequences

comprise any combination of two guide ribonucleic acid sequences comprising RNA sequences which hybridize to two different sequences selected from the group consisting of SEQ ID NOs: 140-297. In some embodiments, the two guide RNA sequences comprise any combination of two guide ribonucleic acid sequences comprising RNA sequences which hybridize to two different offset sequences selected from the group consisting of SEQ ID NOs: 140-297. In some embodiments, the two guide RNA sequences comprise any combination of two guide ribonucleic acid sequences comprising RNA sequences which hybridize to two different sequences comprising at least one nucleotide mismatch compared to a sequence selected from the group consisting of SEQ ID NOs: 140-297. In some embodiments, the two guide RNA sequences comprise any combination of two guide ribonucleic acid sequences comprising RNA sequences which hybridize to two different offset sequences comprising at least one nucleotide mismatch compared to a sequence selected from the group consisting of SEQ ID NOs: 140-297.

[0163] In some embodiments, the two guide RNA sequences comprise any combination of two guide ribonucleic acid sequences selected from the group consisting of SEQ ID NOs: 298-303. In some embodiments, the two guide ribonucleic acid sequences comprise a pair of guide ribonucleic acids selected from the group consisting of SEQ ID NOs: 299 and 303, SEQ ID NOs: 298 and 300, SEQ ID NOs: 299 and 300, SEQ ID NOs: 298 and 303, SEQ ID NOs: 299 and 301, SEQ ID NOs: 298 and 299, SEQ ID NOs: 301 and 303, SEQ ID NOs: 298 and 302, and SEQ ID NOs: 298 and 301. In some embodiments, the two guide RNA sequences comprise any combination of two guide ribonucleic acid sequences comprising RNA sequences which are complementary to two different sequences selected from the group consisting of SEQ ID NOs: 304-333. In some embodiments, the two guide RNA sequences comprise any combination of two guide ribonucleic acid sequences comprising RNA sequences which are complementary to two different offset sequences selected from the group consisting of SEQ ID NOs: 304-333. In some embodiments, the two guide RNA sequences comprise any combination of two guide ribonucleic acid sequences comprising RNA sequences which are complementary to two different sequences comprising at least one nucleotide mismatch compared to a sequence selected from the group consisting of SEQ ID NOs: 304-333. In some

embodiments, the two guide RNA sequences comprise any combination of two guide ribonucleic acid sequences comprising RNA sequences which are complementary to two different offset sequences comprising at least one nucleotide mismatch compared to a sequence selected from the group consisting of SEQ ID NOs: 304-333. In some embodiments, the two guide RNA sequences comprise any combination of two guide ribonucleic acid sequences comprising RNA sequences which hybridize to two different sequences selected from the group consisting of SEQ ID NOs: 304-333. In some embodiments, the two guide RNA sequences comprise any combination of two guide ribonucleic acid sequences comprising RNA sequences which hybridize to two different offset sequences selected from the group consisting of SEQ ID NOs: 304-333. In some embodiments, the two guide RNA sequences comprise any combination of two guide ribonucleic acid sequences comprising RNA sequences which hybridize to two different sequences comprising at least one nucleotide mismatch compared to a sequence selected from the group consisting of SEQ ID NOs: 304-333. In some embodiments, the two guide RNA sequences comprise any combination of two guide ribonucleic acid sequences comprising RNA sequences which hybridize to two different offset sequences comprising at least one nucleotide mismatch compared to a sequence selected from the group consisting of SEQ ID NOs: 304-333.

[0164] In some embodiments, the two ribonucleic acids (e.g., guide RNAs) are complementary to and/or hybridize to sequences on the same strand of a target polynucleotide sequence. In some embodiments, the two ribonucleic acids (e.g., guide RNAs) are complementary to and/or hybridize to sequences on the opposite strands of a target polynucleotide sequence. In some embodiments, the two ribonucleic acids (e.g., guide RNAs) are not complementary to and/or do not hybrize to sequences on the opposite strands of a target polynucleotide sequence. In some embodiments, the two ribonucleic acids (e.g., guide RNAs) are complementary to and/or hybridize to overlapping target motifs of a target polynucleotide sequence. In some embodiments, the two ribonucleic acids (e.g., guide RNAs) are complementary to and/or hybridize to offset target motifs of a target polynucleotide sequence.

[0165] The present invention also contemplates multiplex genomic editing. Those skilled in the art will appreciate that the description above with respect to

genomic editing of a single gene is equally applicable to the multiplex genomic editing embodiments described below.

[0166] In another aspect, the present invention provides a method for simultaneously altering multiple target polynucleotide sequences in a cell.

[0167] An exemplary method for simultaneously altering multiple target polynucleotide sequences in a cell comprises contacting the polynucleotide sequences with a clustered regularly interspaced short palindromic repeats-associated (Cas) protein and multiple ribonucleic acids, wherein the ribonucleic acids direct Cas protein to and hybridize to target motifs of the target polynucleotide sequences, wherein the target polynucleotide sequences are cleaved, and wherein the efficiency of alteration of cells that express Cas protein is from about 50% to about 80%.

[0168] In yet another aspect, the present invention provides a method for treating or preventing a disorder associated with expression of polynucleotide sequences in a subject.

[0169] An exemplary method for treating or preventing a disorder associated with expression of polynucleotide sequences in a subject comprises (a) altering target polynucleotide sequences in a cell *ex vivo* by contacting the polynucleotide sequences with a clustered regularly interspaced short palindromic repeats-associated (Cas) protein and multiple ribonucleic acids, wherein the ribonucleic acids direct Cas protein to and hybridize to target motifs of the target polynucleotide sequences, wherein the target polynucleotide sequences are cleaved, and wherein the efficiency of alteration of cells that express Cas protein is from about 50% to about 80%, and (b) introducing the cell into the subject, thereby treating or preventing a disorder associated with expression of the polynucleotide sequences.

[0170] As used herein, the terms "administering," "introducing" and "transplanting" are used interchangeably in the context of the placement of cells, e.g. cells described herein comprising a target polynucleotide sequence altered according to the methods of the invention into a subject, by a method or route which results in at least partial localization of the introduced cells at a desired site. The cells can be implanted directly to the desired site, or alternatively be administered by any appropriate route which results in delivery to a desired location in the subject where at least a portion of the implanted cells or components of the cells remain viable. The

period of viability of the cells after administration to a subject can be as short as a few hours, e. g. twenty-four hours, to a few days, to as long as several years. In some instances, the cells can also be administered a location other than the desired site, such as in the liver or subcutaneously, for example, in a capsule to maintain the implanted cells at the implant location and avoid migration of the implanted cells.

[0171] For *ex vivo* methods, cells can include autologous cells, i.e., a cell or cells taken from a subject who is in need of altering a target polynucleotide sequence in the cell or cells (i.e., the donor and recipient are the same individual). Autologous cells have the advantage of avoiding any immunologically-based rejection of the cells. Alternatively, the cells can be heterologous, e.g., taken from a donor. The second subject can be of the same or different species. Typically, when the cells come from a donor, they will be from a donor who is sufficiently immunologically compatible with the recipient, i.e., will not be subject to transplant rejection, to lessen or remove the need for immunosuppression. In some embodiments, the cells are taken from a xenogeneic source, i.e., a non-human mammal that has been genetically engineered to be sufficiently immunologically compatible with the recipient, or the recipient's species. Methods for determining immunological compatibility are known in the art, and include tissue typing to assess donor-recipient compatibility for HLA and ABO determinants. See, e.g., *Transplantation Immunology*, Bach and Auchincloss, Eds. (Wiley, John & Sons, Incorporated 1994).

[0172] Any suitable cell culture media can be used for *ex vivo* methods of the invention.

[0173] The terms "subject" and "individual" are used interchangeably herein, and refer to an animal, for example, a human from whom cells can be obtained and/or to whom treatment, including prophylactic treatment, with the cells as described herein, is provided. For treatment of those infections, conditions or disease states which are specific for a specific animal such as a human subject, the term subject refers to that specific animal. The "non-human animals" and “non-human mammals” as used interchangeably herein, includes mammals such as rats, mice, rabbits, sheep, cats, dogs, cows, pigs, and non-human primates. The term "subject" also encompasses any vertebrate including but not limited to mammals, reptiles, amphibians and fish. However, advantageously, the subject is a mammal such as a

human, or other mammals such as a domesticated mammal, e.g. dog, cat, horse, and the like, or production mammal, e.g. cow, sheep, pig, and the like.

[0174] In some embodiments, the alteration results in reduced expression of the target polynucleotide sequences. In some embodiments, the alteration results in a knock out of the target polynucleotide sequences. In some embodiments, the alteration results in correction of the target polynucleotide sequences from undesired sequences to desired sequences. In some embodiments, each alteration is a homozygous alteration. In some embodiments, the efficiency of alteration at each loci is from about 5% to about 80%. In some embodiments, the efficiency of alteration at each loci is from about 10% to about 80%. In some embodiments, the efficiency of alteration at each loci is from about 30% to about 80%. In some embodiments, the efficiency of alteration at each loci is from about 50% to about 80%. In some embodiments, the efficiency of alteration at each loci is from greater than or equal to about 80%.

[0175] In some embodiments, each target polynucleotide sequence is cleaved such that a double-strand break results. In some embodiments, each target polynucleotide sequence is cleaved such that a single-strand break results.

[0176] In some embodiments, the target polynucleotide sequences comprise multiple different portions of B2M. In some embodiments, the target polynucleotide sequences comprise multiple different portions of CCR5. In some embodiments, the target polynucleotide sequences comprise multiple different portions of CXCR4. In some embodiments, the target polynucleotide sequences comprise at least a portion of CCR5 and at least a portion of CXCR4.

[0177] In some embodiments, each target motif is a 20-nucleotide DNA sequence. In some embodiments, each target motif is a 20-nucleotide DNA sequence beginning with G and immediately precedes an NGG motif recognized by the Cas protein. In some embodiments, each target motif is a 20-nucleotide DNA sequence and immediately precedes an NGG motif recognized by the Cas protein. In some embodiments, each target motif is G(N)19NGG. In some embodiments, each target motif is (N)20NGG. In some embodiments, each target motif is selected such that it contains at least two mismatches when compared with all other genomic nucleotide sequences in the cell. In some embodiments, each target motif is selected such that it

contains at least two mismatches when compared with all other genomic nucleotide sequences in the cell.

[0178] In some embodiments, each target motif comprises a different DNA sequence selected from the group consisting of SEQ ID NOs: 1-139. In some embodiments, each target motif comprises a different DNA sequence comprising at least one nucleotide mismatch compared to a DNA sequence selected from the group consisting of SEQ ID NOs: 1-139. In some embodiments, each target motif comprises a DNA sequence comprising at least two nucleotide mismatches compared to a DNA sequence selected from the group consisting of SEQ ID NOs: 1-139. In some embodiments, each target motif comprises a different DNA sequence selected from the group consisting of SEQ ID NOs: 140-297. In some embodiments, each target motif comprises a different DNA sequence comprising at least one nucleotide mismatch compared to a DNA sequence selected from the group consisting of SEQ ID NOs: 140-297. In some embodiments, each target motif comprises a different DNA sequence comprising at least two nucleotide mismatches compared to a DNA sequence selected from the group consisting of SEQ ID NOs: 140-297. In some embodiments, each target motif comprises a different DNA sequence selected from the group consisting of SEQ ID NOs: 304-333. In some embodiments, each target motif comprises a different DNA sequence comprising at least one nucleotide mismatch compared to a DNA sequence selected from the group consisting of SEQ ID NOs: 304-333. In some embodiments, each target motif comprises a different DNA sequence comprising at least two nucleotide mismatches compared to a DNA sequence selected from the group consisting of SEQ ID NOs: 304-333.

[0179] In some embodiments, subsequent to cleavage of the target polynucleotide sequences, homology-directed repair occurs. In some embodiments, homology-directed repair is performed using an exogenously introduced DNA repair template. In some embodiments, exogenously introduced DNA repair template is single-stranded. In some embodiments, exogenously introduced DNA repair template is double-stranded.

[0180] In some embodiments, the Cas protein (e.g., Cas9) is complexed with the multiple ribonucleic acids. In some embodiments, the multiple ribonucleic acids are selected to minimize hybridization with nucleic acid sequences other than the

target polynucleotide sequence (e.g., multiple alterations of a single target polynucleotide sequence). In some embodiments, the multiple ribonucleic acids are selected to minimize hybridization with nucleic acid sequences other than the target polynucleotide sequences (e.g., one or more alterations of multiple target polynucleotide sequences). In some embodiments, each of the multiple ribonucleic acids hybridize to target motifs that contain at least two mismatches when compared with all other genomic nucleotide sequences in the cell. In some embodiments, each of the multiple ribonucleic acids hybridize to target motifs that contain at least one mismatch when compared with all other genomic nucleotide sequences in the cell. In some embodiments, each of the multiple ribonucleic acids are designed to hybridize to target motifs immediately adjacent to deoxyribonucleic acid motifs recognized by the Cas protein. In some embodiments, each of the multiple ribonucleic acids are designed to hybridize to target motifs immediately adjacent to deoxyribonucleic acid motifs recognized by the Cas protein which flank mutant alleles located between the target motifs.

[0181] In some embodiments, each of the multiple ribonucleic acids comprises a different sequence selected from the group consisting of the ribonucleic acid sequences of Fig. 1. In some embodiments, each of the multiple ribonucleic acids comprises a sequence with a single nucleotide mismatch to a different sequence selected from the group consisting of the ribonucleic acid sequences of Fig. 1. In some embodiments, each of the multiple ribonucleic acids comprises a different sequence selected from the group consisting of the ribonucleic acid sequences of Fig. 2. In some embodiments, each of the multiple ribonucleic acids comprises a sequence with a single nucleotide mismatch to a different sequence selected from the group consisting of the ribonucleic acid sequences of Fig. 2. In some embodiments, each of the multiple ribonucleic acids comprises a different sequence selected from the group consisting of the ribonucleic acid sequences of Fig. 1 and the ribonucleic acid sequences of Fig. 2. In some embodiments, each of the multiple ribonucleic acids comprises a sequence with a single nucleotide mismatch to a different sequence selected from the group consisting of the ribonucleic acid sequences of Fig. 1 and the ribonucleic acid sequences of Fig 2.

[0182] In some embodiments, each of the multiple ribonucleic acids comprises a different ribonucleic acid sequence which is complementary to and/or hybridizes to a sequence selected from the group consisting of SEQ ID NOs: 1-139 (Fig. 1). In some embodiments, each of the multiple ribonucleic acids comprises a different ribonucleic acid sequence which is complementary to and/or hybridizes to a sequence with a single nucleotide mismatch to a sequence selected from the group consisting of SEQ ID NOs: 1-139 (Fig. 1). In some embodiments, each of the multiple ribonucleic acids comprises a different ribonucleic acid sequence which is complementary to and/or hybridizes to a sequence selected from the group consisting of SEQ ID NOs: 140-297 (Fig. 2). In some embodiments, each of the multiple ribonucleic acids comprises a different ribonucleic acid sequence which is complementary to and/or hybridizes to a sequence with a single nucleotide mismatch to a sequence selected from the group consisting of SEQ ID NOs: 140-297 (FIG. 2).

[0183] In some embodiments, each of the multiple ribonucleic acids comprises a different sequence selected from the group consisting of the ribonucleic acid sequences of SEQ ID NOs: 298-303. In some embodiments, each of the multiple ribonucleic acids comprises a different ribonucleic acid sequence which is complementary to and/or hybridizes to a sequence selected from the group consisting of SEQ ID NOs: 304-333. In some embodiments, each of the multiple ribonucleic acids comprises a different ribonucleic acid sequence which is complementary to and/or hybridizes to a sequence with a single nucleotide mismatch to a sequence selected from the group consisting of SEQ ID NOs: 304-333.

[0184] It should be appreciated that any of the Cas protein or the ribonucleic acids can be expressed from a plasmid. In some embodiments, any of the Cas protein or the ribonucleic acids are expressed using a promoter optimized for increased expression in stem cells (e.g., human stem cells). In some embodiments, the promoter is selected from the group consisting of a Cytomegalovirus (CMV) early enhancer element and a chicken beta-actin promoter, a chicken beta-actin promoter, an elongation factor-1 alpha promoter, and a ubiquitin promoter.

[0185] In some embodiments, the methods of the present invention further comprise selecting cells that express the Cas protein. The present invention contemplates any suitable method for selecting cells. In some embodiments, selecting

cells comprises FACS. In some embodiments, FACs is used to select cells which co-express Cas and a fluorescent protein selected from the group consisting of green fluorescent protein and red fluorescent protein.

[0186] The present invention contemplates treating and/or preventing a variety of disorders which are associated with expression of a target polynucleotide sequences. It should be appreciated that the methods and compositions described herein can be used to treat or prevent disorders associated with increased expression of a target polynucleotide sequence, as well as decreased expression of a target polynucleotide sequence in a cell. Increased and decreased expression of a target polynucleotide sequence includes circumstances where the expression levels of the target polynucleotide sequence are increased or decreased, respectively, as well as circumstances in which the function and/or level of activity of an expression product of the target polynucleotide sequence increases or decreases, respectively, compared to normal expression and/or activity levels. Those skilled in the art will appreciate that treating or preventing a disorder associated with increased expression of a target polynucleotide sequence can be assessed by determining whether the levels and/or activity of the target polynucleotide sequence (or an expression product thereof) are decreased in a relevant cell after contacting a cell with a composition described herein. The skilled artisan will also appreciate that treating or preventing a disorder associated with decreased expression of a target polynucleotide sequence can be assessed by determining whether the levels and/or activity of the target polynucleotide sequence (or an expression product thereof) are increased in the relevant cell after contacting a cell with a composition described herein.

[0187] In some embodiments, the disorder is a genetic disorder. In some embodiments, the disorder is a monogenic disorder. In some embodiments, the disorder is a multigenic disorder. In some embodiments, the disorder is a disorder associated with one or more SNPs. Exemplary disorders associated with one or more SNPs include a complex disease described in U.S. Patent No. 7,627,436, Alzheimer's disease as described in PCT International Application Publication No. WO/2009/112882, inflammatory diseases as described in U.S. Patent Application Publication No. 2011/0039918, polycystic ovary syndrome as described in U.S. Patent Application Publication No. 2012/0309642, cardiovascular disease as described in

U.S. Patent No. 7,732,139, Huntington's disease as described in U.S. Patent Application Publication No. 2012/0136039, thromboembolic disease as described in European Patent Application Publication No. EP2535424, neurovascular diseases as described in PCT International Application Publication No. WO/2012/001613, psychosis as described in U.S. Patent Application Publication No. 2010/0292211, multiple sclerosis as described in U.S. Patent Application Publication No. 2011/0319288, schizophrenia, schizoaffective disorder, and bipolar disorder as described in PCT International Application Publication No. WO/2006/023719A2, bipolar disorder and other ailments as described in U.S. Patent Application Publication No. U.S. 2011/0104674, colorectal cancer as described in PCT International Application Publication No. WO/2006/104370A1, a disorder associated with a SNP adjacent to the AKT1 gene locus as described in U.S. Patent Application Publication No. U.S. 2006/0204969, an eating disorder as described in PCT International Application Publication No. WO/2003/012143A1, autoimmune disease as described in U.S. Patent Application Publication No. U.S. 2007/0269827, fibrostenosing disease in patients with Crohn's disease as described in U.S. Patent No. 7,790,370, and Parkinson's disease as described in U.S. Patent No. 8,187,811, each of which is incorporated herein by reference in its entirety. Other disorders associated with one or more SNPs which can be treated or prevented according to the methods of the present invention will be apparent to the skilled artisan.

[0188] In some embodiments, the disorder is human immunodeficiency virus (HIV) infection. In some embodiments, the disorder is acquired immunodeficiency syndrome (AIDS).

[0189] The methods of the present invention are capable of altering target polynucleotide sequences in a variety of different cells. In some embodiments, the methods of the present invention are used to alter target polynucleotide sequences in cells *ex vivo* for subsequent introduction into a subject. In some embodiments, the cell is a peripheral blood cell. In some embodiments, the cell is a stem cell or a pluripotent cell. In some embodiments, the cell is a hematopoietic stem cell. In some embodiments, the cell is a CD34+ cell. In some embodiments, the cell is a CD34+ mobilized peripheral blood cell. In some embodiments, the cell is a CD34+ cord blood cell. In some embodiments, the cell is a CD34+ bone marrow cell. In some

embodiments, the cell is a CD34+CD38-Lineage-CD90+CD45RA- cell. In some embodiments, the cell is a CD4+ cell. In some embodiments, the cell is a CD4+ T cell. In some embodiments, the cell is a hepatocyte. In some embodiments, the cell is a human pluripotent cell. In some embodiments, the cell is a primary human cell. In some embodiments, the cell is a primary CD34+ cell. In some embodiments, the cell is a primary CD34+ hematopoietic progenitor cell (HPC). In some embodiments, the cell is a primary CD4+ cell. In some embodiments, the cell is a primary CD4+ T cell. In some embodiments, the cell is an autologous primary cell. In some embodiments, the cell is an autologous primary somatic cell. In some embodiments, the cell is an allogeneic primary cell. In some embodiments, the cell is an allogeneic primary somatic cell. In some embodiments, the cell is a nucleated cell. In some embodiments, the cell is a non-transformed cell. In some embodiments, the cell is not a cancer cell. In some embodiments, the cell is not a tumor cell. In some embodiments, the cell is not a transformed cell.

[0190] In some aspects, the present invention provides a method for altering a target polynucleotide sequence in a cell comprising contacting the polynucleotide sequence in a cell selected from the group consisting of a human pluripotent cell, a primary human cell, and a non-transformed human cell, with a clustered regularly interspaced short palindromic repeats-associated (Cas) protein and from one to two ribonucleic acids, wherein the ribonucleic acids direct Cas protein to and hybridize to a target motif of the target polynucleotide sequence, wherein the target polynucleotide sequence is cleaved, and wherein the efficiency of alteration of cells that express Cas protein is from about 8% to about 80%.

[0191] In some aspects, the present invention provides a method for treating or preventing a disorder associated with expression of a polynucleotide sequence in a subject, the method comprising (a) altering a target polynucleotide sequence in a cell *ex vivo* by contacting the polynucleotide sequence in a cell selected from the group consisting of a human pluripotent cell, a primary human cell, and a non-transformed human cell, with a clustered regularly interspaced short palindromic repeats-associated (Cas) protein and from one to two ribonucleic acids, wherein the ribonucleic acids direct Cas protein to and hybridize to a target motif of the target polynucleotide sequence, wherein the target polynucleotide sequence is cleaved, and

wherein the efficiency of alteration is from about 8% to about 80%, and (b) introducing the cell into the subject, thereby treating or preventing a disorder associated with expression of the polynucleotide sequence.

[0192] In some aspects, the present invention provides a method for simultaneously altering multiple target polynucleotide sequences in a cell comprising contacting the polynucleotide sequences in a cell selected from the group consisting of a human pluripotent cell, a primary human cell, and a non-transformed human cell, with a clustered regularly interspaced short palindromic repeats-associated (Cas) protein and multiple ribonucleic acids, wherein the ribonucleic acids direct Cas protein to and hybridize to target motifs of the target polynucleotide sequences, wherein the target polynucleotide sequences are cleaved, and wherein the efficiency of alteration of cells that express Cas protein is from about 8% to about 80%.

[0193] In some aspects, the present invention provides a method for treating or preventing a disorder associated with expression of polynucleotide sequences in a subject, the method comprising (a) altering target polynucleotide sequences in a cell *ex vivo* by contacting the polynucleotide sequences in a cell selected from the group consisting of a human pluripotent cell, a primary human cell, and a non-transformed human cell, with a clustered regularly interspaced short palindromic repeats-associated (Cas) protein and multiple ribonucleic acids, wherein the ribonucleic acids direct Cas protein to and hybridize to target motifs of the target polynucleotide sequences, wherein the target polynucleotide sequences are cleaved, and wherein the efficiency of alteration of cells that express Cas protein is from about 8% to about 80%, and (b) introducing the cell into the subject, thereby treating or preventing a disorder associated with expression of the polynucleotide sequences.

[0194] The present invention also provides compositions comprising Cas proteins of the present invention or functional portions thereof, nucleic acids encoding the Cas proteins or functional portions thereof, and ribonucleic acid sequences which direct Cas proteins to and hybridize to target motifs of target polynucleotides in a cell.

[0195] In some aspects, the present invention provides a composition comprising at least one ribonucleic acid having a sequence selected from the group consisting of the ribonucleic acid sequences of Fig 1. In some aspects, the present invention provides a composition comprising at least one ribonucleic acid having a

sequence which is complementary to and/or hybridizes to a sequence selected from the group consisting of SEQ ID NOs: 1-139 (Fig. 1).

[0196] In some aspects, the present invention provides a composition comprising at least one ribonucleic acid comprising a sequence with a single nucleotide mismatch to a sequence selected from the group consisting of the ribonucleic acid sequences of Fig 1. In some aspects, the present invention provides a composition comprising at least one ribonucleic acid having a sequence which is complementary to and/or hybridizes to a sequence with a single nucleotide mismatch to a sequence selected from the group consisting of SEQ ID NOs: 1-139 (Fig. 1).

[0197] In some aspects, the present invention provides a composition comprising at least one ribonucleic acid having a sequence selected from the group consisting of the ribonucleic acid sequences of Fig 2. In some aspects, the present invention provides a composition comprising at least one ribonucleic acid having a sequence which is complementary to and/or hybridizes to a sequence selected from the group consisting of SEQ ID NOs: 140-297 (Fig. 2).

[0198] In some aspects, the present invention provides a composition comprising at least one ribonucleic acid comprising a sequence with a single nucleotide mismatch to a sequence selected from the group consisting of the ribonucleic acid sequences of Fig 2. In some aspects, the present invention provides a composition comprising at least one ribonucleic acid having a sequence which is complementary to and/or hybridizes to a sequence with a single nucleotide mismatch to a sequence selected from the group consisting of SEQ ID NOs: 140-297 (Fig. 2).

[0199] In some aspects, the present invention provides a composition comprising at least one ribonucleic acid having a sequence selected from the group consisting of the ribonucleic acid sequences of SEQ ID NOs: 298-303. In some aspects, the present invention provides a composition comprising at least one ribonucleic acid having a sequence which is complementary to and/or hybridizes to a sequence selected from the group consisting of SEQ ID NOs: 304-333. In some aspects, the present invention provides a composition comprising at least one ribonucleic acid having a sequence which is complementary to and/or hybridizes to a sequence comprising at least one nucleotide mismatch compared to a sequence selected from the group consisting of SEQ ID NOs: 304-333. In some aspects, the

present invention provides a composition comprising at least one ribonucleic acid having a sequence selected from the group consisting of the ribonucleic acid sequences of Fig. 1, the ribonucleic acid sequences of Fig. 2, a sequence with a single nucleotide mismatch to a ribonucleic acid sequence of Fig. 1, and a sequence with a single nucleotide mismatch to a ribonucleic acid sequences of Fig. 2.

[0200] In some embodiments, at least one of the ribonucleic acids in the composition is a modified ribonucleic acid as described herein (e.g., a synthetic, modified ribonucleic acid, e.g., comprising one to two modified nucleotides selected from the group consisting of pseudouridine, 5-methylcytodine, 2-thio-uridine, 5-methyluridine-5'-triphosphate, 4-thiouridine-5'-triphosphate, 5,6-dihydrouridine-5'-triphosphate, and 5-azauridine-5'-triphosphate, or any other modified nucleotides or modifications described herein).

[0201] In some embodiments, a composition of the present invention comprises a nucleic acid sequence encoding a Cas protein. In some embodiments, a composition of the present invention comprises nucleic acid sequence encoding Cas9 protein or a functional portion thereof.

[0202] In some embodiments, the nucleic acid encoding the Cas protein (e.g., Cas9) comprises a modified ribonucleic acid as described herein (e.g., a synthetic, modified mRNA described herein, e.g., comprising at least one modified nucleotide selected from the group consisting of pseudouridine, 5-methylcytodine, 2-thio-uridine, 5-methyluridine-5'-triphosphate, 4-thiouridine-5'-triphosphate, 5,6-dihydrouridine-5'-triphosphate, and 5-azauridine-5'-triphosphate or any other modified nucleotides or modifications described herein).

[0203] In some aspects, the present invention provides a composition comprising a chimeric nucleic acid comprising a ribonucleic acid encoding a Cas protein and at least one additional ribonucleic acid having a sequence selected from the group consisting of the ribonucleic acid sequences of Fig. 1. In some aspects, the present invention provides a composition comprising a chimeric nucleic acid comprising a ribonucleic acid encoding a Cas protein and at least one additional ribonucleic acid having a sequence which is complementary to and/or hybridizes to a sequence selected from the group consisting of SEQ ID NOs: 1-139 (Fig. 1). In some aspects, the present invention provides a composition comprising a chimeric nucleic

acid comprising a ribonucleic acid encoding a Cas protein and two additional ribonucleic acid each having a sequence which is complementary to and/or hybridizes to a different sequence selected from the group consisting of SEQ ID NOs: 1-139 (Fig. 1). In some aspects, the present invention provides a composition comprising a chimeric nucleic acid comprising a ribonucleic acid encoding a Cas protein and two additional ribonucleic acid each having a sequence which is complementary to and/or hybridizes to an offset sequence selected from the group consisting of SEQ ID NOs: 1-139 (Fig. 1).In some aspects, the present invention provides a composition comprising a chimeric nucleic acid comprising a ribonucleic acid encoding a Cas protein and at least one additional ribonucleic acid having a sequence selected from the group consisting of the ribonucleic acid sequences of Fig. 2. In some aspects, the present invention provides a composition comprising a chimeric nucleic acid comprising a ribonucleic acid encoding a Cas protein and at least one additional ribonucleic acid having a sequence which is complementary to and/or hybridizes to a sequence selected from the group consisting of SEQ ID NOs: 140-297 (Fig. 2). In some aspects, the present invention provides a composition comprising a chimeric nucleic acid comprising a ribonucleic acid encoding a Cas protein and two additional ribonucleic acids each having a sequence which is complementary to and/or hybridizes to a different sequence selected from the group consisting of SEQ ID NOs: 140-297 (Fig. 2). In some aspects, the present invention provides a composition comprising a chimeric nucleic acid comprising a ribonucleic acid encoding a Cas protein and two additional ribonucleic acids each having a sequence which is complementary to and/or hybridizes to an offset sequence selected from the group consisting of SEQ ID NOs: 140-297 (Fig. 2).

[0204] In some aspects, the present invention provides a composition comprising a chimeric nucleic acid comprising a ribonucleic acid encoding a Cas protein and at least one additional ribonucleic acid sequence comprising a sequence with a single nucleotide mismatch to a sequence selected from the group consisting of the ribonucleic acid sequences of Fig 1. In some aspects, the present invention provides a composition comprising a chimeric nucleic acid comprising a ribonucleic acid encoding a Cas protein and at least one additional ribonucleic acid sequence which is complementary to and/or hybridizes to a sequence with a single nucleotide

mismatch to a sequence selected from the group consisting of SEQ ID NOs: 1-139 (Fig 1). In some aspects, the present invention provides a composition comprising a chimeric nucleic acid comprising a ribonucleic acid encoding a Cas protein and two additional ribonucleic acid sequences each of which are complementary to and/or hybridize to different sequences with single nucleotide mismatches to a sequence selected from the group consisting of SEQ ID NOs: 1-139 (Fig 1). In some aspects, the present invention provides a composition comprising a chimeric nucleic acid comprising a ribonucleic acid encoding a Cas protein and two additional ribonucleic acid sequences each of which are complementary to and/or hybridize to offset sequences with single nucleotide mismatches to a sequence selected from the group consisting of SEQ ID NOs: 1-139 (Fig 1).

[0205] In some aspects, the present invention provides a composition comprising a chimeric nucleic acid comprising a ribonucleic acid encoding a Cas protein and at least one additional ribonucleic acid sequence comprising a sequence with a single nucleotide mismatch to a sequence selected from the group consisting of the ribonucleic acid sequences of Fig 2. In some aspects, the present invention provides a composition comprising a chimeric nucleic acid comprising a ribonucleic acid encoding a Cas protein and at least one additional ribonucleic acid sequence which is complementary to and/or hybridizes to a sequence with a single nucleotide mismatch to a sequence selected from the group consisting of SEQ ID NOs: 140-297 (Fig 1). In some aspects, the present invention provides a composition comprising a chimeric nucleic acid comprising a ribonucleic acid encoding a Cas protein and two additional ribonucleic acid sequences each of which is complementary to and/or hybridizes to a different sequence with a single nucleotide mismatch to a sequence selected from the group consisting of SEQ ID NOs: 140-297 (Fig 1). In some aspects, the present invention provides a composition comprising a chimeric nucleic acid comprising a ribonucleic acid encoding a Cas protein and two additional ribonucleic acid sequences each of which is complementary to and/or hybridizes to an offset sequence with a single nucleotide mismatch to a sequence selected from the group consisting of SEQ ID NOs: 140-297 (Fig 1).

[0206] In some aspects, the present invention provides a composition comprising a chimeric nucleic acid comprising a ribonucleic acid encoding a Cas

protein and at least one additional ribonucleic acid having a sequence selected from the group consisting of the ribonucleic acid sequences of SEQ ID NOs: 298-303.

[0207] In some aspects, the present invention provides a composition comprising a chimeric nucleic acid comprising a ribonucleic acid encoding a Cas protein and at least two additional ribonucleic acid having a sequence selected from the group consisting of the ribonucleic acid sequences of SEQ ID NOs: 298-303.

[0208] In some aspects, the present invention provides a composition comprising a chimeric nucleic acid comprising a ribonucleic acid encoding a Cas protein and two guide ribonucleic acids comprising SEQ ID NOs: 299 and 303. In some aspects, the present invention provides a composition comprising a chimeric nucleic acid comprising a ribonucleic acid encoding a Cas protein and two guide ribonucleic acids comprising SEQ ID NOs: 298 and 300. In some aspects, the present invention provides a composition comprising a chimeric nucleic acid comprising a ribonucleic acid encoding a Cas protein and two guide ribonucleic acids comprising SEQ ID NOs: 299 and 300. In some aspects, the present invention provides a composition comprising a chimeric nucleic acid comprising a ribonucleic acid encoding a Cas protein and two guide ribonucleic acids comprising SEQ ID NOs: 298 and 303. In some aspects, the present invention provides a composition comprising a chimeric nucleic acid comprising a ribonucleic acid encoding a Cas protein and two guide ribonucleic acids comprising SEQ ID NOs: 299 and 301. In some aspects, the present invention provides a composition comprising a chimeric nucleic acid comprising a ribonucleic acid encoding a Cas protein and two guide ribonucleic acids comprising SEQ ID NOs: 298 and 299. In some aspects, the present invention provides a composition comprising a chimeric nucleic acid comprising a ribonucleic acid encoding a Cas protein and two guide ribonucleic acids comprising SEQ ID NOs: 301 and 303. In some aspects, the present invention provides a composition comprising a chimeric nucleic acid comprising a ribonucleic acid encoding a Cas protein and two guide ribonucleic acids comprising SEQ ID NOs: 298 and 302. In some aspects, the present invention provides a composition comprising a chimeric nucleic acid comprising a ribonucleic acid encoding a Cas protein and two guide ribonucleic acids comprising SEQ ID NOs: 298 and 301.

[0209] In some aspects, the present invention provides a composition comprising at least one ribonucleic acid having a sequence which is complementary to and/or hybridizes to a sequence selected from the group consisting of SEQ ID NOs: 304-333. In some aspects, the present invention provides a composition comprising at least two ribonucleic acids each having a sequence which is complementary to and/or hybridizes to a different sequence selected from the group consisting of SEQ ID NOs: 304-333. In some aspects, the present invention provides a composition comprising at least two ribonucleic acids each having a sequence which is complementary to and/or hybridizes to an offset sequence selected from the group consisting of SEQ ID NOs: 304-333. In some aspects, the present invention provides a composition comprising at least one ribonucleic acid having a sequence which is complementary to and/or hybridizes to a sequence comprising at least one nucleotide mismatch compared to a sequence selected from the group consisting of SEQ ID NOs: 304-333. In some aspects, the present invention provides a composition comprising at least two ribonucleic acids each having a sequence which is complementary to and/or hybridizes to a different sequence comprising at least one nucleotide mismatch compared to a sequence selected from the group consisting of SEQ ID NOs: 304-333. In some aspects, the present invention provides a composition comprising at least two ribonucleic acids each having a sequence which is complementary to and/or hybridizes to an offset sequence comprising at least one nucleotide mismatch compared to a sequence selected from the group consisting of SEQ ID NOs: 304-333.

[0210] In some aspects, the present invention provides a composition comprising a chimeric nucleic acid comprising a ribonucleic acid encoding a Cas protein and at least one additional ribonucleic acid having a sequence selected from the group consisting of the ribonucleic acid sequences of Fig. 1, the ribonucleic acid sequences of Fig. 2, a sequence with a single nucleotide mismatch to a ribonucleic acid sequence of Fig. 1, and a sequence with a single nucleotide mismatch to a ribonucleic acid sequences of Fig. 2.

[0211] In some embodiments, a composition of the present invention comprises a nucleic acid sequence encoding a fluorescent protein selected from the group consisting of green fluorescent protein and red fluorescent protein. In some embodiments, a composition of the present invention comprises a promoter operably

linked to the chimeric nucleic acid. In some embodiments, the promoter is optimized for increased expression in human stem cells. In some embodiments, the promoter is optimized for increased expression in primary human cells. In some embodiments, the promoter is selected from the group consisting of a Cytomegalovirus (CMV) early enhancer element and a chicken beta-actin promoter, a chicken beta-actin promoter, an elongation factor-1 alpha promoter, and a ubiquitin promoter.

[0212] In some embodiments, the Cas protein comprises a Cas9 protein or a functional portion thereof.

[0213] The present invention also provides kits for practicing any of the methods of the present invention, as well as kits comprising the compositions of the present invention, and instructions for using the kits for altering target polynucleotide sequences in a cell.

[0214] In some aspects, the present invention comprises a kit for altering a target polynucleotide sequence in a cell comprising a Cas9 protein or a nucleic acid encoding the Cas9 protein, and at least one ribonucleic acid sequence selected from the group consisting of the ribonucleic acid sequences of Fig. 1, the ribonucleic acid sequences of Fig. 2, a sequence with a single nucleotide mismatch to a ribonucleic acid sequence of Fig. 1, and a sequence with a single nucleotide mismatch to a ribonucleic acid sequences of Fig. 2.

[0215] In some aspects, the present invention comprises a kit for altering a target polynucleotide sequence in a cell comprising a Cas9 protein or a nucleic acid encoding the Cas9 protein, and at least one ribonucleic acid sequence selected from the group consisting of the ribonucleic acid sequences of SEQ ID NO: 1-139 (Fig. 1).

[0216] In some aspects, the present invention comprises a kit for altering a target polynucleotide sequence in a cell comprising a Cas9 protein or a nucleic acid encoding the Cas9 protein, and at least one ribonucleic acid sequence which is complementary to and/or hybridizes to a sequence selected from the group consisting of SEQ ID NO: 1-139 (Fig. 1).

[0217] In some aspects, the present invention comprises a kit for altering a target polynucleotide sequence in a cell comprising a Cas9 protein or a nucleic acid encoding the Cas9 protein, and at least one ribonucleic acid sequence which is complementary to and/or hybridizes to a sequence comprising at least one nucleotide

mismatch to a sequence selected from the group consisting of SEQ ID NO: 1-139 (Fig. 1).

[0218] In some aspects, the present invention comprises a kit for altering a target polynucleotide sequence in a cell comprising a Cas9 protein or a nucleic acid encoding the Cas9 protein, and at least two ribonucleic acid sequences selected from the group consisting of the ribonucleic acid sequences of SEQ ID NO: 1-139 (Fig. 1).

[0219] In some aspects, the present invention comprises a kit for altering a target polynucleotide sequence in a cell comprising a Cas9 protein or a nucleic acid encoding the Cas9 protein, and at least two ribonucleic acid sequences each of which is complementary to and/or hybridizes to a different sequence selected from the group consisting of SEQ ID NO: 1-139 (Fig. 1).

[0220] In some aspects, the present invention comprises a kit for altering a target polynucleotide sequence in a cell comprising a Cas9 protein or a nucleic acid encoding the Cas9 protein, and at least two ribonucleic acid sequences each of which is complementary to and/or hybridizes to an offset sequence selected from the group consisting of SEQ ID NO: 1-139 (Fig. 1).

[0221] In some aspects, the present invention comprises a kit for altering a target polynucleotide sequence in a cell comprising a Cas9 protein or a nucleic acid encoding the Cas9 protein, and at least two ribonucleic acid sequences each of which is complementary to and/or hybridizes to a different sequence comprising at least one nucleotide mismatch to a sequence selected from the group consisting of SEQ ID NO: 1-139 (Fig. 1).

[0222] In some aspects, the present invention comprises a kit for altering a target polynucleotide sequence in a cell comprising a Cas9 protein or a nucleic acid encoding the Cas9 protein, and at least two ribonucleic acid sequences each of which is complementary to and/or hybridizes to an offset sequence comprising at least one nucleotide mismatch to a sequence selected from the group consisting of SEQ ID NO: 1-139 (Fig. 1).

[0223] In some aspects, the present invention comprises a kit for altering a target polynucleotide sequence in a cell comprising a Cas9 protein or a nucleic acid encoding the Cas9 protein, and at least one ribonucleic acid sequence selected from

the group consisting of the ribonucleic acid sequences of SEQ ID NO: 140-297 (Fig. 2).

[0224] In some aspects, the present invention comprises a kit for altering a target polynucleotide sequence in a cell comprising a Cas9 protein or a nucleic acid encoding the Cas9 protein, and at least one ribonucleic acid sequence which is complementary to and/or hybridizes to a sequence selected from the group consisting of SEQ ID NO: 140-297 (Fig. 2).

[0225] In some aspects, the present invention comprises a kit for altering a target polynucleotide sequence in a cell comprising a Cas9 protein or a nucleic acid encoding the Cas9 protein, and at least one ribonucleic acid sequence which is complementary to and/or hybridizes to a sequence comprising at least one nucleotide mismatch to a sequence selected from the group consisting of SEQ ID NO: 140-297 (Fig. 2).

[0226] In some aspects, the present invention comprises a kit for altering a target polynucleotide sequence in a cell comprising a Cas9 protein or a nucleic acid encoding the Cas9 protein, and at least two ribonucleic acid sequences selected from the group consisting of the ribonucleic acid sequences of SEQ ID NO: 140-297 (Fig. 2).

[0227] In some aspects, the present invention comprises a kit for altering a target polynucleotide sequence in a cell comprising a Cas9 protein or a nucleic acid encoding the Cas9 protein, and at least two ribonucleic acid sequences each of which is complementary to and/or hybridizes to a different sequence selected from the group consisting of SEQ ID NO: 140-297 (Fig. 2).

[0228] In some aspects, the present invention comprises a kit for altering a target polynucleotide sequence in a cell comprising a Cas9 protein or a nucleic acid encoding the Cas9 protein, and at least two ribonucleic acid sequences each of which is complementary to and/or hybridizes to an offset sequence selected from the group consisting of SEQ ID NO: 140-297 (Fig. 2).

[0229] In some aspects, the present invention comprises a kit for altering a target polynucleotide sequence in a cell comprising a Cas9 protein or a nucleic acid encoding the Cas9 protein, and at least two ribonucleic acid sequences each of which is complementary to and/or hybridizes to a different sequence comprising at least one

nucleotide mismatch to a sequence selected from the group consisting of SEQ ID NO: 140-297 (Fig. 2).

[0230] In some aspects, the present invention comprises a kit for altering a target polynucleotide sequence in a cell comprising a Cas9 protein or a nucleic acid encoding the Cas9 protein, and at least two ribonucleic acid sequences each of which is complementary to and/or hybridizes to an offset sequence comprising at least one nucleotide mismatch to a sequence selected from the group consisting of SEQ ID NO: 140-297 (Fig. 2).

[0231] In some aspects, the present invention comprises a kit for altering a target polynucleotide sequence in a cell comprising a Cas9 protein or a nucleic acid encoding the Cas9 protein, and at least two ribonucleic acid sequences selected from the group consisting of the ribonucleic acid sequences of SEQ ID NO: 298-303. In some embodiments, the at least two ribonucleic acid sequences of SEQ ID NO: 298-303 are complementary to and/or hybridize to offset target sequences.

[0232] In some aspects, the present invention comprises a kit for altering a target polynucleotide sequence in a cell comprising a Cas9 protein or a nucleic acid encoding the Cas9 protein, and at least two ribonucleic acid sequences each of which is complementary to and/or hybridizes to a different sequence selected from the group consisting of SEQ ID NO: 304-333.

[0233] In some aspects, the present invention comprises a kit for altering a target polynucleotide sequence in a cell comprising a Cas9 protein or a nucleic acid encoding the Cas9 protein, and at least two ribonucleic acid sequences each of which is complementary to and/or hybridizes to an offset sequence selected from the group consisting of SEQ ID NO: 304-333.

[0234] In some aspects, the present invention comprises a kit for altering a target polynucleotide sequence in a cell comprising a Cas9 protein or a nucleic acid encoding the Cas9 protein, and at least two ribonucleic acid sequences each of which is complementary to and/or hybridizes to a different sequence comprising at least one nucleotide mismatch to a sequence selected from the group consisting of SEQ ID NO: 304-333.

[0235] In some aspects, the present invention comprises a kit for altering a target polynucleotide sequence in a cell comprising a Cas9 protein or a nucleic acid

encoding the Cas9 protein, and at least two ribonucleic acid sequences each of which is complementary to and/or hybridizes to an offset sequence comprising at least one nucleotide mismatch to a sequence selected from the group consisting of SEQ ID NO: 304-333.

[0236] In some embodiments, the kit comprises one or more cell lines, cultures, or populations selected from the group consisting of human pluripotent cells, primary human cells, and non-transformed cells. In some embodiments, the kit comprises a DNA repair template.

[0237] In some aspects, the invention provides a method of administering cells to a subject in need of such cells, the method comprising: (a) contacting a cell or population of cells *ex vivo* with a Cas protein and two ribonucleic acids which direct Cas protein to and hybridize to a target polynucleotide sequence encoding B2M in the cell or population of cells, wherein the target polynucleotide sequence is cleaved; and (b) administering the resulting cells from (a) to a subject in need of such cells.

[0238] In some aspects, the invention provides a method of administering cells to a subject in need of such cells, the method comprising: (a) contacting a cell or population of cells *ex vivo* with (i) a Cas protein, (ii) at least two ribonucleic acids which direct Cas protein to and hybridize to a target polynucleotide sequence encoding B2M in the cell or population of cells, and (iii) at least two additional ribonucleic acids which direct Cas protein to and hybridize to a target polynucleotide sequence in the cell or population of cells, wherein the target polynucleotide sequences are cleaved; and (b) administering the resulting cell or cells from (a) to a subject in need of such cells.

[0239] B2M is an accessory chain of the MHC class I proteins which is necessary for the expression of MHC class I proteins on the surface of cells. It is believed that engineering cells (e.g., mutant cells) devoid of surface MHC class I may reduce the likelihood that the engineered cells will be detected by cytotoxic T cells when the engineered cells are administered to a host. Accordingly, in some embodiments, cleavage of the target polynucleotide sequence encoding B2M in the cell or population of cells reduces the likelihood that the resulting cell or cells will trigger a host immune response when the cells are administered to the subject.

[0240] In some aspects, the invention provides a method of reducing the likelihood that cells administered to a subject will trigger a host immune response in the subject, the method comprising: (a) contacting a cell or population of cells *ex vivo* with a Cas protein and two ribonucleic acids which direct Cas protein to and hybridize to a target polynucleotide sequence encoding B2M in the cell or population of cells, wherein the target polynucleotide sequence encoding B2M is cleaved, thereby reducing the likelihood that cells administered to the subject will trigger a host immune response in the subject; and (b) administering the resulting cells from (a) to a subject in need of such cells.

[0241] In some aspects, the invention provides a method of reducing the likelihood that cells administered to a subject will trigger a host immune response in the subject, the method comprising: (a) contacting a cell or population of cells *ex vivo* with (i) a Cas protein, (ii) at least two ribonucleic acids which direct Cas protein to and hybridize to a target polynucleotide sequence encoding B2M in the cell or population of cells, wherein the target polynucleotide sequence encoding B2M in the cell or population of cells is cleaved, thereby reducing the likelihood that the cell or population of cells will trigger a host immune response in the subject, and (iii) at least two additional ribonucleic acids which direct Cas protein to and hybridize to a target polynucleotide sequence in the cell or population of cells, wherein the target polynucleotide sequence is cleaved; and (b) administering the resulting cell or cells from (a) to a subject in need of such cells.

[0242] It is contemplated that the methods of administering cells can be adapted for any purpose in which administering such cells is desirable. In some embodiments, the subject in need of administration of cells is suffering from a disorder. For example, the subject may be suffering from a disorder in which the particular cells are decreased in function or number, and it may be desirable to administer functional cells obtained from a healthy or normal individual in which the particular cells are functioning properly and to administer an adequate number of those healthy cells to the individual to restore the function provided by those cells (e.g., hormone producing cells which have decreased in cell number or function, immune cells which have decreased in cell number or function, etc.). In such instances, the healthy cells can be engineered to decrease the likelihood of host

rejection of the healthy cells. In some embodiments, the disorder comprises a genetic disorder. In some embodiments, the disorder comprises an infection. In some embodiments, the disorder comprises HIV or AIDs. In some embodiments, the disorder comprises cancer.

[0243] In some aspects, the invention provides a method of reducing the likelihood that cells administered to a subject will trigger a host immune response in the subject, the method comprising: (a) contacting a cell or population of cells *ex vivo* with a Cas protein and two ribonucleic acids which direct Cas protein to and hybridize to a target polynucleotide sequence encoding B2M in the cell or population of cells, wherein the target polynucleotide sequence encoding B2M is cleaved, thereby reducing the likelihood that cells administered to the subject will trigger a host immune response in the subject; and (b) administering the resulting cells from (a) to a subject in need of such cells.

[0244] In some aspects, the invention provides a method of reducing the likelihood that cells administered to a subject will trigger a host immune response in the subject, the method comprising: (a) contacting a cell or population of cells *ex vivo* with (i) a Cas protein, (ii) at least two ribonucleic acids which direct Cas protein to and hybridize to a target polynucleotide sequence encoding B2M in the cell or population of cells, wherein the target polynucleotide sequence encoding B2M in the cell or population of cells is cleaved, thereby reducing the likelihood that the cell or population of cells will trigger a host immune response in the subject, and (iii) at least two additional ribonucleic acids which direct Cas protein to and hybridize to a target polynucleotide sequence in the cell or population of cells, wherein the target polynucleotide sequence is cleaved; and (b) administering the resulting cell or cells from (a) to a subject in need of such cells.As used herein "nucleic acid," in its broadest sense, includes any compound and/or substance that comprise a polymer of nucleotides linked via a phosphodiester bond. Exemplary nucleic acids include ribonucleic acids (RNAs), deoxyribonucleic acids (DNAs), threose nucleic acids (TNAs), glycol nucleic acids (GNAs), peptide nucleic acids (PNAs), locked nucleic acids (LNAs) or hybrids thereof. They may also include RNAi-inducing agents, RNAi agents, siRNAs, shRNAs, miRNAs, antisense RNAs, ribozymes, catalytic DNA, tRNA, RNAs that induce triple helix formation, aptamers, vectors, etc. In some

embodiments, the nucleic acid encoding the Cas protein is an mRNA. In some embodiments, the Cas protein is encoded by a modified nucleic acid (e.g., a synthetic, modified mRNA described herein).

[0245] The present invention contemplates the use of any nucleic acid modification available to the skilled artisan. The nucleic acids of the present invention can include any number of modifications. In some embodiments, the nucleic acid comprises one or more modifications selected from the group consisting of pyridin-4-one ribonucleoside, 5-aza-uridine, 2-thio-5-aza-uridine, 2-thiouridine, 4-thio-pseudouridine, 2-thio-pseudouridine, 5-hydroxyuridine, 3-methyluridine, 5-carboxymethyl-uridine, 1-carboxymethyl-pseudouridine, 5-propynyl-uridine, 1-propynyl-pseudouridine, 5-taurinomethyluridine, 1-taurinomethyl-pseudouridine, 5-taurinomethyl-2-thio-uridine, 1-taurinomethyl-4-thio-uridine, 5-methyl-uridine, 1-methyl-pseudouridine, 4-thio-1-methyl-pseudouridine, 2-thio-1-methyl-pseudouridine, 1-methyl-1-deaza-pseudouridine, 2-thio-1-methyl-1-deaza-pseudouridine, dihydrouridine, dihydropseudouridine, 2-thio-dihydrouridine, 2-thio-dihydropseudouridine, 2-methoxyuridine, 2-methoxy-4-thio-uridine, 4-methoxy-pseudouridine, 4-methoxy-2-thio-pseudouridine, 5-aza-cytidine, pseudoisocytidine, 3-methyl-cytidine, N4-acetylcytidine, 5-formylcytidine, N4-methylcytidine, 5-hydroxymethylcytidine, 1-methyl-pseudoisocytidine, pyrrolo-cytidine, pyrrolo-pseudoisocytidine, 2-thio-cytidine, 2-thio-5-methyl-cytidine, 4-thio-pseudoisocytidine, 4-thio-1-methyl-pseudoisocytidine, 4-thio-1-methyl-1-deaza-pseudoisocytidine, 1-methyl-1-deaza-pseudoisocytidine, zebularine, 5-aza-zebularine, 5-methyl-zebularine, 5-aza-2-thio-zebularine, 2-thio-zebularine, 2-methoxy-cytidine, 2-methoxy-5-methyl-cytidine, 4-methoxy-pseudoisocytidine, 4-methoxy-1-methyl-pseudoisocytidine, 2-aminopurine, 2,6-diaminopurine, 7-deaza-adenine, 7-deaza-8-aza-adenine, 7-deaza-2-aminopurine, 7-deaza-8-aza-2-aminopurine, 7-deaza-2,6-diaminopurine, 7-deaza-8-aza-2,6-diaminopurine, 1-methyladenosine, N6-methyladenosine, N6-isopentenyladenosine, N6-(cis-hydroxyisopentenyl)adenosine, 2-methylthio-N6-(cis-hydroxyisopentenyl)adenosine, N6-glycinylcarbamoyladenosine, N6-threonylcarbamoyladenosine, 2-methylthio-N6-threonyl carbamoyladenosine, N6,N6-dimethyladenosine, 7-methyladenine, 2-methylthio-adenine, and 2-methoxy-adenine, inosine, 1-methyl-inosine, wyosine,

wybutosine, 7-deaza-guanosine, 7-deaza-8-aza-guanosine, 6-thio-guanosine, 6-thio-7-deaza-guanosine, 6-thio-7-deaza-8-aza-guanosine, 7-methyl-guanosine, 6-thio-7-methyl-guanosine, 7-methylinosine, 6-methoxy-guanosine, 1-methylguanosine, N2-methylguanosine, N2,N2-dimethylguanosine, 8-oxo-guanosine, 7-methyl-8-oxo-guanosine, 1-methyl-6-thio-guanosine, N2-methyl-6-thio-guanosine, and N2,N2-dimethyl-6-thio-guanosine, and combinations thereof.

[0246] Preparation of modified nucleosides and nucleotides used in the manufacture or synthesis of modified RNAs of the present invention can involve the protection and deprotection of various chemical groups. The need for protection and deprotection, and the selection of appropriate protecting groups can be readily determined by one skilled in the art.

[0247] The chemistry of protecting groups can be found, for example, in Greene, et al., Protective Groups in Organic Synthesis, 2d. Ed., Wiley & Sons, 1991, which is incorporated herein by reference in its entirety.

[0248] Modified nucleosides and nucleotides can be prepared according to the synthetic methods described in Ogata et al. Journal of Organic Chemistry 74:2585-2588, 2009; Purmal et al. Nucleic Acids Research 22(1): 72-78, 1994; Fukuhara et al. Biochemistry 1(4): 563-568, 1962; and Xu et al. Tetrahedron 48(9): 1729-1740, 1992, each of which are incorporated by reference in their entirety.

[0249] Modified nucleic acids (e.g., ribonucleic acids) need not be uniformly modified along the entire length of the molecule. Different nucleotide modifications and/or backbone structures may exist at various positions in the nucleic acid. One of ordinary skill in the art will appreciate that the nucleotide analogs or other modification(s) may be located at any position(s) of a nucleic acid such that the function of the nucleic acid is not substantially decreased. A modification may also be a 5′ or 3′ terminal modification. The nucleic acids may contain at a minimum one and at maximum 100% modified nucleotides, or any intervening percentage, such as at least 50% modified nucleotides, at least 80% modified nucleotides, or at least 90% modified nucleotides.

[0250] In some embodiments, at least one of the one to two ribonucleic acids is a modified ribonucleic acid. In some embodiments, each of the one to two ribonucleic acids is a modified ribonucleic acid. In some embodiments, at least one of

the multiple ribonucleic acids is a modified ribonucleic acid. In some embodiments, a plurality of the multiple ribonucleic acids are modified. In some embodiments, each of the multiple ribonucleic acids are modified. Those skilled in the art will appreciate that the modified ribonucleic acids can include one or more of the nucleic acid modification described herein.

[0251] In some aspects, provided herein are synthetic, modified RNA molecules encoding polypeptides, where the synthetic, modified RNA molecules comprise one or more modifications, such that introducing the synthetic, modified RNA molecules to a cell results in a reduced innate immune response relative to a cell contacted with synthetic RNA molecules encoding the polypeptides not comprising the one or more modifications. In some embodiments, the Cas protein comprises a synthetic, modified RNA molecule encoding a Cas protein. In some embodiments, the Cas protein comprises a synthetic, modified RNA molecule encoding a Cas9 protein.

[0252] The synthetic, modified RNAs described herein include modifications to prevent rapid degradation by endo- and exo-nucleases and to avoid or reduce the cell's innate immune or interferon response to the RNA. Modifications include, but are not limited to, for example, (a) end modifications, e.g., 5′ end modifications (phosphorylation dephosphorylation, conjugation, inverted linkages, etc.), 3′ end modifications (conjugation, DNA nucleotides, inverted linkages, etc.), (b) base modifications, e.g., replacement with modified bases, stabilizing bases, destabilizing bases, or bases that base pair with an expanded repertoire of partners, or conjugated bases, (c) sugar modifications (e.g., at the 2′ position or 4′ position) or replacement of the sugar, as well as (d) internucleoside linkage modifications, including modification or replacement of the phosphodiester linkages. To the extent that such modifications interfere with translation (i.e., results in a reduction of 50% or more in translation relative to the lack of the modification—e.g., in a rabbit reticulocyte in vitro translation assay), the modification is not suitable for the methods and compositions described herein. Specific examples of synthetic, modified RNA compositions useful with the methods described herein include, but are not limited to, RNA molecules containing modified or non-natural internucleoside linkages. Synthetic, modified RNAs having modified internucleoside linkages include, among others, those that do

not have a phosphorus atom in the internucleoside linkage. In other embodiments, the synthetic, modified RNA has a phosphorus atom in its internucleoside linkage(s).

[0253] Non-limiting examples of modified internucleoside linkages include phosphorothioates, chiral phosphorothioates, phosphorodithioates, phosphotriesters, aminoalkylphosphotriesters, methyl and other alkyl phosphonates including 3′-alkylene phosphonates and chiral phosphonates, phosphinates, phosphoramidates including 3′-amino phosphoramidate and aminoalkylphosphoramidates, thionophosphoramidates, thionoalkylphosphonates, thionoalkylphosphotriesters, and boranophosphates having normal 3′-5′ linkages, 2′-5′ linked analogs of these, and those) having inverted polarity wherein the adjacent pairs of nucleoside units are linked 3′-5′ to 5′-3′ or 2′-5′ to 5′-2′. Various salts, mixed salts and free acid forms are also included.

[0254] Representative U.S. patents that teach the preparation of the above phosphorus-containing linkages include, but are not limited to, U.S. Pat. Nos. 3,687,808; 4,469,863; 4,476,301; 5,023,243; 5,177,195; 5,188,897; 5,264,423; 5,276,019; 5,278,302; 5,286,717; 5,321,131; 5,399,676; 5,405,939; 5,453,496; 5,455,233; 5,466,677; 5,476,925; 5,519,126; 5,536,821; 5,541,316; 5,550,111; 5,563,253; 5,571,799; 5,587,361; 5,625,050; 6,028,188; 6,124,445; 6,160,109; 6,169,170; 6,172,209; 6,239,265; 6,277,603; 6,326,199; 6,346,614; 6,444,423; 6,531,590; 6,534,639; 6,608,035; 6,683,167; 6,858,715; 6,867,294; 6,878,805; 7,015,315; 7,041,816; 7,273,933; 7,321,029; and U.S. Pat. RE39464, each of which is herein incorporated by reference in its entirety.

[0255] Modified internucleoside linkages that do not include a phosphorus atom therein have internucleoside linkages that are formed by short chain alkyl or cycloalkyl internucleoside linkages, mixed heteroatoms and alkyl or cycloalkyl internucleoside linkages, or one or more short chain heteroatomic or heterocyclic internucleoside linkages. These include those having morpholino linkages (formed in part from the sugar portion of a nucleoside); siloxane backbones; sulfide, sulfoxide and sulfone backbones; formacetyl and thioformacetyl backbones; methylene formacetyl and thioformacetyl backbones; alkene containing backbones; sulfamate backbones; methyleneimino and methylenehydrazino backbones; sulfonate and

sulfonamide backbones; amide backbones; and others having mixed N, O, S and CH2 component parts.

[0256] Representative U.S. patents that teach the preparation of modified oligonucleosides include, but are not limited to, U.S. Pat. Nos. 5,034,506; 5,166,315; 5,185,444; 5,214,134; 5,216,141; 5,235,033; 5,64,562; 5,264,564; 5,405,938; 5,434,257; 5,466,677; 5,470,967; 5,489,677; 5,541,307; 5,561,225; 5,596,086; 5,602,240; 5,608,046; 5,610,289; 5,618,704; 5,623,070; 5,663,312; 5,633,360; 5,677,437; and 5,677,439, each of which is herein incorporated by reference in its entirety.

[0257] Some embodiments of the synthetic, modified RNAs described herein include nucleic acids with phosphorothioate internucleoside linkages and oligonucleosides with heteroatom internucleoside linkage, and in particular —CH2-NH—CH2-, —CH2-N(CH3)-O—CH2-[known as a methylene (methylimino) or MMI], —CH2-O—N(CH3)-CH2-, —CH2-N(CH3)-N(CH3)-CH2- and —N(CH3)-CH2-CH2- [wherein the native phosphodiester internucleoside linkage is represented as —O—P—O—CH2-] of the above-referenced U.S. Pat. No. 5,489,677, and the amide backbones of the above-referenced U.S. Pat. No. 5,602,240, both of which are herein incorporated by reference in their entirety. In some embodiments, the nucleic acid sequences featured herein have morpholino backbone structures of the above-referenced U.S. Pat. No. 5,034,506, herein incorporated by reference in its entirety.

[0258] Synthetic, modified RNAs described herein can also contain one or more substituted sugar moieties. The nucleic acids featured herein can include one of the following at the 2′ position: H (deoxyribose); OH (ribose); F; O—, S—, or N-alkyl; O—, S—, or N-alkenyl; O—, S- or N-alkynyl; or O-alkyl-O-alkyl, wherein the alkyl, alkenyl and alkynyl can be substituted or unsubstituted C1 to C10 alkyl or C2 to C10 alkenyl and alkynyl. Exemplary modifications include O[(CH2)nO]mCH3, O(CH2).nOCH3, O(CH2)nNH2, O(CH2)nCH3, O(CH2)nONH2, and O(CH2)nON[(CH2)nCH3)]2, where n and m are from 1 to about 10. In some embodiments, synthetic, modified RNAs include one of the following at the 2′ position: C1 to C10 lower alkyl, substituted lower alkyl, alkaryl, aralkyl, O-alkaryl or O-aralkyl, SH, SCH3, OCN, Cl, Br, CN, CF3, OCF3, SOCH3, SO2CH3, ONO2, NO2, N3, NH2, heterocycloalkyl, heterocycloalkaryl, aminoalkylamino,

polyalkylamino, substituted silyl, a reporter group, an intercalator, a group for improving the pharmacokinetic properties of an RNA, or a group for improving the pharmacodynamic properties of a synthetic, modified RNA, and other substituents having similar properties. In some embodiments, the modification includes a 2′ methoxyethoxy (2′-O—CH2CH2OCH3, also known as 2′-O-(2-methoxyethyl) or 2′-MOE) (Martin et al., Helv. Chim. Acta, 1995, 78:486-504) i.e., an alkoxy-alkoxy group. Another exemplary modification is 2′-dimethylaminooxyethoxy, i.e., a O(CH2)2ON(CH3)2 group, also known as 2′-DMAOE, and 2′-dimethylaminoethoxyethoxy (also known in the art as 2′-O-dimethylaminoethoxyethyl or 2′-DMAEOE), i.e., 2′-O—CH2-O—CH2-N(CH2)2.

[0259] Other modifications include 2′-methoxy (2′-OCH3), 2′-aminopropoxy (2′-OCH2CH2CH2NH2) and 2′-fluoro (2′-F). Similar modifications can also be made at other positions on the nucleic acid sequence, particularly the 3′ position of the sugar on the 3′ terminal nucleotide or in 2′-5′ linked nucleotides and the 5′ position of 5′ terminal nucleotide. A synthetic, modified RNA can also have sugar mimetics such as cyclobutyl moieties in place of the pentofuranosyl sugar. Representative U.S. patents that teach the preparation of such modified sugar structures include, but are not limited to, U.S. Pat. Nos. 4,981,957; 5,118,800; 5,319,080; 5,359,044; 5,393,878; 5,446,137; 5,466,786; 5,514,785; 5,519,134; 5,567,811; 5,576,427; 5,591,722; 5,597,909; 5,610,300; 5,627,053; 5,639,873; 5,646,265; 5,658,873; 5,670,633; and 5,700,920, certain of which are commonly owned with the instant application, and each of which is herein incorporated by reference in its entirety.

[0260] As non-limiting examples, synthetic, modified RNAs described herein can include at least one modified nucleoside including a 2′-O-methyl modified nucleoside, a nucleoside comprising a 5′ phosphorothioate group, a 2′-amino-modified nucleoside, 2′-alkyl-modified nucleoside, morpholino nucleoside, a phosphoramidate or a non-natural base comprising nucleoside, or any combination thereof.

[0261] In some embodiments of this aspect and all other such aspects described herein, the at least one modified nucleoside is selected from the group consisting of 5-methylcytidine (5mC), N6-methyladenosine (m6A), 3,2′-O-dimethyluridine (m4U), 2-thiouridine (s2U), 2′ fluorouridine, pseudouridine, 2′-O-

methyluridine (Um), 2′ deoxyuridine (2′ dU), 4-thiouridine (s4U), 5-methyluridine (m5U), 2′-O-methyladenosine (m6A), N6,2′-O-dimethyladenosine (m6Am), N6,N6,2′-O-trimethyladenosine (m62Am), 2′-O-methylcytidine (Cm), 7-methylguanosine (m7G), 2′-O-methylguanosine (Gm), N2,7-dimethylguanosine (m2,7G), N2,N2,7-trimethylguanosine (m2,2,7G), and inosine (I).

[0262] Alternatively, a synthetic, modified RNA can comprise at least two modified nucleosides, 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 15, at least 20 or more, up to the entire length of the nucleotide. At a minimum, a synthetic, modified RNA molecule comprising at least one modified nucleoside comprises a single nucleoside with a modification as described herein. It is not necessary for all positions in a given synthetic, modified RNA to be uniformly modified, and in fact more than one of the aforementioned modifications can be incorporated in a single synthetic, modified RNA or even at a single nucleoside within a synthetic, modified RNA. However, it is preferred, but not absolutely necessary, that each occurrence of a given nucleoside in a molecule is modified (e.g., each cytosine is a modified cytosine e.g., 5mC). However, it is also contemplated that different occurrences of the same nucleoside can be modified in a different way in a given synthetic, modified RNA molecule (e.g., some cytosines modified as 5mC, others modified as 2′-O-methylcytidine or other cytosine analog). The modifications need not be the same for each of a plurality of modified nucleosides in a synthetic, modified RNA. Furthermore, in some embodiments of the aspects described herein, a synthetic, modified RNA comprises at least two different modified nucleosides. In some such preferred embodiments of the aspects described herein, the at least two different modified nucleosides are 5-methylcytidine and pseudouridine. A synthetic, modified RNA can also contain a mixture of both modified and unmodified nucleosides.

[0263] As used herein, “unmodified” or “natural” nucleosides or nucleobases include the purine bases adenine (A) and guanine (G), and the pyrimidine bases thymine (T), cytosine (C) and uracil (U). In some embodiments, a synthetic, modified RNA comprises at least one nucleoside (“base”) modification or substitution. Modified nucleosides include other synthetic and natural nucleobases such as inosine, xanthine, hypoxanthine, nubularine, isoguanisine, tubercidine, 2-(halo)adenine, 2-

(alkyl)adenine, 2-(propyl)adenine, 2 (amino)adenine, 2-(aminoalkyl)adenine, 2 (aminopropyl)adenine, 2 (methylthio) N6 (isopentenyl)adenine, 6 (alkyl)adenine, 6 (methyl)adenine, 7 (deaza)adenine, 8 (alkenyl)adenine, 8-(alkyl)adenine, 8 (alkynyl)adenine, 8 (amino)adenine, 8-(halo)adenine, 8-(hydroxyl)adenine, 8 (thioalkyl)adenine, 8-(thiol)adenine, N6-(isopentyl)adenine, N6 (methyl)adenine, N6, N6 (dimethyl)adenine, 2-(alkyl)guanine, 2 (propyl)guanine, 6-(alkyl)guanine, 6 (methyl)guanine, 7 (alkyl)guanine, 7 (methyl)guanine, 7 (deaza)guanine, 8 (alkyl)guanine, 8-(alkenyl)guanine, 8 (alkynyl)guanine, 8-(amino)guanine, 8 (halo)guanine, 8-(hydroxyl)guanine, 8 (thioalkyl)guanine, 8-(thiol)guanine, N (methyl)guanine, 2-(thio)cytosine, 3 (deaza) 5 (aza)cytosine, 3-(alkyl)cytosine, 3 (methyl)cytosine, 5-(alkyl)cytosine, 5-(alkynyl)cytosine, 5 (halo)cytosine, 5 (methyl)cytosine, 5 (propynyl)cytosine, 5 (propynyl)cytosine, 5 (trifluoromethyl)cytosine, 6-(azo)cytosine, N4 (acetyl)cytosine, 3 (3 amino-3 carboxypropyl)uracil, 2-(thio)uracil, 5 (methyl) 2 (thio)uracil, 5 (methylaminomethyl)-2 (thio)uracil, 4-(thio)uracil, 5 (methyl) 4 (thio)uracil, 5 (methylaminomethyl)-4 (thio)uracil, 5 (methyl) 2,4 (dithio)uracil, 5 (methylaminomethyl)-2,4 (dithio)uracil, 5 (2-aminopropyl)uracil, 5-(alkyl)uracil, 5-(alkynyl)uracil, 5-(allylamino)uracil, 5 (aminoallyl)uracil, 5 (aminoalkyl)uracil, 5 (guanidiniumalkyl)uracil, 5 (1,3-diazole-1-alkyl)uracil, 5-(cyanoalkyl)uracil, 5-(dialkylaminoalkyl)uracil, 5 (dimethylaminoalkyl)uracil, 5-(halo)uracil, 5-(methoxy)uracil, uracil-5 oxyacetic acid, 5 (methoxycarbonylmethyl)-2-(thio)uracil, 5 (methoxycarbonyl-methyl)uracil, 5 (propynyl)uracil, 5 (propynyl)uracil, 5 (trifluoromethyl)uracil, 6 (azo)uracil, dihydrouracil, N3 (methyl)uracil, 5-uracil (i.e., pseudouracil), 2 (thio)pseudouracil,4 (thio)pseudouracil,2,4-(dithio)psuedouracil,5-(alkyl)pseudouracil, 5-(methyl)pseudouracil, 5-(alkyl)-2-(thio)pseudouracil, 5-(methyl)-2-(thio)pseudouracil, 5-(alkyl)-4 (thio)pseudouracil, 5-(methyl)-4 (thio)pseudouracil, 5-(alkyl)-2,4 (dithio)pseudouracil, 5-(methyl)-2,4 (dithio)pseudouracil, 1 substituted pseudouracil, 1 substituted 2(thio)-pseudouracil, 1 substituted 4 (thio)pseudouracil, 1 substituted 2,4-(dithio)pseudouracil, 1 (aminocarbonylethylenyl)-pseudouracil, 1 (aminocarbonylethylenyl)-2(thio)-pseudouracil, 1 (aminocarbonylethylenyl)-4 (thio)pseudouracil, 1 (aminocarbonylethylenyl)-2,4-(dithio)pseudouracil, 1

(aminoalkylaminocarbonylethylenyl)-pseudouracil, 1 (aminoalkylamino-carbonylethylenyl)-2(thio)-pseudouracil, 1 (aminoalkylaminocarbonylethylenyl)-4 (thio)pseudouracil, 1 (aminoalkylaminocarbonylethylenyl)-2,4-(dithio)pseudouracil, 1,3-(diaza)-2-(oxo)-phenoxazin-1-yl, 1-(aza)-2-(thio)-3-(aza)-phenoxazin-1-yl, 1,3-(diaza)-2-(oxo)-phenthiazin-1-yl, 1-(aza)-2-(thio)-3-(aza)-phenthiazin-1-yl, 7-substituted 1,3-(diaza)-2-(oxo)-phenoxazin-1-yl, 7-substituted 1-(aza)-2-(thio)-3-(aza)-phenoxazin-1-yl, 7-substituted 1,3-(diaza)-2-(oxo)-phenthiazin-1-yl, 7-substituted 1-(aza)-2-(thio)-3-(aza)-phenthiazin-1-yl, 7-(aminoalkylhydroxy)-1,3-(diaza)-2-(oxo)-phenoxazin-1-yl, 7-(aminoalkylhydroxy)-1-(aza)-2-(thio)-3-(aza)-phenoxazin-1-yl, 7-(aminoalkylhydroxy)-1,3-(diaza)-2-(oxo)-phenthiazin-1-yl, 7-(aminoalkylhydroxy)-1-(aza)-2-(thio)-3-(aza)-phenthiazin-1-yl, 7-(guanidiniumalkylhydroxy)-1,3-(diaza)-2-(oxo)-phenoxazin-1-yl, 7-(guanidiniumalkylhydroxy)-1-(aza)-2-(thio)-3-(aza)-phenoxazin-1-yl, 7-(guanidiniumalkylhydroxy)-1,3-(diaza)-2-(oxo)-phenthiazin-1-yl, 7-(guanidiniumalkylhydroxy)-1-(aza)-2-(thio)-3-(aza)-phenthiazin-1-yl, 1,3,5-(triaza)-2,6-(dioxa)-naphthalene, inosine, xanthine, hypoxanthine, nubularine, tubercidine, isoguanisine, inosinyl, 2-aza-inosinyl, 7-deaza-inosinyl, nitroimidazolyl, nitropyrazolyl, nitrobenzimidazolyl, nitroindazolyl, aminoindolyl, pyrrolopyrimidinyl, 3-(methyl)isocarbostyrilyl, 5-(methyl)isocarbostyrilyl, 3-(methyl)-7-(propynyl)isocarbostyrilyl, 7-(aza)indolyl, 6-(methyl)-7-(aza)indolyl, imidizopyridinyl, 9-(methyl)-imidizopyridinyl, pyrrolopyrizinyl, isocarbostyrilyl, 7-(propynyl)isocarbostyrilyl, propynyl-7-(aza)indolyl, 2,4,5-(trimethyl)phenyl, 4-(methyl)indolyl, 4,6-(dimethyl)indolyl, phenyl, napthalenyl, anthracenyl, phenanthracenyl, pyrenyl, stilbenzyl, tetracenyl, pentacenyl, difluorotolyl, 4-(fluoro)-6-(methyl)benzimidazole, 4-(methyl)benzimidazole, 6-(azo)thymine, 2-pyridinone, 5 nitroindole, 3 nitropyrrole, 6-(aza)pyrimidine, 2 (amino)purine, 2,6-(diamino)purine, 5 substituted pyrimidines, N2-substituted purines, N6-substituted purines, 06-substituted purines, substituted 1,2,4-triazoles, pyrrolo-pyrimidin-2-on-3-yl, 6-phenyl-pyrrolo-pyrimidin-2-on-3-yl, para-substituted-6-phenyl-pyrrolo-pyrimidin-2-on-3-yl, ortho-substituted-6-phenyl-pyrrolo-pyrimidin-2-on-3-yl, bis-ortho-substituted-6-phenyl-pyrrolo-pyrimidin-2-on-3-yl, para-(aminoalkylhydroxy)-6-phenyl-pyrrolo-pyrimidin-2-on-3-yl, ortho-(aminoalkylhydroxy)-6-phenyl-pyrrolo-pyrimidin-2-on-3-

yl, bis-ortho-(aminoalkylhydroxy)-6-phenyl-pyrrolo-pyrimidin-2-on-3-yl, pyridopyrimidin-3-yl, 2-oxo-7-amino-pyridopyrimidin-3-yl, 2-oxo-pyridopyrimidine-3-yl, or any O-alkylated or N-alkylated derivatives thereof. Modified nucleosides also include natural bases that comprise conjugated moieties, e.g. a ligand. As discussed herein above, the RNA containing the modified nucleosides must be translatable in a host cell (i.e., does not prevent translation of the polypeptide encoded by the modified RNA). For example, transcripts containing s2U and m6A are translated poorly in rabbit reticulocyte lysates, while pseudouridine, m5U, and m5C are compatible with efficient translation. In addition, it is known in the art that 2′-fluoro-modified bases useful for increasing nuclease resistance of a transcript, leads to very inefficient translation. Translation can be assayed by one of ordinary skill in the art using e.g., a rabbit reticulocyte lysate translation assay.

[0264] Further modified nucleobases include those disclosed in U.S. Pat. No. 3,687,808, those disclosed in Modified Nucleosides in Biochemistry, Biotechnology and Medicine, Herdewijn, P. ed. Wiley-VCH, 2008; those disclosed in Int. Appl. No. PCT/US09/038,425, filed Mar. 26, 2009; those disclosed in The Concise Encyclopedia Of Polymer Science And Engineering, pages 858-859, Kroschwitz, J. L, ed. John Wiley & Sons, 1990, and those disclosed by Englisch et al., Angewandte Chemie, International Edition, 1991, 30, 613.

[0265] Representative U.S. patents that teach the preparation of certain of the above noted modified nucleobases as well as other modified nucleobases include, but are not limited to, the above noted U.S. Pat. No. 3,687,808, as well as U.S. Pat. Nos. 4,845,205; 5,130,30; 5,134,066; 5,175,273; 5,367,066; 5,432,272; 5,457,187; 5,457,191; 5,459,255; 5,484,908; 5,502,177; 5,525,711; 5,552,540; 5,587,469; 5,594,121, 5,596,091; 5,614,617; 5,681,941; 6,015,886; 6,147,200; 6,166,197; 6,222,025; 6,235,887; 6,380,368; 6,528,640; 6,639,062; 6,617,438; 7,045,610; 7,427,672; and 7,495,088, each of which is herein incorporated by reference in its entirety, and U.S. Pat. No. 5,750,692, also herein incorporated by reference in its entirety.

[0266] Another modification for use with the synthetic, modified RNAs described herein involves chemically linking to the RNA one or more ligands, moieties or conjugates that enhance the activity, cellular distribution or cellular uptake

of the RNA. The synthetic, modified RNAs described herein can further comprise a 5′ cap. In some embodiments of the aspects described herein, the synthetic, modified RNAs comprise a 5′ cap comprising a modified guanine nucleotide that is linked to the 5′ end of an RNA molecule using a 5′-5′ triphosphate linkage. As used herein, the term "5′ cap" is also intended to encompass other 5′ cap analogs including, e.g., 5′ diguanosine cap, tetraphosphate cap analogs having a methylene-bis(phosphonate) moiety (see e.g., Rydzik, A M et al., (2009) Org Biomol Chem 7(22):4763-76), dinucleotide cap analogs having a phosphorothioate modification (see e.g., Kowalska, J. et al., (2008) RNA 14(6):1119-1131), cap analogs having a sulfur substitution for a non-bridging oxygen (see e.g., Grudzien-Nogalska, E. et al., (2007) RNA 13(10): 1745-1755), N7-benzylated dinucleoside tetraphosphate analogs (see e.g., Grudzien, E. et al., (2004) RNA 10(9):1479-1487), or anti-reverse cap analogs (see e.g., Jemielity, J. et al., (2003) RNA 9(9): 1108-1122 and Stepinski, J. et al., (2001) RNA 7(10):1486-1495). In one such embodiment, the 5′ cap analog is a 5′ diguanosine cap. In some embodiments, the synthetic, modified RNA does not comprise a 5′ triphosphate.

[0267] The 5′ cap is important for recognition and attachment of an mRNA to a ribosome to initiate translation. The 5′ cap also protects the synthetic, modified RNA from 5′ exonuclease mediated degradation. It is not an absolute requirement that a synthetic, modified RNA comprise a 5′ cap, and thus in other embodiments the synthetic, modified RNAs lack a 5′ cap. However, due to the longer half-life of synthetic, modified RNAs comprising a 5′ cap and the increased efficiency of translation, synthetic, modified RNAs comprising a 5′ cap are preferred herein.

[0268] The synthetic, modified RNAs described herein can further comprise a 5′ and/or 3′ untranslated region (UTR). Untranslated regions are regions of the RNA before the start codon (5′) and after the stop codon (3′), and are therefore not translated by the translation machinery. Modification of an RNA molecule with one or more untranslated regions can improve the stability of an mRNA, since the untranslated regions can interfere with ribonucleases and other proteins involved in RNA degradation. In addition, modification of an RNA with a 5′ and/or 3′ untranslated region can enhance translational efficiency by binding proteins that alter ribosome binding to an mRNA. Modification of an RNA with a 3′ UTR can be used

to maintain a cytoplasmic localization of the RNA, permitting translation to occur in the cytoplasm of the cell. In one embodiment, the synthetic, modified RNAs described herein do not comprise a 5′ or 3′ UTR. In another embodiment, the synthetic, modified RNAs comprise either a 5′ or 3′ UTR. In another embodiment, the synthetic, modified RNAs described herein comprise both a 5′ and a 3′ UTR. In one embodiment, the 5′ and/or 3′ UTR is selected from an mRNA known to have high stability in the cell (e.g., a murine alpha-globin 3′ UTR). In some embodiments, the 5′ UTR, the 3′ UTR, or both comprise one or more modified nucleosides.

[0269] In some embodiments, the synthetic, modified RNAs described herein further comprise a Kozak sequence. The "Kozak sequence" refers to a sequence on eukaryotic mRNA having the consensus (gcc)gccRccAUGG , where R is a purine (adenine or guanine) three bases upstream of the start codon (AUG), which is followed by another 'G'. The Kozak consensus sequence is recognized by the ribosome to initiate translation of a polypeptide. Typically, initiation occurs at the first AUG codon encountered by the translation machinery that is proximal to the 5′ end of the transcript. However, in some cases, this AUG codon can be bypassed in a process called leaky scanning. The presence of a Kozak sequence near the AUG codon will strengthen that codon as the initiating site of translation, such that translation of the correct polypeptide occurs. Furthermore, addition of a Kozak sequence to a synthetic, modified RNA will promote more efficient translation, even if there is no ambiguity regarding the start codon. Thus, in some embodiments, the synthetic, modified RNAs described herein further comprise a Kozak consensus sequence at the desired site for initiation of translation to produce the correct length polypeptide. In some such embodiments, the Kozak sequence comprises one or more modified nucleosides.

[0270] In some embodiments, the synthetic, modified RNAs described herein further comprise a "poly (A) tail", which refers to a 3′ homopolymeric tail of adenine nucleotides, which can vary in length (e.g., at least 5 adenine nucleotides) and can be up to several hundred adenine nucleotides). The inclusion of a 3′ poly(A) tail can protect the synthetic, modified RNA from degradation in the cell, and also facilitates extra-nuclear localization to enhance translation efficiency. In some embodiments, the poly(A) tail comprises between 1 and 500 adenine nucleotides; in other embodiments the poly(A) tail comprises at least 5, at least 10, at least 20, at least 30, at least 40, at

least 50, at least 60, at least 70, at least 80, at least 90, at least 100, at least 110, at least 120, at least 130, at least 140, at least 150, at least 160, at least 170, at least 180, at least 190, at least 200, at least 225, at least 250, at least 275, at least 300, at least 325, at least 350, at least 375, at least 400, at least 425, at least 450, at least 475, at least 500 adenine nucleotides or more. In one embodiment, the poly(A) tail comprises between 1 and 150 adenine nucleotides. In another embodiment, the poly(A) tail comprises between 90 and 120 adenine nucleotides. In some such embodiments, the poly(A) tail comprises one or more modified nucleosides.

[0271] It is contemplated that one or more modifications to the synthetic, modified RNAs described herein permit greater stability of the synthetic, modified RNA in a cell. To the extent that such modifications permit translation and either reduce or do not exacerbate a cell's innate immune or interferon response to the synthetic, modified RNA with the modification, such modifications are specifically contemplated for use herein. Generally, the greater the stability of a synthetic, modified RNA, the more protein can be produced from that synthetic, modified RNA. Typically, the presence of AU-rich regions in mammalian mRNAs tend to destabilize transcripts, as cellular proteins are recruited to AU-rich regions to stimulate removal of the poly(A) tail of the transcript. Loss of a poly(A) tail of a synthetic, modified RNA can result in increased RNA degradation. Thus, in one embodiment, a synthetic, modified RNA as described herein does not comprise an AU-rich region. In particular, it is preferred that the 3′ UTR substantially lacks AUUUA sequence elements.

[0272] In one embodiment, a ligand alters the cellular uptake, intracellular targeting or half-life of a synthetic, modified RNA into which it is incorporated. In some embodiments a ligand provides an enhanced affinity for a selected target, e.g., molecule, cell or cell type, intracellular compartment, e.g., mitochondria, cytoplasm, peroxisome, lysosome, as, e.g., compared to a composition absent such a ligand. Preferred ligands do not interfere with expression of a polypeptide from the synthetic, modified RNA.

[0273] The ligand can be a substance, e.g., a drug, which can increase the uptake of the synthetic, modified RNA or a composition thereof into the cell, for example, by disrupting the cell's cytoskeleton, e.g., by disrupting the cell's

microtubules, microfilaments, and/or intermediate filaments. The drug can be, for example, taxol, vincristine, vinblastine, cytochalasin, nocodazole, japlakinolide, latrunculin A, phalloidin, swinholide A, indanocine, or myoservin.

[0274] In another aspect, the ligand is a moiety, e.g., a vitamin, which is taken up by a host cell. Exemplary vitamins include vitamin A, E, and K. Other exemplary vitamins include B vitamin, e.g., folic acid, B12, riboflavin, biotin, pyridoxal or other vitamins or nutrients taken up, for example, by cancer cells. Also included are HSA and low density lipoprotein (LDL).

[0275] In another aspect, the ligand is a cell-permeation agent, preferably a helical cell-permeation agent. Preferably, the agent is amphipathic. An exemplary agent is a peptide such as tat or antennopedia. If the agent is a peptide, it can be modified, including a peptidylmimetic, invertomers, non-peptide or pseudo-peptide linkages, and use of D-amino acids. The helical agent is preferably an alpha-helical agent, which preferably has a lipophilic and a lipophobic phase.

[0276] A "cell permeation peptide" is capable of permeating a cell, e.g., a microbial cell, such as a bacterial or fungal cell, or a mammalian cell, such as a human cell. A microbial cell-permeating peptide can be, for example, an α-helical linear peptide (e.g., LL-37 or Ceropin P1), a disulfide bond-containing peptide (e.g., α-defensin, β-defensin or bactenecin), or a peptide containing only one or two dominating amino acids (e.g., PR-39 or indolicidin). For example, a cell permeation peptide can be a bipartite amphipathic peptide, such as MPG, which is derived from the fusion peptide domain of HIV-1 gp41 and the NLS of SV40 large T antigen (Simeoni et al., Nucl. Acids Res. 31:2717-2724, 2003).

[0277] The synthetic, modified RNAs described herein can be synthesized and/or modified by methods well established in the art, such as those described in "Current Protocols in Nucleic Acid Chemistry," Beaucage, S. L. et al. (Edrs.), John Wiley & Sons, Inc., New York, N.Y., USA, which is hereby incorporated herein by reference in its entirety. Transcription methods are described further herein in the Examples.

[0278] In one embodiment of the aspects described herein, a template for a synthetic, modified RNA is synthesized using "splint-mediated ligation," which allows for the rapid synthesis of DNA constructs by controlled concatenation of long

oligos and/or dsDNA PCR products and without the need to introduce restriction sites at the joining regions. It can be used to add generic untranslated regions (UTRs) to the coding sequences of genes during T7 template generation. Splint mediated ligation can also be used to add nuclear localization sequences to an open reading frame, and to make dominant-negative constructs with point mutations starting from a wild-type open reading frame. Briefly, single-stranded and/or denatured dsDNA components are annealed to splint oligos which bring the desired ends into conjunction, the ends are ligated by a thermostable DNA ligase and the desired constructs amplified by PCR. A synthetic, modified RNA is then synthesized from the template using an RNA polymerase in vitro. After synthesis of a synthetic, modified RNA is complete, the DNA template is removed from the transcription reaction prior to use with the methods described herein.

[0279] In some embodiments of these aspects, the synthetic, modified RNAs are further treated with an alkaline phosphatase.

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[0280] One skilled in the art readily appreciates that the present invention is well adapted to carry out the objects and obtain the ends and advantages mentioned, as well as those inherent therein. The details of the description and the examples herein are representative of certain embodiments, are exemplary, and are not intended as limitations on the scope of the invention. Modifications therein and other uses will occur to those skilled in the art. These modifications are encompassed within the spirit of the invention. It will be readily apparent to a person skilled in the art that varying substitutions and modifications may be made to the invention disclosed herein without departing from the scope and spirit of the invention.

[0281] The articles “a” and “an” as used herein in the specification and in the claims, unless clearly indicated to the contrary, should be understood to include the plural referents. Claims or descriptions that include “or” between one or more members of a group are considered satisfied if one, more than one, or all of the group members are present in, employed in, or otherwise relevant to a given product or process unless indicated to the contrary or otherwise evident from the context. The invention includes embodiments in which exactly one member of the group is present

in, employed in, or otherwise relevant to a given product or process. The invention also includes embodiments in which more than one, or all of the group members are present in, employed in, or otherwise relevant to a given product or process. Furthermore, it is to be understood that the invention provides all variations, combinations, and permutations in which one or more limitations, elements, clauses, descriptive terms, etc., from one or more of the listed claims is introduced into another claim dependent on the same base claim (or, as relevant, any other claim) unless otherwise indicated or unless it would be evident to one of ordinary skill in the art that a contradiction or inconsistency would arise. It is contemplated that all embodiments described herein are applicable to all different aspects of the invention where appropriate. It is also contemplated that any of the embodiments or aspects can be freely combined with one or more other such embodiments or aspects whenever appropriate. Where elements are presented as lists, e.g., in Markush group or similar format, it is to be understood that each subgroup of the elements is also disclosed, and any element(s) can be removed from the group. It should be understood that, in general, where the invention, or aspects of the invention, is/are referred to as comprising particular elements, features, etc., certain embodiments of the invention or aspects of the invention consist, or consist essentially of, such elements, features, etc. For purposes of simplicity those embodiments have not in every case been specifically set forth in so many words herein. It should also be understood that any embodiment or aspect of the invention can be explicitly excluded from the claims, regardless of whether the specific exclusion is recited in the specification. For example, any one or more active agents, additives, ingredients, optional agents, types of organism, disorders, subjects, or combinations thereof, can be excluded.

[0282] Where the claims or description relate to a composition of matter, it is to be understood that methods of making or using the composition of matter according to any of the methods disclosed herein, and methods of using the composition of matter for any of the purposes disclosed herein are aspects of the invention, unless otherwise indicated or unless it would be evident to one of ordinary skill in the art that a contradiction or inconsistency would arise. Where the claims or description relate to a method, e.g., it is to be understood that methods of making compositions useful for performing the method, and products produced according to

the method, are aspects of the invention, unless otherwise indicated or unless it would be evident to one of ordinary skill in the art that a contradiction or inconsistency would arise.

[0283] Where ranges are given herein, the invention includes embodiments in which the endpoints are included, embodiments in which both endpoints are excluded, and embodiments in which one endpoint is included and the other is excluded. It should be assumed that both endpoints are included unless indicated otherwise. Furthermore, it is to be understood that unless otherwise indicated or otherwise evident from the context and understanding of one of ordinary skill in the art, values that are expressed as ranges can assume any specific value or subrange within the stated ranges in different embodiments of the invention, to the tenth of the unit of the lower limit of the range, unless the context clearly dictates otherwise. It is also understood that where a series of numerical values is stated herein, the invention includes embodiments that relate analogously to any intervening value or range defined by any two values in the series, and that the lowest value may be taken as a minimum and the greatest value may be taken as a maximum. Numerical values, as used herein, include values expressed as percentages. For any embodiment of the invention in which a numerical value is prefaced by "about" or "approximately", the invention includes an embodiment in which the exact value is recited. For any embodiment of the invention in which a numerical value is not prefaced by "about" or "approximately", the invention includes an embodiment in which the value is prefaced by "about" or "approximately".

[0284] As used herein "A and/or B", where A and B are different claim terms, generally means at least one of A, B, or both A and B. For example, one sequence which is complementary to and/or hybridizes to another sequence includes (i) one sequence which is complementary to the other sequence even though the one sequence may not necessarily hybridize to the other sequence under all conditions, (ii) one sequence which hybridizes to the other sequence even if the one sequence is not perfectly complementary to the other sequence, and (iii) sequences which are both complementary to and hybridize to the other sequence.

[0285] "Approximately" or "about" generally includes numbers that fall within a range of 1% or in some embodiments within a range of 5% of a number or in

some embodiments within a range of 10% of a number in either direction (greater than or less than the number) unless otherwise stated or otherwise evident from the context (except where such number would impermissibly exceed 100% of a possible value). It should be understood that, unless clearly indicated to the contrary, in any methods claimed herein that include more than one act, the order of the acts of the method is not necessarily limited to the order in which the acts of the method are recited, but the invention includes embodiments in which the order is so limited. It should also be understood that unless otherwise indicated or evident from the context, any product or composition described herein may be considered "isolated".

[0286] As used herein the term "comprising" or "comprises" is used in reference to compositions, methods, and respective component(s) thereof, that are essential to the invention, yet open to the inclusion of unspecified elements, whether essential or not.

[0287] As used herein the term "consisting essentially of" refers to those elements required for a given embodiment. The term permits the presence of additional elements that do not materially affect the basic and novel or functional characteristic(s) of that embodiment of the invention.

[0288] The term "consisting of" refers to compositions, methods, and respective components thereof as described herein, which are exclusive of any element not recited in that description of the embodiment.

* * *

Examples

[0289] Example 1

[0290] Transcription activator-like effector nucleases (TALENs) bind as a pair around a genomic site, in which a double-strand break (DSB) is introduced by a dimer of FokI nuclease domains. The use of a TALEN genome-editing system to rapidly and efficiently generate mutant alleles of 15 different genes in human pluripotent stem cells (hPSCs) as a means of performing rigorous disease modeling was recently reported (Ding et al., Cell Stem Cell 12:238-251 (2013)); the proportions of clones bearing at least one mutant allele ranged from 2%-34%.

[0291] As described below, the relative efficacies of CRISPRs and TALENs targeting the same genomic sites in the same hPSC lines was assessed with the use of

the same delivery platform described previously (Ding et al., Cell Stem Cell 12:238-251 (2013)). In the TALEN genome-editing system, the CAG promoter was used to co-translate (via a viral 2A peptide) each TALEN with green fluorescent protein (GFP) or red fluorescent protein (RFP). For CRISPRs, a human codon-optimized Cas9 gene was subcloned with a C-terminal nuclear localization signal (Mali et al., Science 339:823-826 (2013)) into the same CAG expression plasmid with GFP, and the guide RNA (gRNA) was separately expressed from a plasmid with the human U6 polymerase III promoter (Mali et al., Science 339:823-826 (2013)). The 20-nucleotide protospacer sequence for each gRNA was introduced using polymerase chain reaction (PCR)-based methods. Whether using TALENs or CRISPRs, equal amounts of the two plasmids were co-electroporated into hPSCs (either 25 μg of each plasmid, or 12.5 μg of each plasmid along with 25 μg of a DNA repair template if attempting knock-in) followed by fluorescence-activated cell sorting (FACS) after 24-48 hours, clonal expansion of single cells, and screening for mutations at the genomic target site via PCR.

[0292] gRNAs were designed matching G(N)19NGG sequences in seven loci in six genes (AKT2, CELSR2, CIITA, GLUT4, LINC00116, and SORT1) previously successfully targeted with TALENs (Ding et al., Cell Stem Cell 12:238-251 (2013)) and one additional locus in LDLR. In this system, CRISPRs consistently and substantially outperformed TALENs across loci and hPSC lines (see Table S1). The TALENs yielded clones with at least one mutant allele at efficiencies of 0%-34%, but matched CRISPRs yielded mutant clones at efficiencies of 51%-79% (Table S1). Just as with TALENs, CRISPRs produced a variety of indels of sizes ranging from one nucleotide to several dozen nucleotides in size, centered on the predicted cleavage sites, suggesting that non-homologous end-joining mutagenesis occurs in the same way regardless of whether CRISPRs or TALENs are used. Moreover, CRISPRs readily generated homozygous mutant clones (7%-25% of all clones; Table S1) as discerned by sequencing.

[0293] Knock-in of E17K mutations into AKT2 was also attempted using a 67-nucleotide single-stranded DNA oligonucleotide as previously described (Ding et al., Cell Stem Cell 12:238-251 (2013)). Although the predicted CRISPR cleavage site

lay 11 and 13 nucleotides from the point mutations, respectively, the CRISPR yielded knock-in clones at a rate of 11%, whereas TALENs yielded only 1.6% (Table S1).

Table S1. Targeting Efficiency of CRISPRs Versus TALENs in Human Pluripotent Stem Cells

Gene	Chromosome: Position (Start of Target Sequence)	Target Sequence[a]	Cell Line[b]	TALENs	CRISPRs	
				Efficiency (Mutants/Clones Screened)[c]	Efficiency (Mutants/Clones Screened)[c]	Efficiency of Homozygous Mutants
AKT2	chr19:40762982	TCCCTTCCTGCCTCATTTCAGGTGAATACATCAAGACCTGGAGGCCA	HUES 9	8.9% (17/192)		
AKT2	chr19:40762982	TCCCTTCCTGCC|TCATTTCAGGTGAATACATCAAGACCTGGAGGCCA	HUES 9		60.6% (86/142)	12.7% (18/142)
CELSR2	chr1:109817568	TGCTGGCTCGGCTGCCCTGAGGTTGCTCAATCAAGCACAGGTTTCAA	HUES 1	3.5% (18/506)		
CELSR2	chr1:109817568	TGCTGGCTCGGCTGCCCTGAGGTTGCTCAATCAAG|CACAGGTTTCAA	HUES 1		66.2% (45/68)	7.4% (5/68)
CIITA	chr16:10989200	TAACAGCGATGCTGACCCCCTGTGCCTCTACCACTTCTATGACCAGA	BJ-RiPS	12.7% (37/292)		
CIITA	chr16:10989206	CGATGCTGACCCCCTGTGCCTCTACCACTT|CTATGACCAGATGGACC	BJ-RiPS		78.7% (96/122)	11.5% (14/122)
GLUT4	chr17:7186601	TGGTCCTTGCTGTGTTCTCTGCGGTGCTTGGCTCCCTGCAGTTTGGGTA	HUES 9	33.5% (52/155)		
GLUT4	chr17:7186601	TGGTCCTTGCTGTGTTCT|CTGCGGTGCTTGGCTCCCTGCAGTTTGGGTA	HUES 9		66.5% (123/185)	24.9% (46/185)
LDLR	chr19:11210899	TGGGCGACAGATGCGAAAGAAACGAGTTCCAGTGCCAAGACGGGAAA	HUES 9	0% (0/568)		
LDLR	chr19:11210917	GAAACGAGTTCCAGTGCCAAGACGGGAAATGCATCTCCTAC|AAGTGG	HUES 9		51.1% (90/176)	8.0% (14/176)
LINC00116	chr2:110970090	TCAGAGAGGACACTGCAGTTGTCCGTGCTAGTAGCCTTCGCTTCTGGA	HUES 9	29.5% (26/88)		
LINC00116	chr2:110970090	TCAGAGAGGACACTGCAGTTGTCCGTGCTAGTAGCCTTCGC|TTCTGGA	HUES 9		57.4% (93/162)	8.6% (14/162)
SORT1 exon 2	chr1:109912203	TGATGATCTCAGAGGCTCAGTATCCTTGTCCTGGGTTGGAGATAGCA	HUES 1	22.2% (128/576)		
SORT1 exon 2	chr1:109912203	TGATGATCTCAGAGGCTCAGTATCCTTG|TCCTGGGTTGGAGATAGCA	HUES 1		68.5% (100/146)	13.0% (19/146)
SORT1 exon 3	chr1:109910069	TGGTAATTATGACTTTTGGACAGTCCAAGCTATATCGAAGGTGAGATCA	HUES 9	10.9% (21/192)		
SORT1 exon 3	chr1:109910069	TGGTAATTATGACTTTTG|GACAGTCCAAGCTATAT|CGAAGGTGAGATCA	HUES 9		75.9% (148/195)	10.3% (20/195)
AKT2 E17K	chr19:40762982	TCCCTTCCTGCCTCATTTCAGGT*GAA*TACATCAAGACCTGGAGGCCA	HUES 9	1.6% (3/192)[d]		
AKT2 E17K	chr19:40762982	TCCCTTCCTGCC|TCATTTCAGGT*GAA*TACATCAAGACCTGGAGGCCA	HUES 9		10.6% (10/94)[d]	1.1% (1/94)[d]
AKT2 off-target	chr5:22683972	CTATGCCCTGCC|TCATTTCAGGTGAA*GA*TGAAATCCCTGGAGCTTGG	HUES 9		0% (0/142)	0% (0/142)

[a] For TALENs, the binding sites are indicated with underlines, with the cleavage site predicted to be midway between the binding sites; for CRISPRs, the protospacer is underlined, the NGG motif is in bold (may be on the antisense strand), and the predicted cleavage site is indicated with "|"; for the *AKT2* E17K target sequence, the sites of the knock-in mutations are indicated in bold/italics; for the *AKT2* off-target site, the two mismatches in the protospacer are indicated in bold/italics

[b] HUES 1 and HUES 9 are human embryonic stem cell lines; BJ-RiPS is an induced pluripotent stem cell line

[c] Mutants include single heterozygotes, compound heterozygotes, and homozygous mutants; TALEN data is from Table 1 of Ding et al. (2013), with the exception of *LDLR*

[d] Successfully inserted E17K knock-in mutations into an *AKT2* allele(s) using single-stranded DNA oligonucleotide (refer to Figure 3 of Ding et al., 2013)

[0294] It is worth noting that the requirement for a G(N)19NGG target sequence somewhat limits site selection. Because either DNA strand can be targeted, a target sequence occurs on average every 32 basepairs. This is no barrier for gene knockout, where any coding sequence can be targeted, but it may present difficulties when trying to knock in or correct a mutation at a specific location. However, the requirement for a G at the start of the protospacer is dictated by the use of the U6 promoter to express the gRNA, and alternative CRISPR/Cas systems can relieve this requirement (Cong et al., Science 339:819-823 (2013)). This allows for the use of (N)20NGG target sequences, which are found on average every 8 basepairs.

[0295] In addition, the extent of CRISPR off-target effects remains to be defined and is highly sequence-dependent. Previous analyses have suggested that one-nucleotide mismatches in the first half of the protospacer are better tolerated than mismatches in second half (Jinek et al., Science 337:816-821 (2012); Cong et al., Science 339:819-823 (2013)). For the AKT2 sequence, there is a two-mismatch sequence differing at nucleotides 1 and 3, in the more "tolerant" half of the protospacer. Zero clones were obtained with mutations at this potential off-target site, as compared to 61% at the on-target site (Table S1). For one of the SORT1 sequences, use of a different human pluripotent stem cell line in which a single nucleotide polymorphism results in a one-nucleotide mismatch at the target site yielded mutant clones at an efficiency of 42%, compared to 66% in the original cell line. Thus, judicious selection of target sites is necessary to minimize systematic off-target effects; target sites with perfect-match or single-nucleotide-mismatch sequences elsewhere in the genome should be avoided.

[0296] From a practical standpoint, CRISPRs are easier to implement than TALENs. Each TALEN pair must be constructed de novo, whereas for CRISPRs the Cas9 component is fixed and the gRNA requires only swapping of the 20-nucleotide protospacer. Given this consideration and the demonstration herein of substantially increased efficiency as a result of replacing TALENs with CRISPRs in an otherwise identical system, CRISPRs appear to be a very powerful and broadly applicable tool for genome editing, particularly in a therapeutic context.

[0297] Example 2: Efficient targeting of clinically relevant genes in primary somatic cells

[0298] Work described herein shows for the first time that the CRISPR/Cas9 system can be used to edit the genome of somatic cells (e.g., primary) with high efficiency by using a double guide strategy. The inventors posit that this work will help bring genome editing in clinically relevant primary cells into reality.

[0299] The advent of genome editing tools that allow one to target any desired genomic site has greatly advanced the investigation of human biology and disease. In particular, the CRISPR/Cas9 system has become the gold standard in targeted genome editing technology, due to its flexibility and high efficacy. This system is constituted by the Cas9 nuclease from the microbial type II CRISPR/Cas system, which is targeted to specific genomic loci by a 20-nucleotide region in a synthetic guide RNA molecule. Similar to other targeted nucleases (ZFNs and TALENs), Cas9 induces double strand breaks (DSBs) that are repaired mainly by error-prone non-homologous end joining (NHEJ) (Cong et al., 2013; Jinek et al., 2013; Mali et al., 2013).

[0300] Implementation of the CRISPR/Cas9 system has made it possible to achieve unprecedentedly high targeting efficiencies in immortalized cell lines (Cong et al., 2013; Jinek et al., 2013; Mali et al., 2013), human pluripotent stem cells (Ding et al., 2013) and even zygotes of mice (Wang et al., 2013), rats (Li et al., 2013) and, most recently, monkeys (Niu et al., 2014), leading to the generation of knock-out or knock-in animals in very short periods of time when compared to classical strategies.

[0301] However, it remains to be proven whether CRISPR/Cas9 technology can be used to edit the genome of clinically relevant primary somatic cells with high efficiency, an essential step for the full realization of the promise of genome editing for regenerative medicine and transplantation therapies.

[0302] The inventors sought to test the amenability of the CRISPR/Cas9 system to edit clinically relevant genes in primary somatic cells. For this purpose the inventors chose to target two therapy-related genes: *CCR5*, a co-receptor for HIV, in CD34+ hematopoietic progenitor cells (HPCs), and *B2M*, the accessory chain of MHC class I molecules, in CD4+ T cells. The inventors found that a single guide strategy yielded very low to undetectable mutational rates in HPCs and T cells,

despite high efficiencies in immortalized cell lines such 293T and K562. In contrast, surprisingly and unexpectedly a double guide strategy with a pair of gRNAs with different offsets targeting the locus of interest resulted in up to 40% homozygous deletion efficiency in HPCs and T cells. These results establish a novel approach through which the CRISPR/Cas9 system can be used to edit the genome in clinically relevant somatic cells with high efficiency.

[0303] RESULTS

[0304] *Efficient and rapid genome editing using the CRISPR/Cas9 system in cell lines*

[0305] The inventors transfected HEK293T cells with Cas9 and a series of CRISPR guide RNAs targeting the B2M locus and measured cutting efficiency based on SURVEYOR assays (Fig. 4), as well as flow cytometry, taking advantage of the fact that B2M is a surface antigen. These experiments were performed only 72h post-transfection, in order to account for the half-life of B2M on the cell membrane. Of note, B2M surface expression was abrogated in up to 60% of transfected HEK293T cells (Fig. 4). In addition, the inventors observed a wide variation of efficiency between individual guide RNAs, even if targeting the same exon. For instance, variation between single guide cutting efficiencies was several-fold amongst the seven guide RNAs binding within the 67 bp long protein coding portion of the first exon of B2M (Fig. 1X), strongly suggesting that CRISPR cutting efficiency is primarily guide sequence-dependent.

[0306] *Primary somatic cells are refractory to CRISPR/Cas9 targeting*

[0307] Next, the inventors tested the CRISPR/Cas9 system in primary cells. We chose two clinically relevant immune cell types: primary $CD34^+$ hematopoietic progenitor cells (HPCs) and primary $CD4^+$ T cells isolated from peripheral blood. Surprisingly, the same guide RNAs that resulted in up to 60% cutting efficiency in a cell line (B2M in 293T cells, Fig. 4) revealed ineffective in somatic cells (Fig. 4). The inventors speculate that such dramatic drop in targeting efficiency in primary cells is due to either a lower expression level of Cas9 nuclease in nucleofected cells, enhanced DNA repair mechanisms, or a combination of both.

[0308] *Double guide strategy dramatically increases targeting efficiency in primary cells*

[0309] The inventors sought to determine whether genome editing efficacy in clinically relevant primary cells using the CRISPR/Cas9 system could be improved, hoping to achieve targeting efficiencies high enough to be potentially used in therapy. The inventors devised a double guide strategy, where two CRISPR guide RNAs targeting the same locus were delivered to cells simultaneously.

[0310] Addition of another guide RNA targeting the HPRT locus almost invariably resulted in increased mutation efficiency compared with the first guide RNA alone. Cells deficient in *HPRT* were selected by resistance to 6-thioguanine (6-TG). The use of additional gRNAs invariably resulted in increased *HPRT* mutant frequency. In an embodiment, the target polynucleotide sequence comprises a HPRT gene sequence.

[0311] Different guide RNA pairs were tested for each locus, and the most active one was used for further studies with primary cells. FIGS. 4A-4E demonstrate that the single guide strategy achieves high efficiency genome editing in cell lines, but not in clinically relevant primary somatic cells. In the two systems we used, the double guide strategy consistently and substantially outperformed the traditional single guide strategy in primary somatic cells. These results are demonstrated in FIGS. 5A-5E, which show that the double guide strategy achieves genome editing with high efficiency in clinically relevant cells.

[0312] DISCUSSION

[0313] One of the major focuses in the field of CRISPR/Cas9 genome editing field is the search for parameters that modulate cutting efficiency by Cas9. The data described herein suggest that this phenomenon appears to be mostly determined by gRNA sequence, as gRNAs matching very close or even partially overlapping sequences within the same exon result in significantly different targeting efficiencies (Fig. 4).

[0314] In a previous report, an approach combining a Cas9 nickase mutant with paired guide RNAs to introduce targeted double-strand breaks has been used to drastically reduce CRISPR off-target effects without sacrificing on-target efficiency (Ran et al., 2013). In our hands, however, this strategy did not yield a significant mutation rate (Max & Pankaj). We thus combined WT Cas9 with pairs of gRNAs to increase cutting efficiency in cell types refractory to targeting – primary somatic cells.

[0315] B2M is an accessory chain of the MHC class I proteins, being necessary for their expression on the cells surface. Engineering cells devoid of surface MHC class I, hence invisible to cytotoxic T cells, is of utmost importance in transplantation and adoptive cell therapy.

[0316] Altogether, our data shows that the CRISPR/Cas9 system can be used to edit the genome of clinically relevant primary somatic cells with significant efficiencies by using a double guide strategy. We predict that this strategy has the potential to be a general approach to target genes in somatic cells with a high enough efficiency that it becomes relevant for potential translation into therapeutics.

[0317] SOME EXPERIMENTAL PROCEDURES

[0318] *Flow cytometry.* Cells were stained with mouse monoclonal anti-B2M antibody 2M2 (Biolegend).

[0319] *Primary blood cell electroporation*. Primary CD4+ T cells were isolated from leukopacs (MGH) using RosetteSep CD4 T cell enrichment cocktail (Stem Cell Technologies) and electroporated with endotoxin-free DNA using Amaxa T cell nucleofection kit (Lonza).

[0320] *6-TG selection for HPRT deficiency.* 5*10^6 cells were used per electroporation, with 25ugCas9 and 12.5ug of each gRNA. For the Cas9 control a non-cutting gRNA was used to keep the total DNA amount the same. FACS sorting ended up being relatively similar at 5-8% GFP 48 hours after EP. Cells were plated out at 40,000 per 10cm plate per sample, and grown until colonies could clearly be seen. 30 uM 6-Thioguanine (6-TG) in mTESR (e.g., at a concentration of 30 μm) and was used as selection medium for 8-9 days and colonies were counted again. The results are shown in Table 1 below.

[0321] Table 1

gRNA	Starting colonies	Final colonies	Percentage	Percentage - Cas9 background
Cas9	105	17	0.161904762	0.00
1	121	55	0.454545455	0.29
3	118	67	0.56779661	0.41
5	124	76	0.612903226	0.45
7	125	27	0.216	0.05
9	131	29	0.221374046	0.06
11	93	63	0.677419355	0.52

1+5	64	43	0.671875	0.51
1+3	77	45	0.584415584	0.42
1+7	55	19	0.345454545	0.18
1+9	60	26	0.433333333	0.27
1+11	52	32	0.615384615	0.45
3+5	69	46	0.666666667	0.50
3+7	55	33	0.6	0.44
3+11	38	30	0.789473684	0.63
7+11	72	41	0.569444444	0.41

[0322] Table 2 below shows the results from Table 1 above ranked according to editing efficiency.

[0323] *Table 2*

gRNA	Percentage
3+11	0.63
11	0.52
1+5	0.51
3+5	0.50
1+11	0.45
5	0.45
3+7	0.44
1+3	0.42
7+11	0.41
3	0.41
1	0.29
1+9	0.27
1+7	0.18
9	0.06
7	0.05
Cas9	0.00

[0324] gRNAs used in the experiments are shown below:

[0325] 1-gtcttgctcgagatgtgatg (SEQ ID NO: 298)

[0326] 3-taaattctttgctgacctgc (SEQ ID NO: 299)

[0327] 5-tagatccattcctatgactg (SEQ ID NO: 300)

[0328] 7-cttcagtctgataaaatcta (SEQ ID NO: 301)

[0329] 9-tttgatgtaatccagcaggt (SEQ ID NO: 302)

[0330] 11-cacagagggctacaatgtga (SEQ ID NO: 303)

[0331]

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CLAIMS

What is claimed is:

1. A method for altering a target polynucleotide sequence in a cell comprising contacting the polynucleotide sequence with a clustered regularly interspaced short palindromic repeats-associated (Cas) protein and from one to two ribonucleic acids, wherein the ribonucleic acids direct Cas protein to and hybridize to a target motif of the target polynucleotide sequence, wherein the target polynucleotide sequence is cleaved, and wherein the efficiency of alteration of cells that express Cas protein is from about 50% to about 80%.

2. A method for treating or preventing a disorder associated with expression of a polynucleotide sequence in a subject, the method comprising (a) altering a target polynucleotide sequence in a cell *ex vivo* by contacting the polynucleotide sequence with a clustered regularly interspaced short palindromic repeats-associated (Cas) protein and from one to two ribonucleic acids, wherein the ribonucleic acids direct Cas protein to and hybridize to a target motif of the target polynucleotide sequence, wherein the target polynucleotide sequence is cleaved, and wherein the efficiency of alteration of cells that express Cas protein is from about 50% to about 80%, and (b) introducing the cell into the subject, thereby treating or preventing a disorder associated with expression of the polynucleotide sequence.

3. A method for simultaneously altering multiple target polynucleotide sequences in a cell comprising contacting the polynucleotide sequences with a clustered regularly interspaced short palindromic repeats-associated (Cas) protein and multiple ribonucleic acids, wherein the ribonucleic acids direct Cas protein to and hybridize to target motifs of the target polynucleotide sequences, wherein the target polynucleotide sequences are cleaved, and wherein the efficiency of alteration of cells that express Cas protein is from about 50% to about 80%.

4. A method for treating or preventing a disorder associated with expression of polynucleotide sequences in a subject, the method comprising (a) altering target polynucleotide sequences in a cell *ex vivo* by contacting the polynucleotide sequences with a clustered regularly interspaced short palindromic repeats-associated (Cas) protein and multiple ribonucleic acids, wherein the ribonucleic acids direct Cas protein to and hybridize to target motifs of the target polynucleotide sequences, wherein the target polynucleotide sequences are cleaved, and wherein the efficiency of alteration of cells that express Cas protein is from about 50% to about 80%, and (b) introducing the cell into the subject, thereby treating or preventing a disorder associated with expression of the polynucleotide sequences.

5. A method according to any one of claims 1-4, wherein the Cas protein is *Streptococcus pyogenes* Cas9 protein or a functional portion thereof.

6. The method according to claim 5, wherein the functional portion comprises a combination of operably linked Cas9 protein functional domains selected from the group consisting of a DNA binding domain, at least one RNA binding domain, a helicase domain, and an endonuclease domain.

7. The method according to claim 6, wherein the functional domains form a complex.

8. A method according to any one of claims 1-4, wherein the Cas protein is Cas9 protein from any bacterial species or functional portion thereof.

9. The method according to claim 8, wherein the functional portion comprises a combination of operably linked Cas9 protein functional domains selected from the group consisting of a DNA binding domain, at least one RNA binding domain, a helicase domain, and an endonuclease domain.

10. The method according to claim 9, wherein the functional domains form a complex.

11. A method according to any one of claims 1-2, wherein the Cas protein is complexed with the one to two ribonucleic acids.

12. A method according to any one of claims 3-4, wherein the Cas protein is complexed with the multiple ribonucleic acids.

13. A method according to any one of claims 1-2, wherein the target motif is a 20-nucleotide DNA sequence.

14. A method according to any one of claims 3-4, wherein each target motif is a 20-nucleotide DNA sequence.

15. A method according to any one of claims 1-2, wherein the target motif is a 20-nucleotide DNA sequence beginning with G and immediately precedes an NGG motif recognized by the Cas protein.

16. A method according to any one of claims 3-4, wherein each target motif is a 20-nucleotide DNA sequence beginning with G and immediately precedes an NGG motif recognized by the Cas protein.

17. A method according to any one of claims 1-2, wherein the target motif is a 20-nucleotide DNA sequence and immediately precedes an NGG motif recognized by the Cas protein.

18. A method according to any one of claims 3-4, wherein each target motif is a 20-nucleotide DNA sequence and immediately precedes an NGG motif recognized by the Cas protein.

19. A method according to any one of claims 1-2, wherein the target motif is $G(N)_{19}NGG$.

20. A method according to any one of claims 3-4, wherein each target motif is $G(N)_{19}NGG$.

21. A method according to any one of claims 1-2, wherein the target motif is $(N)_{20}NGG$.

22. A method according to any one of claims 3-4, wherein each target motif is $(N)_{20}NGG$.

23. A method according to any one of claims 1-2, wherein the target polynucleotide sequence is cleaved such that a double-strand break results.

24. A method according to any one of claims 3-4, wherein each target polynucleotide sequence is cleaved such that a double-strand break results.

25. A method according to any one of claims 1-2, wherein the target polynucleotide sequence is cleaved such that a single-strand break results.

26. A method according to any one of claims 3-4, wherein each target polynucleotide sequence is cleaved such that a single-strand break results.

27. A method according to any one of claims 1-4, wherein the alteration is an indel.

28. A method according to any one of claims 1-2, wherein the alteration results in reduced expression of the target polynucleotide sequence.

29. A method according to any one of claims 3-4, wherein the alteration results in reduced expression of the target polynucleotide sequences.

30. A method according to any one of claims 1-2, wherein the alteration results in a knock out of the target polynucleotide sequence.

31. A method according to any one of claims 3-4, wherein the alteration results in a knock out of the target polynucleotide sequences.

32. A method according to any one of claims 1-2, wherein the alteration results in correction of the target polynucleotide sequence from an undesired sequence to a desired sequence.

33. A method according to any one of claims 3-4, wherein the alteration results in correction of the target polynucleotide sequences from undesired sequences to desired sequences.

34. A method according to any one of claims 1-2, wherein the alteration is a homozygous alteration.

35. A method according to any one of claims 3-4, wherein each alteration is a homozygous alteration.

36. A method according to any one of claims 1-2, wherein subsequent to cleavage of the target polynucleotide sequence, homology-directed repair occurs.

37. A method according to claim 36, wherein homology-directed repair is performed using an exogenously introduced DNA repair template.

38. A method according to claim 37, wherein the exogenously introduced DNA repair template is single-stranded.

39. A method according to claim 37, wherein the exogenously introduced DNA repair template is double-stranded.

40. A method according to any one of claims 3-4, wherein subsequent to cleavage of the target polynucleotide sequences, homology-directed repair occurs.

41. A method according to claim 40, wherein homology-directed repair is performed using an exogenously introduced DNA repair template.

42. A method according to claim 41, wherein the exogenously introduced DNA repair template is single-stranded.

43. A method according to claim 41, wherein the exogenously introduced DNA repair template is double-stranded.

44. A method according to any one of claims 1-4, wherein the cell is a peripheral blood cell.

45. A method according to any one of claims 1-4, wherein the cell is a stem cell or a pluripotent cell.

46. A method according to any one of claims 1-4, wherein the cell is a hematopoietic stem cell.

47. A method according to any one of claims 1-4, wherein the cell is a $CD34^+$ cell.

48. A method according to any one of claims 1-4, wherein the cell is a $CD34^+$ mobilized peripheral blood cell.

49. A method according to any one of claims 1-4, wherein the cell is a $CD34^+$ cord blood cell.

50. A method according to any one of claims 1-4, wherein the cell is a $CD34^+$ bone marrow cell.

51. A method according to any one of claims 1-4, wherein the cell is a $CD34^+CD38\text{-}Lineage\text{-}CD90^+CD45RA^-$ cell.

52. A method according to any one of claims 1-4, wherein the cell is a hepatocyte.

53. A method according to any one of claims 1-2, wherein the target polynucleotide sequence is CCR5.

54. A method according to claim 53, wherein at least one of the one to two ribonucleic acids comprises a sequence selected from the group consisting of the ribonucleic acid sequences of Fig. 1.

55. A method according to claim 53, wherein at least one of the one to two ribonucleic acids comprises a sequence with a single nucleotide mismatch to a sequence selected from the group consisting of the ribonucleic acid sequences of Fig. 1.

56. A method according to any one of claims 1-2, wherein the target polynucleotide sequence is CXCR4.

57. A method according to claim 56, wherein at least one of the one to two ribonucleic acids comprises a sequence selected from the group consisting of the ribonucleic acid sequences of Fig. 2.

58. A method according to claim 56, wherein at least one of the one to two ribonucleic acids comprises a sequence with a single nucleotide mismatch to a sequence selected from the group consisting of the ribonucleic acid sequences of Fig. 2.

59. A method according to any one of claims 3-4, wherein the target polynucleotide sequences comprise multiple different portions of CCR5.

60. A method according to claim 59, wherein each of the multiple ribonucleic acids comprises a different sequence selected from the group consisting of the ribonucleic acid sequences of Fig. 1.

61. A method according to claim 59, wherein each of the multiple ribonucleic acids comprises a sequence with a single nucleotide mismatch to a different sequence selected from the group consisting of the ribonucleic acid sequences of Fig. 1.

62. A method according to any one of claims 3-4, wherein the target polynucleotide sequences comprise multiple different portions of CXCR4.

63. A method according to claim 62, wherein each of the multiple ribonucleic acids comprises a different sequence selected from the group consisting of the ribonucleic acid sequences of Fig. 2.

64. A method according to claim 59, wherein each of the multiple ribonucleic acids comprises a sequence with a single nucleotide mismatch to a different sequence selected from the group consisting of the ribonucleic acid sequences of Fig. 2.

65. A method according to any one of claims 3-4, wherein the target polynucleotide sequences comprise at least a portion of CCR5 and at least a portion of CXCR4.

66. A method according to claim 65, wherein each of the multiple ribonucleic acids comprises a different sequence selected from the group consisting of the ribonucleic acid sequences of Fig. 1 and the ribonucleic acid sequences of Fig. 2.

67. A method according to claim 65, wherein each of the multiple ribonucleic acids comprises a sequence with a single nucleotide mismatch to a different sequence selected from the group consisting of the ribonucleic acid sequences of Fig. 1 and the ribonucleic acid sequences of Fig 2.

68. A method according to claims 2 or 4, wherein the disorder is a genetic disorder.

69. A method according to claims 2 or 4, wherein the disorder is a monogenic disorder.

70. A method according to claims 2 or 4, wherein the disorder is human immunodeficiency virus (HIV) infection.

71. A method according to claims 2 or 4, wherein the disorder is acquired immunodeficiency syndrome (AIDS).

72. A method according to any one of claims 1-2, wherein the one to two ribonucleic acids are designed to hybridize to a target motif immediately adjacent to a deoxyribonucleic acid motif recognized by the Cas protein.

73. A method according to any one of claims 1-2, wherein each of the one to two ribonucleic acids are designed to hybridize to target motifs immediately adjacent to deoxyribonucleic acid motifs recognized by the Cas protein which flank a mutant allele located between the target motifs.

74. A method according to any one of claims 3-4, wherein the multiple ribonucleic acids are designed to hybridize to target motifs immediately adjacent to deoxyribonucleic acid motifs recognized by the Cas protein.

75. A method according to any one of claims 3-4, wherein the multiple ribonucleic acids are designed to hybridize to target motifs immediately adjacent to deoxyribonucleic acid motifs recognized by the Cas protein which flank mutant alleles located between the target motifs.

76. A method according to any one of claims 1-2, wherein the one to two ribonucleic acids are selected to minimize hybridization with nucleic acid sequences other than the target polynucleotide sequence.

77. A method according to any one of claims 3-4, wherein the multiple ribonucleic acids are selected to minimize hybridization with nucleic acid sequences other than the target polynucleotide sequence.

78. A method according to any one of claims 1-2, wherein the target motif is selected such that it contains at least two mismatches when compared with all other genomic nucleotide sequences in the cell.

79. A method according to any one of claims 3-4, wherein each target motif is selected such that it contains at least two mismatches when compared with all other genomic nucleotide sequences in the cell.

80. A method according to any one of claims 1-2, wherein the target motif is selected such that it contains at least one mismatch when compared with all other genomic nucleotide sequences in the cell.

81. A method according to any one of claims 3-4, wherein the target motif is selected such that it contains at least one mismatch when compared with all other genomic nucleotide sequences in the cell.

82. A method according to any one of claims 1-2, wherein the one to two ribonucleic acids hybridize to a target motif that it contains at least two mismatches when compared with all other genomic nucleotide sequences in the cell.

83. A method according to any one of claims 3-4, wherein each of the multiple ribonucleic acids hybridize to target motifs that contain at least two mismatches when compared with all other genomic nucleotide sequences in the cell.

84. A method according to any one of claims 1-2, wherein the one to two ribonucleic acids hybridize to a target motif that contains at least one mismatch when compared with all other genomic nucleotide sequences in the cell.

85. A method according to any one of claims 3-4, wherein each of the multiple ribonucleic acids hybridize to target motifs that contain at least one mismatch when compared with all other genomic nucleotide sequences in the cell.

86. A method according to any one of claims 3-4, wherein the efficiency of alteration at each loci is from about 50% to about 80%.

87. A method according to any one of claims 1-4, wherein the Cas protein is encoded by a modified nucleic acid.

88. A method according to claim 87, wherein the modified nucleic acid comprises a ribonucleic acid containing at least one modified nucleotide selected from the group consisting of pseudouridine, 5-methylcytodine, 2-thio-uridine, 5-methyluridine-5'-triphosphate, 4-thiouridine-5'-triphosphate, 5,6-dihydrouridine-5'-triphosphate, and 5-azauridine-5'-triphosphate.

89. A method according to any one of claims 1-4, wherein at least one of the ribonucleic acids is a modified ribonucleic acid comprising one to two modified nucleotides selected from the group consisting of pseudouridine, 5-methylcytodine, 2-thio-uridine, 5-methyluridine-5'-triphosphate, 4-thiouridine-5'-triphosphate, 5,6-dihydrouridine-5'-triphosphate, and 5-azauridine-5'-triphosphate.

90. A method according to any one of claims 1-4, wherein any of the Cas protein or the ribonucleic acids are expressed from a plasmid.

91. A method according to any one of claims 1-4, wherein any of the Cas protein or the ribonucleic acids are expressed using a promoter optimized for increased expression in stem cells.

92. A method according to claim 91, wherein the promoter is selected from the group consisting of a Cytomegalovirus (CMV) early enhancer element and a chicken beta-actin promoter, a chicken beta-actin promoter, an elongation factor-1 alpha promoter, and a ubiquitin promoter.

93. A method according to any one of claims 1-4, further comprising selecting cells that express the Cas protein.

94. A method according to claim 93, wherein selecting cells comprises FACS.

95. A method according to claim 94, wherein FACs is used to select cells which co-express Cas and a fluorescent protein selected from the group consisting of green fluorescent protein and red fluorescent protein.

96. A method for altering a target polynucleotide sequence in a cell comprising contacting the polynucleotide sequence in a cell selected from the group consisting of a human pluripotent cell, a primary human cell, and a non-transformed human cell, with a clustered regularly interspaced short palindromic repeats-associated (Cas) protein and from one to two ribonucleic acids, wherein the ribonucleic acids direct Cas protein to and hybridize to a target motif of the target polynucleotide sequence, wherein the target polynucleotide sequence is cleaved, and wherein the efficiency of alteration of cells that express Cas protein is from about 8% to about 80%.

97. A method for treating or preventing a disorder associated with expression of a polynucleotide sequence in a subject, the method comprising (a) altering a target polynucleotide sequence in a cell *ex vivo* by contacting the polynucleotide sequence in a cell selected from the group consisting of a human pluripotent cell, a primary human cell, and a non-transformed human cell, with a clustered regularly interspaced short palindromic repeats-associated (Cas) protein and from one to two ribonucleic acids, wherein the ribonucleic acids direct Cas protein to and hybridize to a target motif of the target polynucleotide sequence, wherein the target polynucleotide sequence is cleaved, and wherein the efficiency of alteration is from about 8% to about 80%, and (b) introducing the cell into the subject, thereby treating or preventing a disorder associated with expression of the polynucleotide sequence.

98. A method for simultaneously altering multiple target polynucleotide sequences in a cell comprising contacting the polynucleotide sequences in a cell selected from the group consisting of a human pluripotent cell, a primary human cell, and a non-transformed human cell, with a clustered regularly interspaced short palindromic repeats-associated (Cas) protein and multiple ribonucleic acids, wherein the ribonucleic acids direct Cas protein to and hybridize to target motifs of the target polynucleotide sequences, wherein the target

polynucleotide sequences are cleaved, and wherein the efficiency of alteration of cells that express Cas protein is from about 8% to about 80%.

99. A method for treating or preventing a disorder associated with expression of polynucleotide sequences in a subject, the method comprising (a) altering target polynucleotide sequences in a cell *ex vivo* by contacting the polynucleotide sequences in a cell selected from the group consisting of a human pluripotent cell, a primary human cell, and a non-transformed human cell, with a clustered regularly interspaced short palindromic repeats-associated (Cas) protein and multiple ribonucleic acids, wherein the ribonucleic acids direct Cas protein to and hybridize to target motifs of the target polynucleotide sequences, wherein the target polynucleotide sequences are cleaved, and wherein the efficiency of alteration of cells that express Cas protein is from about 8% to about 80%, and (b) introducing the cell into the subject, thereby treating or preventing a disorder associated with expression of the polynucleotide sequences.

100. A composition comprising at least one ribonucleic acid having a sequence selected from the group consisting of the ribonucleic acid sequences of Fig 1.

101. A composition comprising at least one ribonucleic acid comprising a sequence with a single nucleotide mismatch to a sequence selected from the group consisting of the ribonucleic acid sequences of Fig 1.

102. A composition comprising at least one ribonucleic acid having a sequence selected from the group consisting of the ribonucleic acid sequences of Fig 2.

103. A composition comprising at least one ribonucleic acid comprising a sequence with a single nucleotide mismatch to a sequence selected from the group consisting of the ribonucleic acid sequences of Fig 2.

104. A composition comprising at least one ribonucleic acid having a sequence selected from the group consisting of the ribonucleic acid sequences of Fig. 1,

the ribonucleic acid sequences of Fig. 2, a sequence with a single nucleotide mismatch to a ribonucleic acid sequence of Fig. 1, and a sequence with a single nucleotide mismatch to a ribonucleic acid sequences of Fig. 2.

105. A composition according to any one of claims 100-104, wherein at least one of the ribonucleic acids is a modified ribonucleic acid comprising one to two modified nucleotides selected from the group consisting of pseudouridine, 5-methylcytodine, 2-thio-uridine, 5-methyluridine-5'-triphosphate, 4-thiouridine-5'-triphosphate, 5,6-dihydrouridine-5'-triphosphate, and 5-azauridine-5'-triphosphate.

106. A composition according to any one of claims 100-104, further comprising a nucleic acid sequence encoding a Cas protein.

107. A composition according to any one of claims 100-104, further comprising a nucleic acid sequence encoding a Cas9 protein or a functional portion thereof.

108. A composition according to claim 107, wherein the nucleic acid comprises a modified ribonucleic acid comprising at least one modified nucleotide selected from the group consisting of pseudouridine, 5-methylcytodine, 2-thio-uridine, 5-methyluridine-5'-triphosphate, 4-thiouridine-5'-triphosphate, 5,6-dihydrouridine-5'-triphosphate, and 5-azauridine-5'-triphosphate.

109. A composition comprising a chimeric nucleic acid comprising a ribonucleic acid encoding a Cas protein and at least one additional ribonucleic acid having a sequence selected from the group consisting of the ribonucleic acid sequences of Fig. 1.

110. A composition comprising a chimeric nucleic acid comprising a ribonucleic acid encoding a Cas protein and at least one additional ribonucleic acid having a sequence selected from the group consisting of the ribonucleic acid sequences of Fig. 2.

111. A composition comprising a chimeric nucleic acid comprising a ribonucleic acid encoding a Cas protein and at least one additional ribonucleic acid sequence comprising a sequence with a single nucleotide mismatch to a sequence selected from the group consisting of the ribonucleic acid sequences of Fig 1.

112. A composition comprising a chimeric nucleic acid comprising a ribonucleic acid encoding a Cas protein and at least one additional ribonucleic acid sequence comprising a sequence with a single nucleotide mismatch to a sequence selected from the group consisting of the ribonucleic acid sequences of Fig 2.

113. A composition comprising a chimeric nucleic acid comprising a ribonucleic acid encoding a Cas protein and at least one additional ribonucleic acid having a sequence selected from the group consisting of the ribonucleic acid sequences of Fig. 1, the ribonucleic acid sequences of Fig. 2, a sequence with a single nucleotide mismatch to a ribonucleic acid sequence of Fig. 1, and a sequence with a single nucleotide mismatch to a ribonucleic acid sequences of Fig. 2.

114. A composition according to any one of claims 109-113, further comprising a nucleic acid sequence encoding a fluorescent protein selected from the group consisting of green fluorescent protein and red fluorescent protein.

115. A composition according to any one of claims 109-113, further comprising a promoter operably linked to the chimeric nucleic acid.

116. A composition according to claim 115, wherein the promoter is optimized for increased expression in human stem cells.

117. A composition according to claim 116, wherein the promoter is selected from the group consisting of a Cytomegalovirus (CMV) early enhancer element and a chicken beta-actin promoter, a chicken beta-actin promoter, an elongation factor-1 alpha promoter, and a ubiquitin promoter.

118. A composition according to any one of claims 109-113, wherein the chimeric nucleic acid comprises at least one modified nucleotide selected from the group consisting of pseudouridine, 5-methylcytodine, 2-thio-uridine, 5-methyluridine-5'-triphosphate, 4-thiouridine-5'-triphosphate, 5,6-dihydrouridine-5'-triphosphate, and 5-azauridine-5'-triphosphate.

119. A composition according to any one of claims 109-113, wherein the Cas protein comprises a Cas9 protein or a functional portion thereof.

120. A kit for altering a target polynucleotide sequence in a cell comprising a Cas9 protein or a nucleic acid encoding the Cas9 protein, and at least one ribonucleic acid sequence selected from the group consisting of the ribonucleic acid sequences of Fig. 1, the ribonucleic acid sequences of Fig. 2, a sequence with a single nucleotide mismatch to a ribonucleic acid sequence of Fig. 1, and a sequence with a single nucleotide mismatch to a ribonucleic acid sequences of Fig. 2.

121. A kit according to claim 120 further comprising one or more cell lines, cultures, or populations selected from the group consisting of human pluripotent cells, primary human cells, and non-transformed cells.

122. A kit according to claim 120 further comprising a DNA repair template.

123. A method according to any one of claims 1-4, wherein the cell comprises a primary cell.

124. A method according to any one of claims 1-4 and 123, wherein the cell comprises a primary somatic cell.

125. A method according to any one of claims 1-4 and 123 or 124, wherein the cell comprises an autologous primary somatic cell.

126. A method according to any one of claims 1-4 and 123-125, wherein the cell comprises an allogeneic primary somatic cell.

127. A method according to any one of claims 1-2 and 123-126, wherein the target polynucleotide sequence is B2M.

128. A method according to any one of claims 1-2 and 123-127, wherein at least one of the one to two ribonucleic acids comprises a sequence optimized to target the B2M gene.

129. A method according to any one of claims 1-2 and 123-128, wherein at least one of the one to two ribonucleic acids comprises a sequence with a single nucleotide mismatch to a sequence optimized to target the B2M gene.

130. A method according to any one of claims 3-4 and 123-126, wherein the target polynucleotide sequences comprises multiple different portions of B2M.

131. A method according to any one of claims 3-4, 123-126 and 130, wherein each of the multiple ribonucleic acids comprises a different sequence optimized to target the B2M gene.

132. A method according to any one of claims 3-4, 123-126 and 130-131, wherein each of the multiple ribonucleic acids comprises a sequence with a single nucleotide mismatch to a different sequence optimized to target the B2M gene.

133. A method according to any one of claims 1-132, wherein the one to two ribonucleic acids comprise two guide ribonucleic acid sequences.

134. A method according to any one of claims 1-2, wherein the one to two ribonucleic acids comprise two guide ribonucleic acid sequences.

135. A method according to claim 134, wherein the target polynucleotide sequence comprises CCR5.

136. A method according to claims 134 or 135, wherein the cell comprises a primary CD34+ hematopoietic progenitor cell.

137. A method according to any one of claims 134-136, wherein the two guide ribonucleic acid sequences comprise any combination of two guide ribonucleic acid sequences which are complementary to a different sequence selected from the group consisting of SEQ ID NOs: 1-139.

138. A method according to any one of claims 134-136, wherein the two guide ribonucleic acid sequences comprise any combination of two guide ribonucleic acid sequences which are complementary to offset sequences selected from the group consisting of SEQ ID NOs: 1-139.

139. A method according to any one of claims 134-137, wherein the two guide ribonucleic acid sequences comprise any combination of two guide ribonucleic acid sequences which hybridize to and target Cas protein to offset target sites in CCR5 selected from the group consisting of SEQ ID NOs: 1-139.

140. A method according to claims 134-139, wherein the two guide ribonucleic acid sequences comprise any combination of two guide ribonucleic acid sequences from SEQ ID NOs: 298-303.

141. A method according to any one of claims 134-140, wherein the two guide ribonucleic acid sequences comprise a pair of guide ribonucleic acids selected from the group consisting of SEQ ID NOs: 299 and 303, SEQ ID NOs: 298 and 300, SEQ ID NOs: 299 and 300, SEQ ID NOs: 298 and 303, SEQ ID NOs: 299 and 301, SEQ ID NOs: 298 and 299, SEQ ID NOs: 301 and 303, SEQ ID NOs: 298 and 302, and SEQ ID NOs: 298 and 301.

142. A method according to claims 134-141, wherein the two guide ribonucleic acid sequences comprise any combination of two guide ribonucleic acid sequences which are complementary to a different sequence selected from the group consisting of SEQ ID NOs: 304-333.

143. A method according to any one of claims 134-142, wherein the two guide ribonucleic acid sequences comprise any combination of two guide

ribonucleic acid sequences which are complementary to offset sequences selected from the group consisting of SEQ ID NOs: 304-333.

144. A method according to any one of claims 134-143, wherein the two guide ribonucleic acid sequences comprise any combination of two guide ribonucleic acid sequences which hybridize to and target Cas protein to offset target sites in CCR5 selected from the group consisting of SEQ ID NOs: 304-333

145. A method according to claim 134, wherein the target polynucleotide sequence comprises CXCR4.

146. A method according to claims 145, wherein the cell comprises a primary CD34+ hematopoietic progenitor cell.

147. A method according to claims 145 or 146, wherein the two guide ribonucleic acid sequences comprise any combination of two guide ribonucleic acid sequences which are complementary to a different sequence selected from the group consisting of SEQ ID NOs: 140-297.

148. A method according to any one of claims 145-147, wherein the two guide ribonucleic acid sequences comprise any combination of two guide ribonucleic acid sequences which are complementary to offset sequences selected from the group consisting of SEQ ID NOs: 140-297.

149. A method according to any one of claims 145-148, wherein the two guide ribonucleic acid sequences comprise any combination of two guide ribonucleic acid sequences which hybridize to and target Cas protein to offset target sites in CXCR4 selected from the group consisting of SEQ ID NOs: 140-297.

150. A method according to claim 134, wherein the target polynucleotide sequence comprises B2M.

151. A method according to claims 150, wherein the cell comprises a primary cell.

152. A method according to claims 150 or 151, wherein the two guide ribonucleic acid sequences comprise any combination of two guide ribonucleic acid sequences which are complementary to different sequences in the B2M gene.

153. A method according to any one of claims 150-152, wherein the two guide ribonucleic acid sequences comprise any combination of two guide ribonucleic acid sequences which are complementary to offset sequences in the B2M gene.

154. A method according to any one of claims 150-153, wherein the two guide ribonucleic acid sequences comprise any combination of two guide ribonucleic acid sequences which hybridize to and target Cas protein to offset target sites in B2M.

155. A method for altering a target polynucleotide sequence in a primary cell comprising contacting the polynucleotide sequence with a clustered regularly interspaced short palindromic repeats-associated (Cas) protein and at least two ribonucleic acids, wherein the at least two ribonucleic acids comprise guide ribonucleic acids which direct Cas protein to and hybridize to a target motif of the target polynucleotide sequence, wherein the target polynucleotide sequence is cleaved, and wherein the efficiency of alteration of cells that express Cas protein is from about 50% to about 80%.

156. A method for treating or preventing a disorder associated with expression of a polynucleotide sequence in a subject, the method comprising (a) altering a target polynucleotide sequence in a primary cell *ex vivo* by contacting the polynucleotide sequence with a clustered regularly interspaced short palindromic repeats-associated (Cas) protein and at least two ribonucleic acids, wherein the at least two ribonucleic acids comprise guide ribonucleic acids which direct Cas protein to and hybridize to a target motif of the target polynucleotide sequence, wherein the target polynucleotide sequence is cleaved, and wherein the efficiency of alteration of cells that express Cas

protein is from about 50% to about 80%, and (b) introducing the cell into the subject, thereby treating or preventing a disorder associated with expression of the polynucleotide sequence.

157. A method for simultaneously altering multiple target polynucleotide sequences in a primary cell comprising contacting the polynucleotide sequences with a clustered regularly interspaced short palindromic repeats-associated (Cas) protein and multiple ribonucleic acids, wherein the ribonucleic acids comprise guide ribonucleic acids which direct Cas protein to and hybridize to target motifs of the target polynucleotide sequences, wherein the target polynucleotide sequences are cleaved, and wherein the efficiency of alteration of cells that express Cas protein is from about 50% to about 80%.

158. A method for treating or preventing a disorder associated with expression of polynucleotide sequences in a subject, the method comprising (a) altering target polynucleotide sequences in a primary cell *ex vivo* by contacting the polynucleotide sequences with a clustered regularly interspaced short palindromic repeats-associated (Cas) protein and multiple ribonucleic acids, wherein the ribonucleic acids comprise guide ribonucleic acids which direct Cas protein to and hybridize to target motifs of the target polynucleotide sequences, wherein the target polynucleotide sequences are cleaved, and wherein the efficiency of alteration of cells that express Cas protein is from about 50% to about 80%, and (b) introducing the cell into the subject, thereby treating or preventing a disorder associated with expression of the polynucleotide sequences.

159. A method according to any one of claims 155-158, wherein the Cas protein is *Streptococcus pyogenes* Cas9 protein or a functional portion thereof.

160. The method according to claim 159, wherein the functional portion comprises a combination of operably linked Cas9 protein functional domains selected from the group consisting of a DNA binding domain, at least one RNA binding domain, a helicase domain, and an endonuclease domain.

161. The method according to claim 160, wherein the functional domains form a complex.

162. A method according to any one of claims 155-158, wherein the Cas protein is Cas9 protein from any bacterial species or functional portion thereof.

163. The method according to claim 162, wherein the functional portion comprises a combination of operably linked Cas9 protein functional domains selected from the group consisting of a DNA binding domain, at least one RNA binding domain, a helicase domain, and an endonuclease domain.

164. The method according to claim 163, wherein the functional domains form a complex.

165. A method according to any one of claims 155-156, wherein the Cas protein is complexed with the one to two ribonucleic acids.

166. A method according to any one of claims 155-158, wherein the Cas protein is complexed with the multiple ribonucleic acids.

167. A method according to any one of claims 155-156, wherein the target motif is a 20-nucleotide DNA sequence.

168. A method according to any one of claims 157-158, wherein each target motif is a 20-nucleotide DNA sequence.

169. A method according to any one of claims 155-156, wherein the target motif is a 20-nucleotide DNA sequence beginning with G and immediately precedes an NGG motif recognized by the Cas protein.

170. A method according to any one of claims 157-158, wherein each target motif is a 20-nucleotide DNA sequence beginning with G and immediately precedes an NGG motif recognized by the Cas protein.

171. A method according to any one of claims 155-156, wherein the target motif is a 20-nucleotide DNA sequence and immediately precedes an NGG motif recognized by the Cas protein.

172. A method according to any one of claims 157-158, wherein each target motif is a 20-nucleotide DNA sequence and immediately precedes an NGG motif recognized by the Cas protein.

173. A method according to any one of claims 155-156, wherein the target motif is $G(N)_{19}NGG$.

174. A method according to any one of claims 157-158, wherein each target motif is $G(N)_{19}NGG$.

175. A method according to any one of claims 155-156, wherein the target motif is $(N)_{20}NGG$.

176. A method according to any one of claims 157-158, wherein each target motif is $(N)_{20}NGG$.

177. A method according to any one of claims 167, 169, 171, 173, or 175, wherein the target motif comprises a sequence selected from the group consisting of SEQ ID NOs: 1-297 or 304-333.

178. A method according to any one of claims 168, 170, 172, 174, or 176, wherein the target motif comprises a sequence selected from the group consisting of SEQ ID NOs: 1-297 or 304-333.

179. A method according to any one of claims 155-156, wherein the target polynucleotide sequence is cleaved such that a double-strand break results.

180. A method according to any one of claims 157-158, wherein each target polynucleotide sequence is cleaved such that a double-strand break results.

181. A method according to any one of claims 155-156, wherein the target polynucleotide sequence is cleaved such that a single-strand break results.

182. A method according to any one of claims 157-158, wherein each target polynucleotide sequence is cleaved such that a single-strand break results.

183. A method according to any one of claims 155-158, wherein the alteration is an indel.

184. A method according to any one of claims 155-156, wherein the alteration results in reduced expression of the target polynucleotide sequence.

185. A method according to any one of claims 157-158, wherein the alteration results in reduced expression of the target polynucleotide sequences.

186. A method according to any one of claims 155-156, wherein the alteration results in a knock out of the target polynucleotide sequence.

187. A method according to any one of claims 157-158, wherein the alteration results in a knock out of the target polynucleotide sequences.

188. A method according to any one of claims 155-156, wherein the alteration results in correction of the target polynucleotide sequence from an undesired sequence to a desired sequence.

189. A method according to any one of claims 157-158, wherein the alteration results in correction of the target polynucleotide sequences from undesired sequences to desired sequences.

190. A method according to any one of claims 155-156, wherein the alteration is a homozygous alteration.

191. A method according to any one of claims 157-158, wherein each alteration is a homozygous alteration.

192. A method according to any one of claims 155-156, wherein subsequent to cleavage of the target polynucleotide sequence, homology-directed repair occurs.

193. A method according to claim 192, wherein homology-directed repair is performed using an exogenously introduced DNA repair template.

194. A method according to claim 193, wherein the exogenously introduced DNA repair template is single-stranded.

195. A method according to claim 193, wherein the exogenously introduced DNA repair template is double-stranded.

196. A method according to any one of claims 157-158, wherein subsequent to cleavage of the target polynucleotide sequences, homology-directed repair occurs.

197. A method according to claim 196, wherein homology-directed repair is performed using an exogenously introduced DNA repair template.

198. A method according to claim 197, wherein the exogenously introduced DNA repair template is single-stranded.

199. A method according to claim 197, wherein the exogenously introduced DNA repair template is double-stranded.

200. A method according to any one of claims 155-158, wherein the cell is a peripheral blood cell.

201. A method according to any one of claims 155-158, wherein the cell is a stem cell or a pluripotent cell.

202. A method according to any one of claims 155-158, wherein the cell is a hematopoietic stem cell.

203. A method according to any one of claims 155-158, wherein the cell is a $CD34^+$ cell.

204. A method according to any one of claims 155-158, wherein the cell is a $CD34^+$ mobilized peripheral blood cell.

205. A method according to any one of claims 155-158, wherein the cell is a $CD34^+$ cord blood cell.

206. A method according to any one of claims 155-158, wherein the cell is a $CD34^+$ bone marrow cell.

207. A method according to any one of claims 155-158, wherein the cell is a $CD34^+CD38\text{-}Lineage\text{-}CD90^+CD45RA^-$ cell.

208. A method according to any one of claims 155-158, wherein the cell is a hepatocyte.

209. A method according to any one of claims 155-158, wherein the cell is a primary cell.

210. A method according to any one of claims 155-156, wherein the target polynucleotide sequence is CCR5.

211. A method according to claim 210, wherein the two ribonucleic acids comprise a different sequence selected from the group consisting of SEQ ID NOs: 298-303.

212. A method according to claim 210, wherein the two guide ribonucleic acid sequences comprise a pair of guide ribonucleic acids selected from the group consisting of SEQ ID NOs: 299 and 303, SEQ ID NOs: 298 and 300, SEQ ID NOs: 299 and 300, SEQ ID NOs: 298 and 303, SEQ ID NOs: 299 and 301, SEQ ID NOs: 298 and 299, SEQ ID NOs: 301 and 303, SEQ ID NOs: 298 and 302, and SEQ ID NOs: 298 and 301.

213. A method according to claim 210, wherein the two ribonucleic acids comprise sequences which are complementary to and/or hybridize to different sequences selected from the group consisting of SEQ ID NOs: 1-139 and 304-333.

214. A method according to claim 210, wherein the two ribonucleic acids comprise sequences which are complementary to and/or hybridize to different sequences with a single nucleotide mismatch to a sequence selected from the group consisting of SEQ ID NOs: 1-139 and 304-333.

215. A method according to claim 210, wherein the two ribonucleic acids comprise sequences which are complementary to and/or hybridizes to offset sequences selected from the group consisting of SEQ ID NOs: 1-139 and 304-333.

216. A method according to claim 210, wherein the two ribonucleic acids comprise sequences which are complementary to and/or hybridize to offsets sequences with a single nucleotide mismatch to a sequence selected from the group consisting of SEQ ID NOs: 1-139 and 304-333.

217. A method according to any one of claims 155-156, wherein the target polynucleotide sequence is CXCR4.

218. A method according to claim 217, wherein the two ribonucleic acids comprise sequences which are complementary to and/or hybridize to different sequences selected from the group consisting of SEQ ID NO: 140-297.

219. A method according to claim 217, wherein the two ribonucleic acids comprise sequences which are complementary to and/or hybridize to different sequences with a single nucleotide mismatch to a sequence selected from the group consisting of SEQ ID NO: 140-297.

220. A method according to claim 217, wherein the two ribonucleic acids comprise sequences which are complementary to and/or hybridize to offset sequences selected from the group consisting of SEQ ID NO: 140-297.

221. A method according to claim 217, wherein the two ribonucleic acids comprise sequences which are complementary to and/or hybridize to offset sequences with a single nucleotide mismatch to a sequence selected from the group consisting of SEQ ID NO: 140-297.

222. A method according to any one of claims 157-158, wherein the target polynucleotide sequences comprise multiple different portions of CCR5.

223. A method according to claim 222, wherein each of the multiple ribonucleic acids comprises a sequence which is complementary to and/or hybridizes to a different sequence selected from the group consisting of SEQ ID NOs: 1-139 and 304-333.

224. A method according to claim 222, wherein each of the multiple ribonucleic acids comprises a sequence which is complementary to and/or hybridizes to an offset sequence selected from the group consisting of SEQ ID NOs: 1-139 and 304-333.

225. A method according to claim 222, wherein each of the multiple ribonucleic acids comprises a sequence which is complementary to and/or hybridizes to a sequence with a single nucleotide mismatch to a different sequence selected from the group consisting of SEQ ID NOs: 1-139 and 304-333.

226. A method according to claim 222, wherein each of the multiple ribonucleic acids comprises a sequence which is complementary to and/or hybridizes to a sequence with a single nucleotide mismatch to an offset sequence selected from the group consisting of SEQ ID NOs: 1-139 and 304-333.

227. A method according to any one of claims 157-158, wherein the target polynucleotide sequences comprise multiple different portions of CXCR4.

228. A method according to claim 227, wherein each of the multiple ribonucleic acids comprises a sequence which is complementary to and/or hybridizes to a

different sequence selected from the group consisting of SEQ ID NOs: 140-297 and 304-333.

229. A method according to claim 227, wherein each of the multiple ribonucleic acids comprises a sequence which is complementary to and/or hybridizes to an offset sequence selected from the group consisting of SEQ ID NOs: 140-297 and 304-333.

230. A method according to claim 227, wherein each of the multiple ribonucleic acids comprises a sequence which is complementary to and/or hybridizes to a sequence with a single nucleotide mismatch to a different sequence selected from the group consisting of SEQ ID NOs: 140-297 and 304-333.

231. A method according to claim 227, wherein each of the multiple ribonucleic acids comprises a sequence which is complementary to and/or hybridizes to a sequence with a single nucleotide mismatch to an offset sequence selected from the group consisting of SEQ ID NOs: 140-297 and 304-333.

232. A method according to any one of claims 157-158, wherein the target polynucleotide sequences comprise at least a portion of CCR5 and at least a portion of CXCR4.

233. A method according to claim 232, wherein each of the multiple ribonucleic acids comprises a sequence which is complementary to and/or hybridizes to a different sequence selected from the group consisting of SEQ ID NOs: 1-297 and 304-333.

234. A method according to claim 232, wherein each of the multiple ribonucleic acids comprises a sequence which is complementary to and/or hybridizes to an offset sequence selected from the group consisting of SEQ ID NOs: 1-297 and 304-333.

235. A method according to claim 232, wherein each of the multiple ribonucleic acids comprises a sequence which is complementary to and/or hybridizes to a

sequence with a single nucleotide mismatch to a different sequence selected from the group consisting of SEQ ID NOs: 1-297 and 304-333.

236. A method according to claim 232, wherein each of the multiple ribonucleic acids comprises a sequence which is complementary to and/or hybridizes to a sequence with a single nucleotide mismatch to an offset sequence selected from the group consisting of SEQ ID NOs: 1-297 and 304-333.

237. A method according to claim 232, wherein the multiple ribonucleic acids comprise at least two ribonucleic acid sequences which are complementary to and/or hybridize to offset sequences selected from the group consisting of SEQ ID NOs: 1-139 and 304-333, and at least two ribonucleic acid sequences which are complementary to and/or hybridize to offset sequences selected from the group consisting of SEQ ID NOs: 140-297.

238. A method according to claim 232, wherein the multiple ribonucleic acids comprises at least two ribonucleic acid sequences which are complementary to and/or hybridize to different sequences with a single nucleotide mismatch to an offset sequence selected from the group consisting of SEQ ID NOs: 1-139 and 304-333, and at least two ribonucleic acid sequences which are complementary to and/or hybridize to different sequences with a single nucleotide mismatch to an offset sequence selected from the group consisting of SEQ ID NOs: 140-297.

239. A method according to claims 156 or 158, wherein the disorder is a genetic disorder.

240. A method according to claims 156 or 158, wherein the disorder is a monogenic disorder.

241. A method according to claims 156 or 158, wherein the disorder is human immunodeficiency virus (HIV) infection.

242. A method according to claims 156 or 158, wherein the disorder is acquired immunodeficiency syndrome (AIDS).

243. A method according to any one of claims 155-156, wherein the two ribonucleic acids are designed to hybridize to a target motif immediately adjacent to a deoxyribonucleic acid motif recognized by the Cas protein.

244. A method according to any one of claims 155-156, wherein each of the two ribonucleic acids are designed to hybridize to target motifs immediately adjacent to deoxyribonucleic acid motifs recognized by the Cas protein which flank a mutant allele located between the target motifs.

245. A method according to any one of claims 157-158, wherein the multiple ribonucleic acids are designed to hybridize to target motifs immediately adjacent to deoxyribonucleic acid motifs recognized by the Cas protein.

246. A method according to any one of claims 157-158, wherein the multiple ribonucleic acids are designed to hybridize to target motifs immediately adjacent to deoxyribonucleic acid motifs recognized by the Cas protein which flank mutant alleles located between the target motifs.

247. A method according to any one of claims 155-156, wherein the two ribonucleic acids are selected to minimize hybridization with nucleic acid sequences other than the target polynucleotide sequence.

248. A method according to any one of claims 157-158, wherein the multiple ribonucleic acids are selected to minimize hybridization with nucleic acid sequences other than the target polynucleotide sequence.

249. A method according to any one of claims 155-156, wherein the target motif is selected such that it contains at least two mismatches when compared with all other genomic nucleotide sequences in the cell.

250. A method according to any one of claims 157-158, wherein each target motif is selected such that it contains at least two mismatches when compared with all other genomic nucleotide sequences in the cell.

251. A method according to any one of claims 155-156, wherein the target motif is selected such that it contains at least one mismatch when compared with all other genomic nucleotide sequences in the cell.

252. A method according to any one of claims 157-158, wherein the target motif is selected such that it contains at least one mismatch when compared with all other genomic nucleotide sequences in the cell.

253. A method according to any one of claims 155-156, wherein the two ribonucleic acids hybridize to a target motif that contains at least two mismatches when compared with all other genomic nucleotide sequences in the cell.

254. A method according to any one of claims 157-158, wherein each of the multiple ribonucleic acids hybridize to target motifs that contain at least two mismatches when compared with all other genomic nucleotide sequences in the cell.

255. A method according to any one of claims 155-156, wherein the two ribonucleic acids hybridize to a target motif that contains at least one mismatch when compared with all other genomic nucleotide sequences in the cell.

256. A method according to any one of claims 157-158, wherein each of the multiple ribonucleic acids hybridize to target motifs that contain at least one mismatch when compared with all other genomic nucleotide sequences in the cell.

257. A method according to any one of claims 157-158, wherein the efficiency of alteration at each loci is from about 50% to about 80%.

258. A method according to any one of claims 155-158, wherein the Cas protein is encoded by a modified nucleic acid.

259. A method according to claim 258, wherein the modified nucleic acid comprises a ribonucleic acid containing at least one modified nucleotide selected from the group consisting of pseudouridine, 5-methylcytodine, 2-thio-uridine, 5-methyluridine-5'-triphosphate, 4-thiouridine-5'-triphosphate, 5,6-dihydrouridine-5'-triphosphate, and 5-azauridine-5'-triphosphate.

260. A method according to any one of claims 155-156, wherein at least one of the two ribonucleic acids is a modified ribonucleic acid comprising one to two modified nucleotides selected from the group consisting of pseudouridine, 5-methylcytodine, 2-thio-uridine, 5-methyluridine-5'-triphosphate, 4-thiouridine-5'-triphosphate, 5,6-dihydrouridine-5'-triphosphate, and 5-azauridine-5'-triphosphate.

261. A method according to any one of claims 155-156, wherein the two ribonucleic acids comprise modified ribonucleic acids comprising one to two modified nucleotides selected from the group consisting of pseudouridine, 5-methylcytodine, 2-thio-uridine, 5-methyluridine-5'-triphosphate, 4-thiouridine-5'-triphosphate, 5,6-dihydrouridine-5'-triphosphate, and 5-azauridine-5'-triphosphate.

262. A method according to any one of claims 155-158, wherein any of the Cas protein or the ribonucleic acids are expressed from a plasmid.

263. A method according to any one of claims 155-158, wherein any of the Cas protein or the ribonucleic acids are expressed using a promoter optimized for increased expression in stem cells.

264. A method according to claim 263, wherein the promoter is selected from the group consisting of a Cytomegalovirus (CMV) early enhancer element and a

chicken beta-actin promoter, a chicken beta-actin promoter, an elongation factor-1 alpha promoter, and a ubiquitin promoter.

265. A method according to any one of claims 155-158, further comprising selecting cells that express the Cas protein.

266. A method according to claim 265, wherein selecting cells comprises FACS.

267. A method according to claim 265, wherein FACs is used to select cells which co-express Cas and a fluorescent protein.

268. A method for altering a target polynucleotide sequence in a cell comprising contacting the polynucleotide sequence in a cell selected from the group consisting of a human pluripotent cell, a primary human cell, and a non-transformed human cell, with a clustered regularly interspaced short palindromic repeats-associated (Cas) protein and two ribonucleic acids, wherein the ribonucleic acids direct Cas protein to and hybridize to a target motif of the target polynucleotide sequence, wherein the target polynucleotide sequence is cleaved, and wherein the efficiency of alteration of cells that express Cas protein is from about 8% to about 80%.

269. A method for treating or preventing a disorder associated with expression of a polynucleotide sequence in a subject, the method comprising (a) altering a target polynucleotide sequence in a cell *ex vivo* by contacting the polynucleotide sequence in a cell selected from the group consisting of a human pluripotent cell, a primary human cell, and a non-transformed human cell, with a clustered regularly interspaced short palindromic repeats-associated (Cas) protein and two ribonucleic acids, wherein the ribonucleic acids direct Cas protein to and hybridize to a target motif of the target polynucleotide sequence, wherein the target polynucleotide sequence is cleaved, and wherein the efficiency of alteration is from about 8% to about 80%, and (b) introducing the cell into the subject, thereby treating or

preventing a disorder associated with expression of the polynucleotide sequence.

270. A method for simultaneously altering multiple target polynucleotide sequences in a cell comprising contacting the polynucleotide sequences in a cell selected from the group consisting of a human pluripotent cell, a primary human cell, and a non-transformed human cell, with a clustered regularly interspaced short palindromic repeats-associated (Cas) protein and multiple ribonucleic acids, wherein the ribonucleic acids direct Cas protein to and hybridize to target motifs of the target polynucleotide sequences, wherein the target polynucleotide sequences are cleaved, and wherein the efficiency of alteration of cells that express Cas protein is from about 8% to about 80%.

271. A method for treating or preventing a disorder associated with expression of polynucleotide sequences in a subject, the method comprising (a) altering target polynucleotide sequences in a cell *ex vivo* by contacting the polynucleotide sequences in a cell selected from the group consisting of a human pluripotent cell, a primary human cell, and a non-transformed human cell, with a clustered regularly interspaced short palindromic repeats-associated (Cas) protein and multiple ribonucleic acids, wherein the ribonucleic acids direct Cas protein to and hybridize to target motifs of the target polynucleotide sequences, wherein the target polynucleotide sequences are cleaved, and wherein the efficiency of alteration of cells that express Cas protein is from about 8% to about 80%, and (b) introducing the cell into the subject, thereby treating or preventing a disorder associated with expression of the polynucleotide sequences.

272. A composition comprising at least two ribonucleic acids each comprising a different sequence selected from the group consisting of SEQ ID NOs: 298-303.

273. A composition comprising at least two ribonucleic acids each comprising a sequence which is complementary to and/or hybridizes to a different sequence selected from the group consisting of SEQ ID NOs: 1-139 and 304-333.

274. A composition comprising at least two ribonucleic acids each comprising a sequence which is complementary to and/or hybridizes to a sequence with a single nucleotide mismatch to a sequence selected from the group consisting of SEQ ID NOs: 1-139 and 304-333.

275. A composition comprising at least two ribonucleic acids each comprising a sequence which is complementary to and/or hybridizes to a different sequence selected from the group consisting of SEQ ID NOs: 140-297.

276. A composition comprising at least two ribonucleic acids each comprising a sequence which is complementary to and/or hybridizes to a sequence with a single nucleotide mismatch to a sequence selected from the group consisting of SEQ ID NOs: 140-297.

277. A composition according to any one of claims 272-276, wherein at least one of the two ribonucleic acids is a modified ribonucleic acid comprising one to two modified nucleotides selected from the group consisting of pseudouridine, 5-methylcytodine, 2-thio-uridine, 5-methyluridine-5'-triphosphate, 4-thiouridine-5'-triphosphate, 5,6-dihydrouridine-5'-triphosphate, and 5-azauridine-5'-triphosphate.

278. A composition according to any one of claims 272-277, wherein the two ribonucleic acids comprise modified ribonucleic acids comprising one to two modified nucleotides selected from the group consisting of pseudouridine, 5-methylcytodine, 2-thio-uridine, 5-methyluridine-5'-triphosphate, 4-thiouridine-5'-triphosphate, 5,6-dihydrouridine-5'-triphosphate, and 5-azauridine-5'-triphosphate.

279. A composition according to any one of claims 272-278, further comprising a nucleic acid sequence encoding a Cas protein.

280. A composition according to any one of claims 272-279, further comprising a nucleic acid sequence encoding a Cas9 protein or a functional portion thereof.

281. A composition according to claims 279 or 280, wherein the nucleic acid comprises a modified ribonucleic acid comprising at least one modified nucleotide selected from the group consisting of pseudouridine, 5-methylcytodine, 2-thio-uridine, 5-methyluridine-5'-triphosphate, 4-thiouridine-5'-triphosphate, 5,6-dihydrouridine-5'-triphosphate, and 5-azauridine-5'-triphosphate.

282. A composition comprising a chimeric nucleic acid comprising a ribonucleic acid encoding a Cas protein and at least two additional ribonucleic acids each having a sequence selected from the group consisting of the ribonucleic acid sequences of SEQ ID NOs: 298-303.

283. A composition comprising a chimeric nucleic acid comprising a ribonucleic acid encoding a Cas protein and at least two additional ribonucleic acids each having a sequence which is complementary to and/or hybridizes to a different sequence selected from the group consisting of SEQ ID NOs: 1-139 and 304-333.

284. A composition comprising a chimeric nucleic acid comprising a ribonucleic acid encoding a Cas protein and at least two additional ribonucleic acids each having a sequence which is complementary to and/or hybridizes to a different sequence selected from the group consisting of SEQ ID NOs: 140-297.

285. A composition comprising a chimeric nucleic acid comprising a ribonucleic acid encoding a Cas protein and at least two additional ribonucleic acid sequences each comprising a sequence which is complementary to and/or hybridizes to a sequence with a single nucleotide mismatch to a sequence selected from the group consisting of SEQ ID NOs: 1-139 and 304-333.

286. A composition comprising a chimeric nucleic acid comprising a ribonucleic acid encoding a Cas protein and at least two additional ribonucleic acid sequences each comprising a sequence which is complementary to and/or hybridizes to a sequence with a single nucleotide mismatch to a sequence selected from the group consisting of SEQ ID NOs: 140-297.

287. A composition according to any one of claims 272-286, further comprising a nucleic acid sequence encoding a detectable marker.

288. A composition according to any one of claims 272-287, further comprising a nucleic acid sequence encoding a fluorescent protein.

289. A composition according to any one of claims 272-288, further comprising a promoter operably linked to the chimeric nucleic acid.

290. A composition according to claim 289, wherein the promoter is optimized for increased expression in human stem cells.

291. A composition according to claims 289 or 290, wherein the promoter is selected from the group consisting of a Cytomegalovirus (CMV) early enhancer element and a chicken beta-actin promoter, a chicken beta-actin promoter, an elongation factor-1 alpha promoter, and a ubiquitin promoter.

292. A composition according to any one of claims 282-291, wherein the chimeric nucleic acid comprises at least one modified nucleotide selected from the group consisting of pseudouridine, 5-methylcytodine, 2-thio-uridine, 5-methyluridine-5'-triphosphate, 4-thiouridine-5'-triphosphate, 5,6-dihydrouridine-5'-triphosphate, and 5-azauridine-5'-triphosphate.

293. A composition according to any one of claims 282-292, wherein the Cas protein comprises a Cas9 protein or a functional portion thereof.

294. A kit for altering a target polynucleotide sequence in a cell comprising a Cas9 protein or a nucleic acid encoding the Cas9 protein, and at least two

ribonucleic acids each comprising a different sequence selected from the group consisting of the ribonucleic acid sequences of SEQ ID NOs: 298-303.

295. A kit for altering a target polynucleotide sequence in a cell comprising a Cas9 protein or a nucleic acid encoding the Cas9 protein, and at least two ribonucleic acids each comprising a sequence which is complementary to and/or hybridizes to a different sequence selected from the group consisting of SEQ ID NOs: 1-139 and 304-333.

296. A kit for altering a target polynucleotide sequence in a cell comprising a Cas9 protein or a nucleic acid encoding the Cas9 protein, and at least two ribonucleic acids each comprising a sequence which is complementary to and/or hybridizes to a different sequence selected from the group consisting of SEQ ID NOs: 140-297.

297. A kit according to any one of claims 294-296 further comprising one or more cell lines, cultures, or populations selected from the group consisting of human pluripotent cells, primary human cells, and non-transformed cells.

298. A kit according to any one of claims 294-297 further comprising a DNA repair template.

299. A method of administering cells to a subject in need of such cells, the method comprising: (a) contacting a cell or population of cells *ex vivo* with a Cas protein and two ribonucleic acids which direct Cas protein to and hybridize to a target polynucleotide sequence encoding B2M in the cell or population of cells, wherein the target polynucleotide sequence is cleaved; and (b) administering the resulting cells from (a) to a subject in need of such cells.

300. A method of administering cells to a subject in need of such cells, the method comprising: (a) contacting a cell or population of cells *ex vivo* with (i) a Cas protein, (ii) at least two ribonucleic acids which direct Cas protein to and hybridize to a target polynucleotide sequence encoding B2M in the cell or population of cells, and (iii) at least two additional ribonucleic acids which

direct Cas protein to and hybridize to a target polynucleotide sequence in the cell or population of cells, wherein the target polynucleotide sequences are cleaved; and (b) administering the resulting cell or cells from (a) to a subject in need of such cells.

301. The method of claims 299 or 300, wherein cleavage of the target polynucleotide sequence encoding B2M in the cell or population of cells reduces the likelihood that the resulting cell or cells will trigger a host immune response when the cells are administered to the subject.

302. The method of any one of claims 299 to 301, wherein the target polynucleotide sequence comprises CCR5.

303. The method of any one of claims 299 to 302, wherein the at least two ribonucleic acids comprise two different sequences selected from the group consisting of SEQ ID NOs: 298-303.

304. The method of any one of claims 299 to 303, wherein the at least two ribonucleic acids each comprise sequences which are complementary to and/or hybridize to a different sequence selected from the group consisting of SEQ ID NOs: 1-139 and 304-333.

305. The method of any one of claims 299 to 304, wherein the at least two ribonucleic acids each comprise sequences which are complementary to and/or hybridize to sequences comprising at least one nucleotide mismatch to different sequences selected from the group consisting of SEQ ID NOs: 1-139 and 304-333.

306. The method of any one of claims 299 to 305, wherein the target polynucleotide sequence comprises CXCR4.

307. The method of any one of claims 299 to 306, wherein the at least two ribonucleic acids each comprise sequences which are complementary to and/or

hybridize to a different sequence selected from the group consisting of SEQ ID NOs: 140-297.

308. The method of any one of claims 299 to 307, wherein the at least two ribonucleic acids each comprise sequences which are complementary to and/or hybridize to sequences comprising at least one nucleotide mismatch to different sequences selected from the group consisting of SEQ ID NOs: 140-297.

309. The method of any one of claims 299 to 308, wherein the cell or population of cells comprises primary cells.

310. The method of any one of claims 299 to 309, wherein the subject in need of administration of cells is suffering from a disorder.

311. The method of claim 310, wherein the disorder comprises a genetic disorder.

312. The method of claim 310, wherein the disorder comprises an infection.

313. The method of claim 310, wherein the disorder comprises HIV or AIDs.

314. The method of claim 310, wherein the disorder comprises cancer.

315. A method of reducing the likelihood that cells administered to a subject will trigger a host immune response in the subject, the method comprising: (a) contacting a cell or population of cells *ex vivo* with a Cas protein and two ribonucleic acids which direct Cas protein to and hybridize to a target polynucleotide sequence encoding B2M in the cell or population of cells, wherein the target polynucleotide sequence encoding B2M is cleaved, thereby reducing the likelihood that cells administered to the subject will trigger a host immune response in the subject; and (b) administering the resulting cells from (a) to a subject in need of such cells.

316. A method of reducing the likelihood that cells administered to a subject will trigger a host immune response in the subject, the method comprising: (a) contacting a cell or population of cells *ex vivo* with (i) a Cas protein, (ii) at least two ribonucleic acids which direct Cas protein to and hybridize to a target polynucleotide sequence encoding B2M in the cell or population of cells, wherein the target polynucleotide sequence encoding B2M in the cell or population of cells is cleaved, thereby reducing the likelihood that the cell or population of cells will trigger a host immune response in the subject, and (iii) at least two additional ribonucleic acids which direct Cas protein to and hybridize to a target polynucleotide sequence in the cell or population of cells, wherein the target polynucleotide sequence is cleaved; and (b) administering the resulting cell or cells from (a) to a subject in need of such cells.

317. The method of any one of claims 315 to 316, wherein the target polynucleotide sequence comprises CCR5.

318. The method of any one of claims 315 to 317, wherein the at least two ribonucleic acids comprise two different sequences selected from the group consisting of SEQ ID NOs: 298-303.

319. The method of any one of claims 315 to 318, wherein the at least two ribonucleic acids each comprise sequences which are complementary to and/or hybridize to a different sequence selected from the group consisting of SEQ ID NOs: 1-139 and 304-333.

320. The method of any one of claims 315 to 319, wherein the at least two ribonucleic acids each comprise sequences which are complementary to and/or hybridize to sequences comprising at least one nucleotide mismatch to different sequences selected from the group consisting of SEQ ID NOs: 1-139 and 304-333.

321. The method of any one of claims 315 to 320, wherein the target polynucleotide sequence comprises CXCR4.

322. The method of any one of claims 315 to 321, wherein the at least two ribonucleic acids each comprise sequences which are complementary to and/or hybridize to a different sequence selected from the group consisting of SEQ ID NOs: 140-297.

323. The method of any one of claims 315 to 322, wherein the at least two ribonucleic acids each comprise sequences which are complementary to and/or hybridize to sequences comprising at least one nucleotide mismatch to different sequences selected from the group consisting of SEQ ID NOs: 140-297.

324. The method of any one of claims 315 to 323, wherein the cell or population of cells comprises primary cells.

325. The method of any one of claims 315 to 324, wherein the subject in need of administration of cells is suffering from a disorder.

326. The method of claim 325, wherein the disorder comprises a genetic disorder.

327. The method of claim 325, wherein the disorder comprises an infection.

328. The method of claim 325, wherein the disorder comprises HIV or AIDs.

329. The method of claim 325, wherein the disorder comprises cancer.

site_type	site_start	site_end	site_strand	relative_start	site_sequence	genome wide hits with 1 mismatches	genome wide hits with 2 mismatches	genome wide hits with 3 mismatches
(N20)NGG	46414444	46414445	1	50	TGACATCAATTATTATACATCGG (SEQ ID NO: 1)	1	4	28
(N20)NGG	46414510	46414511	1	116	CCTGCCTCCGGCTCTACTCACTGG (SEQ ID NO: 2)	2	2	25
(N20)NGG	46414523	46414524	1	129	TACTCACTGGTGTTCATCTTTGG (SEQ ID NO: 3)	2	3	25
(N20)NGG	46414531	46414532	1	137	GGTGTTCATCTTTGGTTTTTGTGG (SEQ ID NO: 4)	2	2	34
(N20)NGG	46414532	46414533	1	138	GTGTTCATCTTTGGTTTTTGTGGG (SEQ ID NO: 5)	2	3	64
(N20)NGG	46414543	46414544	1	149	TGGTTTTTGTGGGCAACATGCTGG (SEQ ID NO: 6)	2	4	32
(N20)NGG	46414572	46414573	1	178	TCATCCTGATAAAACTGCAAAAGG (SEQ ID NO: 7)	1	1	35
(N20)NGG	46414609	46414610	1	215	TGACATCTACCTGCTCAACCTGG (SEQ ID NO: 8)	2	5	37
(N20)NGG	46414650	46414651	1	256	TCCTTCTTACTGTCCCCTTCTGG (SEQ ID NO: 9)	1	2	35
(N20)NGG	46414651	46414652	1	257	CCTTCTTACTGTCCCCTTCTGGG (SEQ ID NO: 10)	2	3	44
(N20)NGG	46414674	46414675	1	280	CTCACTATGCTGCCGCCCAGTGG (SEQ ID NO: 11)	1	2	16
(N20)NGG	46414675	46414676	1	281	TCACTATGCTGCCGCCCAGTGGG (SEQ ID NO: 12)	1	1	7
(N20)NGG	46414682	46414683	1	288	GCTGCCGCCCAGTGGGACTTTGG (SEQ ID NO: 13)	1	2	10
(N20)NGG	46414709	46414710	1	315	ACAATGTGTCAACTCTTGACAGG (SEQ ID NO: 14)	1	1	6
(N20)NGG	46414710	46414711	1	316	CAATGTGTCAACTCTTGACAGGG (SEQ ID NO: 15)	1	2	11

FIG. 1

site_type	site_start	site_end	site_ strand	relative_ start	site_sequence	genome wide hits with 1 mismatches	genome wide hits with 2 mismatches	genome wide hits with 3 mismatches
(N20)NGG	46414724	46414725	1	330	TTGACAGGGCTCTATTTTATAGG (SEQ ID NO: 16)	1	1	12
(N20)NGG	46414736	46414737	1	342	TATTTTATAGGCTTCTTCTCTGG (SEQ ID NO: 17)	1	2	31
(N20)NGG	46414770	46414771	1	376	TCATCCTCCTGACAATCGATAGG (SEQ ID NO: 18)	2	2	7
(N20)NGG	46414777	46414778	1	383	CCTGACAATCGATAGGTACCTGG (SEQ ID NO: 19)	2	3	4
(N20)NGG	46414812	46414813	1	418	CTGTGTTTGCTTTAAAAGCCAGG (SEQ ID NO: 20)	2	6	36
(N20)NGG	46414816	46414817	1	422	GTTTGCTTTAAAAGCCAGGACGG (SEQ ID NO: 21)	2	5	30
(N20)NGG	46414826	46414827	1	432	AAAGCCAGGACGGTCACCTTTGG (SEQ ID NO: 22)	2	3	16
(N20)NGG	46414827	46414828	1	433	AAGCCAGGACGGTCACCTTTGGG (SEQ ID NO: 23)	2	2	10
(N20)NGG	46414828	46414829	1	434	AGCCAGGACGGTCACCTTTGGGG (SEQ ID NO: 24)	2	4	10
(N20)NGG	46414831	46414832	1	437	CAGGACGGTCACCTTTGGGGTGG (SEQ ID NO: 25)	2	2	10
(N20)NGG	46414851	46414852	1	457	TGGTGACAAGTGTGATCACTTGG (SEQ ID NO: 26)	2	3	33
(N20)NGG	46414852	46414853	1	458	GGTGACAAGTGTGATCACTTGGG (SEQ ID NO: 27)	1	2	20
(N20)NGG	46414855	46414856	1	461	GACAAGTGTGATCACTTGGGTGG (SEQ ID NO: 28)	1	2	11
(N20)NGG	46414858	46414859	1	464	AAGTGTGATCACTTGGGTGGTGG (SEQ ID NO: 29)	1	4	20
(N20)NGG	46414880	46414881	1	486	GCTGTGTTTGCGTCTCTCCCAGG (SEQ ID NO: 30)	1	4	22

FIG. 1 cont.

site_type	site_start	site_end	site_strand	relative_start	site_sequence	genome wide hits with 1 mismatches	genome wide hits with 2 mismatches	genome wide hits with 3 mismatches
(N20)NGG	46414910	46414911	1	516	TTTACCAGATCTCAAAAAGAAGG (SEQ ID NO: 31)	1	3	27
(N20)NGG	46414962	46414963	1	568	CATACAGTCAGTATCAATTCTGG (SEQ ID NO: 32)	1	3	16
(N20)NGG	46414996	46414997	1	602	GACATTAAAGATAGTCATCTTGG (SEQ ID NO: 33)	1	3	31
(N20)NGG	46414997	46414998	1	603	ACATTAAAGATAGTCATCTTGGG (SEQ ID NO: 34)	1	2	25
(N20)NGG	46414998	46414999	1	604	CATTAAAGATAGTCATCTTGGGG (SEQ ID NO: 35)	1	1	25
(N20)NGG	46415002	46415003	1	608	AAAGATAGTCATCTTGGGGCTGG (SEQ ID NO: 36)	1	1	20
(N20)NGG	46415023	46415024	1	629	GGTCCTGCCCGCTGCTTGTCATGG (SEQ ID NO: 37)	1	4	21
(N20)NGG	46415038	46415039	1	644	TGTCATGGTCATCTGCTACTCGG (SEQ ID NO: 38)	1	2	18
(N20)NGG	46415039	46415040	1	645	GTCATGGTCATCTGCTACTCGGG (SEQ ID NO: 39)	2	4	17
(N20)NGG	46415061	46415062	1	667	GAATCCTAAAAACTCTGCTTCGG (SEQ ID NO: 40)	1	4	26
(N20)NGG	46415082	46415083	1	688	GGTGTCGAAATGAGAAGAAGAAGAGG (SEQ ID NO: 41)	2	3	22
(N20)NGG	46415088	46415089	1	694	GAAATGAGAAGAAGAAGAGGCACAG G (SEQ ID NO: 42)	1	7	81
(N20)NGG	46415089	46415090	1	695	AAATGAGAAGAAGAGGCACAGG G (SEQ ID NO: 43)	1	8	109
(N20)NGG	46415097	46415098	1	703	AGAAGAGGCACAGGGCTGTGAG G (SEQ ID NO: 44)	1	3	70
(N20)NGG	46415136	46415137	1	742	TGATTGTTTATTTTCTCTTCTGG (SEQ ID NO: 45)	2	10	122

FIG. 1 cont.

site_type	site_start	site_end	site_strand	relative_start	site_sequence	genome wide hits with 1 mismatches	genome wide hits with 2 mismatches	genome wide hits with 3 mismatches
(N20)NGG	46415137	46415138	1	743	GATTGTTTATTTTCTCTTCTGGG (SEQ ID NO: 46)	1	7	142
(N20)NGG	46415176	46415177	1	782	CCTTCTCCTGAACACCTTCCAGG (SEQ ID NO: 47)	2	3	32
(N20)NGG	46415186	46415187	1	792	AACACCTTCCAGGAATTCTTTGG (SEQ ID NO: 48)	2	2	32
(N20)NGG	46415214	46415215	1	820	ATAATTGCAGTAGCTCTAACAGG (SEQ ID NO: 49)	1	1	15
(N20)NGG	46415218	46415219	1	824	TTGCAGTAGCTCTAACAGGTTGG (SEQ ID NO: 50)	1	2	12
(N20)NGG	46415233	46415234	1	839	CAGGTTGGACCAAGCTATGCAGG (SEQ ID NO: 51)	1	1	14
(N20)NGG	46415249	46415250	1	855	ATGCAGGTGACACAGAGACTCTTGG (SEQ ID NO: 52)	2	2	24
(N20)NGG	46415250	46415251	1	856	TGCAGGTGACAGAGACTCTTGGG (SEQ ID NO: 53)	2	5	30
(N20)NGG	46415294	46415295	1	900	CCCATCATCTATGCCTTTGTCGG (SEQ ID NO: 54)	2	3	19
(N20)NGG	46415295	46415296	1	901	CCATCATCTATGCCTTTGTCGGG (SEQ ID NO: 55)	1	4	16
(N20)NGG	46415296	46415297	1	902	CATCATCTATGCCTTTGTCGGGG (SEQ ID NO: 56)	1	3	9
(N20)NGG	46415383	46415384	1	989	CTGTTCTATTTTCCAGCAAGAGG (SEQ ID NO: 57)	1	1	24
(N20)NGG	46415423	46415424	1	1029	TCAGTTTACACCCGATCCACTGG (SEQ ID NO: 58)	1	1	2
(N20)NGG	46415424	46415425	1	1030	CAGTTTACACCCGATCCACTGGG (SEQ ID NO: 59)	1	1	3
(N20)NGG	46415425	46415426	1	1031	AGTTTACACCCGATCCACTGGGG (SEQ ID NO: 60)	1	1	3

FIG. 1 cont.

site_type	site_start	site_end	site_ strand	relative_ start	site_sequence	genome wide hits with 1 mismatches	genome wide hits with 2 mismatches	genome wide hits with 3 mismatches
(N20)NGG	46415431	46415432	1	1037	CACCCGATCCACTGGGGAGCAGG (SEQ ID NO: 61)	1	2	19
(N20)NGG	46415443	46415444	1	1049	TGGGGAGCAGGAAATATCTGTGG (SEQ ID NO: 62)	1	6	34
(N20)NGG	46415444	46415445	1	1050	GGGGAGCAGGAAATATCTGTGG G (SEQ ID NO: 63)	1	3	31
(N20)NGG	46414414	46414415	-1	20	TAATAATTGATGTCATAGATTGG (SEQ ID NO: 64)	1	2	38
(N20)NGG	46414447	46414448	-1	53	TTCACATTGATTTTTTGGCAGGG (SEQ ID NO: 65)	1	5	40
(N20)NGG	46414448	46414449	-1	54	CTTCACATTGATTTTTTGGCAGG (SEQ ID NO: 66)	1	1	28
(N20)NGG	46414452	46414453	-1	58	TTTGCTTCACATTGATTTTTTGG (SEQ ID NO: 67)	2	3	79
(N20)NGG	46414481	46414482	-1	87	GTAGAGCGGAGGCAGGAGGCGG G (SEQ ID NO: 68)	1	7	89
(N20)NGG	46414482	46414483	-1	88	AGTAGAGCGGAGGCAGGAGGCG G (SEQ ID NO: 69)	3	6	72
(N20)NGG	46414485	46414486	-1	91	GTGAGTAGAGCGGAGGCAGGAG G (SEQ ID NO: 70)	1	2	44
(N20)NGG	46414488	46414489	-1	94	CCAGTGAGTAGAGCGGAGGCAG G (SEQ ID NO: 71)	2	3	19
(N20)NGG	46414492	46414493	-1	98	AACACCAGTGAGTAGAGCGGAG G (SEQ ID NO: 72)	2	4	14
(N20)NGG	46414495	46414496	-1	101	ATGAACACCAGTGAGTAGAGCGG (SEQ ID NO: 73)	2	3	21
(N20)NGG	46414548	46414549	-1	154	TTTTGCAGTTTATCAGGATGAGG (SEQ ID NO: 74)	1	4	33
(N20)NGG	46414554	46414555	-1	160	TCAGCCTTTTGCAGTTTATCAGG (SEQ ID NO: 75)	1	1	26

FIG. 1 cont.

site_type	site_start	site_end	site_strand	relative_start	site_sequence	genome wide hits with 1 mismatches	genome wide hits with 2 mismatches	genome wide hits with 3 mismatches
					CAGAGATGGCCAGGTTGAGCAG			
(N20)NGG	46414596	46414597	+	202	G (SEQ ID NO: 76)	2	12	67
					AAAACAGGTCAGAGATGGCCAGG			
(N20)NGG	46414605	46414606	+	211	(SEQ ID NO: 77)	1	12	88
					AAGGAAAAACAGGTCAGAGATGG			
(N20)NGG	46414610	46414611	+	216	(SEQ ID NO: 78)	1	5	123
					GGACAGTAAGAAGGAAAAAACAGG			
(N20)NGG	46414620	46414621	+	226	(SEQ ID NO: 79)	1	5	97
					CCCAGAAGGGGACAGTAAGAAG			
(N20)NGG	46414629	46414630	+	235	G (SEQ ID NO: 80)	2	3	38
					CAGCATAGTGAGCCCAGAAGGG			
(N20)NGG	46414641	46414642	+	247	G (SEQ ID NO: 81)	1	3	41
					GCAGCATAGTGAGCCCCAGAAGG			
(N20)NGG	46414642	46414643	+	248	G (SEQ ID NO: 82)	1	2	30
					GGCAGCATAGTGAGCCCCAGAAG			
(N20)NGG	46414643	46414644	+	249	G (SEQ ID NO: 83)	1	1	31
					ATTTCCAAAGTCCCACTGGGCGG			
(N20)NGG	46414664	46414665	+	270	(SEQ ID NO: 84)	1	4	23
					TGTATTTCCAAAGTCCCACTGGG			
(N20)NGG	46414667	46414668	+	273	(SEQ ID NO: 85)	1	1	22
					TTGTATTTCCAAAGTCCCACTGG			
(N20)NGG	46414668	46414669	+	274	(SEQ ID NO: 86)	1	2	23
					GGTACCTATCGATTGTCAGGAGG			
(N20)NGG	46414752	46414753	+	358	(SEQ ID NO: 87)	2	3	3
					CCAGGTACCTATCGATTGTCAGG			
(N20)NGG	46414755	46414756	+	361	(SEQ ID NO: 88)	2	3	5
					ACACAGCATGGACGACAGCCAGG			
(N20)NGG	46414773	46414774	+	379	(SEQ ID NO: 89)	1	6	30
					CTTTTAAAGCAAACACAGCATGG			
(N20)NGG	46414785	46414786	+	391	(SEQ ID NO: 90)	2	4	62

FIG. 1 cont.

site_type	site_start	site_end	site_strand	relative_start	site_sequence	genome wide hits with 1 mismatches	genome wide hits with 2 mismatches	genome wide hits with 3 mismatches
(N20)NGG	46414808	46414809	-1	414	CACCCCAAAGGTGACCGTCCTGG (SEQ ID NO: 91)	2	2	5
(N20)NGG	46414820	46414821	-1	426	CACACTTGTCACCACCCCAAAGG (SEQ ID NO: 92)	2	2	19
(N20)NGG	46414875	46414876	-1	481	ATCTGGTAAAGATGATTCCTGGG (SEQ ID NO: 93)	1	4	29
(N20)NGG	46414876	46414877	-1	482	GATCTGGTAAAGATGATTCCTGG (SEQ ID NO: 94)	1	1	22
(N20)NGG	46414892	46414893	-1	498	AAGACCTTCTTTTTGAGATCTGG (SEQ ID NO: 95)	1	2	35
(N20)NGG	46414922	46414923	-1	528	GTATGGAAAATGAGAGCTGCAGG (SEQ ID NO: 96)	1	4	23
(N20)NGG	46414939	46414940	-1	545	CAGAATTGATACTGACTGTATGG (SEQ ID NO: 97)	1	2	11
(N20)NGG	46414971	46414972	-1	577	AGATGACTATCTTTAATGTCTGG (SEQ ID NO: 98)	1	5	25
(N20)NGG	46415004	46415005	-1	610	TGACCATGACAAGCAGCGGCAGG (SEQ ID NO: 99)	1	2	8
(N20)NGG	46415008	46415009	-1	614	CAGATGACCATGACAAGCAGCGG (SEQ ID NO: 100)	1	3	22
(N20)NGG	46415043	46415044	-1	649	GACACCGAAGCAGAGTTTTTAGG (SEQ ID NO: 101)	1	2	14
(N20)NGG	46415108	46415109	-1	714	GAGAAAATAAACAATCATGATGG (SEQ ID NO: 102)	2	9	88
(N20)NGG	46415140	46415141	-1	746	AGGAGAAGGACAATGTTGTAGGG (SEQ ID NO: 103)	1	1	31
(N20)NGG	46415141	46415142	-1	747	CAGGAGAAGGACAATGTTGTAGG (SEQ ID NO: 104)	1	2	34
(N20)NGG	46415154	46415155	-1	760	CCTGGAAGGTGTTCAGGAGAAGG (SEQ ID NO: 105)	2	6	38

FIG. 1 cont.

site_type	site_start	site_end	site_ strand	relative_ start	site_sequence	genome wide hits with 1 mismatches	genome wide hits with 2 mismatches	genome wide hits with 3 mismatches
(N20)NGG	46415160	46415161	-1	766	AGAATTCCTGGAAGGTGTTCAGG (SEQ ID NO: 106)	2	2	19
(N20)NGG	46415168	46415169	-1	774	CAGGCCAAAGAATTCCTGGAAGG (SEQ ID NO: 107)	2	3	32
(N20)NGG	46415172	46415173	-1	778	TATTCAGGCCAAAGAATTCCTGG (SEQ ID NO: 108)	1	3	15
(N20)NGG	46415187	46415188	-1	793	TAGAGCTACTGCAATTATTCAGG (SEQ ID NO: 109)	1	2	28
(N20)NGG	46415220	46415221	-1	826	TCTCTGTCACCTGCATAGCTTGG (SEQ ID NO: 110)	1	8	124
(N20)NGG	46415271	46415272	-1	877	CGACAAAGGCATAGATGATGGGG (SEQ ID NO: 111)	1	1	13
(N20)NGG	46415272	46415273	-1	878	CCGACAAAGGCATAGATGATGGG (SEQ ID NO: 112)	1	2	7
(N20)NGG	46415273	46415274	-1	879	CCCGACAAAGGCATAGATGATGG (SEQ ID NO: 113)	1	3	7
(N20)NGG	46415285	46415286	-1	891	TCTGAACTTCTCCCCCGACAAAGG (SEQ ID NO: 114)	1	2	8
(N20)NGG	46415313	46415314	-1	919	GCTTTTGGAAGAAGACTAAGAGG (SEQ ID NO: 115)	1	10	90
(N20)NGG	46415328	46415329	-1	934	AGCGTTTGGCAATGTGCTTTTGG (SEQ ID NO: 116)	1	1	11
(N20)NGG	46415342	46415343	-1	948	ACAGCATTTGCAGAAGCGTTTGG (SEQ ID NO: 117)	1	2	18
(N20)NGG	46415373	46415374	-1	979	CTCGCTCGGGAGCCTCTTGCTGG (SEQ ID NO: 118)	1	2	6
(N20)NGG	46415386	46415387	-1	992	TAAACTGAGCTTGCTCGCTCGGG (SEQ ID NO: 119)	1	2	6
(N20)NGG	46415387	46415388	-1	993	GTAAACTGAGCTTGCTCGCTCGG (SEQ ID NO: 120)	1	2	5

FIG. 1 cont.

site_type	site_start	site_end	site_ strand	relative_ start	site_sequence	genome wide hits with 1 mismatches	genome wide hits with 2 mismatches	genome wide hits with 3 mismatches
(N20)NGG	46415411	46415412	-1	1017	TTCCTGCTCCCCAGTGGATCGGG (SEQ ID NO: 121)	1	3	39
(N20)NGG	46415412	46415413	-1	1018	TTTCCTGCTCCCCAGTGGATCGG (SEQ ID NO: 122)	1	5	30
(N20)NGG	46415417	46415418	-1	1023	AGATATTTCCTGCTCCCCAGTGG (SEQ ID NO: 123)	1	1	39
(N21)NNAGAAW	46414909	46414910	1	515	ATCATCTTTACCAGATCTCAAAAA GAAG (SEQ ID NO: 124)	1	1	2
(N21)NNAGAAW	46415076	46415077	1	682	AACTCTGCTTCGGTGTCGAAATG AGAAG (SEQ ID NO: 125)	2	2	2
(N21)NNAGAAW	46415079	46415080	1	685	TCTGCTTCGGTGTCGAAATGAGA AGAAG (SEQ ID NO: 126)	1	2	2
(N21)NNAGAAW	46415301	46415302	1	907	CATCATCTATGCCCTTTGTCGCGGGA GAAG (SEQ ID NO: 127)	1	2	2
(N21)NNAGAAW	46415309	46415310	1	915	ATGCCTTTGTCGGGGAGAAGTTC AGAAA (SEQ ID NO: 128)	1	2	3
BTTCTNN(N21)	46414630	46414631	-1	236	AGTGAGCCCAGAAGGGGACAGT AAGAAG (SEQ ID NO: 129)	1	1	2
BTTCTNN(N21)	46414644	46414645	-1	250	CTGGGCGGCAGCATAGTGAGCCC AGAAG (SEQ ID NO: 130)	1	1	1
BTTCTNN(N21)	46414725	46414726	-1	331	GATGATGAAGAAGATTCCAGAGA AGAAG (SEQ ID NO: 131)	1	1	3
BTTCTNN(N21)	46414728	46414729	-1	334	GAGGATGATGAAGAAGATTCCAG AGAAG (SEQ ID NO: 132)	1	3	5
BTTCTNN(N21)	46414740	46414741	-1	346	ATCGATTGTCAGGAGGATGATGA AGAAG (SEQ ID NO: 133)	2	2	2
BTTCTNN(N21)	46415125	46415126	-1	731	CAATGTTGTAGGGAGCCCAGAAG AGAAA (SEQ ID NO: 134)	1	1	4
BTTCTNN(N21)	46415130	46415131	-1	736	AAGGACAATGTTGTAGGGAGCCC AGAAG (SEQ ID NO: 135)	1	1	2

FIG. 1 cont.

site_type	site_start	site_end	site_ strand	relative_ start	site_sequence	genome wide hits with 1 mismatches	genome wide hits with 2 mismatches	genome wide hits with 3 mismatches
BTTCTNN(N21)	46415155	46415156	-1	761	AGAATTCCTGGAAGGTGTTCAGG AGAAG (SEQ ID NO: 136)	2	2	2
BTTCTNN(N21)	46415322	46415323	-1	928	GCGTTTGGCAATGTGCTTTTGGA AGAAG (SEQ ID NO: 137)	1	1	2
BTTCTNN(N21)	46415349	46415350	-1	955	CTGCAAAATAGAACACATTTGC AGAAG (SEQ ID NO: 138)	1	1	5
BTTCTNN(N21)	46415363	46415364	-1	969	TCGGGAGCCTCTTGCTGGAAAAT AGAAC (SEQ ID NO: 139)	1	1	1

FIG. 1 cont.

site_type	site_start	site_end	site_strand	relative_start	site_sequence	genome wide hits with 1 mismatches	genome wide hits with 2 mismatches	genome wide hits with 3 mismatches
(N20)NGG	136872475	136872476	1	1034	ACTTGAAGACTCAGACTCAGTGG (SEQ ID NO: 140)	1	1	24
(N20)NGG	136872508	136872509	1	1001	ATGTCCACCTCGCTTTCCTTTCG (SEQ ID NO: 141)	1	1	12
(N20)NGG	136872513	136872514	1	996	CACCTCGCTTTCCTTTGGAGAGG (SEQ ID NO: 142)	1	2	12
(N20)NGG	136872522	136872523	1	987	TTCCTTTGGAGAGGATCTTGAGG (SEQ ID NO: 143)	1	4	31
(N20)NGG	136872526	136872527	1	983	TTTGGAGAGGATCTTGAGGCTGG (SEQ ID NO: 144)	1	2	16
(N20)NGG	136872544	136872545	1	965	GCTGGACCCTCTGCTCACAGAGG (SEQ ID NO: 145)	1	3	38
(N20)NGG	136872558	136872559	1	951	TCACAGAGGTGAGTGCGTGCTGG (SEQ ID NO: 146)	1	1	38
(N20)NGG	136872559	136872560	1	950	CACAGAGGTGAGTGCGTGCTGGG (SEQ ID NO: 147)	1	1	26
(N20)NGG	136872565	136872566	1	944	GGTGAGTGCGTGCTGGGCAGAGG (SEQ ID NO: 148)	1	7	50
(N20)NGG	136872577	136872578	1	932	CTGGGCAGAGGTTTTAAATTTGG (SEQ ID NO: 149)	1	6	47
(N20)NGG	136872585	136872586	1	924	AGGTTTTAAATTTGGCTCCAAGG (SEQ ID NO: 150)	1	2	33
(N20)NGG	136872597	136872598	1	912	TGGCTCCAAGGAAAGCATAGAGG (SEQ ID NO: 151)	1	1	18
(N20)NGG	136872601	136872602	1	908	TCCAAGGAAAGCATAGAGGATGG (SEQ ID NO: 152)	1	5	45
(N20)NGG	136872602	136872603	1	907	CCAAGGAAAGCATAGAGGATGGG (SEQ ID NO: 153)	1	2	31

FIG. 2

site_type	site_start	site_end	site_strand	relative_start	site_sequence	genome wide hits with 1 mismatches	genome wide hits with 2 mismatches	genome wide hits with 3 mismatches
(N20)NGG	136872603	136872604	1	906	CAAGGAAAGCATAGAGGATGGGG (SEQ ID NO: 154)	1	3	53
(N20)NGG	136872618	136872619	1	891	GGATGGGGTTCAGACAACAGTGG (SEQ ID NO: 155)	1	2	16
(N20)NGG	136872630	136872631	1	879	GACAACAGTGGAAGAAAGCTAGG (SEQ ID NO: 156)	1	6	101
(N20)NGG	136872631	136872632	1	878	ACAACAGTGGAAGAAAGCTAGGG (SEQ ID NO: 157)	1	1	24
(N20)NGG	136872637	136872638	1	872	GTGGAAGAAAGCTAGGGCCTCGG (SEQ ID NO: 158)	1	3	37
(N20)NGG	136872643	136872644	1	866	GAAAGCTAGGGCCTCGGTGATGG (SEQ ID NO: 159)	1	1	8
(N20)NGG	136872699	136872700	1	810	ACCCTTGCTTGATGATTTCCAGG (SEQ ID NO: 160)	1	1	10
(N20)NGG	136872702	136872703	1	807	CTTGCTTGATGATTTCCAGGAGG (SEQ ID NO: 161)	1	3	26
(N20)NGG	136872709	136872710	1	800	GATGATTTCCAGGAGGATGAAGG (SEQ ID NO: 162)	1	29	73
(N20)NGG	136872737	136872738	1	772	ATGCTGATCCCAATGTAGTAAGG (SEQ ID NO: 163)	1	2	7
(N20)NGG	136872748	136872749	1	761	AATGTAGTAAGGCAGCCAACAGG (SEQ ID NO: 164)	1	1	9
(N20)NGG	136872762	136872763	1	747	GCCAACAGGCGAAGAAAGCCAGG (SEQ ID NO: 165)	1	1	5
(N20)NGG	136872768	136872769	1	741	AGGCGAAGAAAGCCAGGATGAGG (SEQ ID NO: 166)	2	4	38
(N20)NGG	136872778	136872779	1	731	AGCCAGGATGAGGATGACTGTGG (SEQ ID NO: 167)	1	4	38

FIG. 2 cont.

site_type	site_start	site_end	site_strand	relative_start	site_sequence	genome wide hits with 1 mismatches	genome wide hits with 2 mismatches	genome wide hits with 3 mismatches
(N20)NGG	136872786	136872787	1	723	TGAGGATGACTGTGGTCTTGAGG (SEQ ID NO: 168)	1	2	34
(N20)NGG	136872787	136872788	1	722	GAGGATGACTGTGGTCTTGAGGG (SEQ ID NO: 169)	1	2	20
(N20)NGG	136872801	136872802	1	708	TCTTGAGGGCCTTGCGCTTCTGG (SEQ ID NO: 170)	1	1	9
(N20)NGG	136872804	136872805	1	705	TGAGGGCCTTGCGCTTCTGGTGG (SEQ ID NO: 171)	1	2	12
(N20)NGG	136872811	136872812	1	698	CTTGCGCTTCTGGTGGCCCTTGG (SEQ ID NO: 172)	1	2	21
(N20)NGG	136872826	136872827	1	683	GCCCTTGGAGTGTGACAGCTTGG (SEQ ID NO: 173)	1	4	17
(N20)NGG	136872847	136872848	1	662	GGAGATGATAATGCAATAGCAGG (SEQ ID NO: 174)	1	1	16
(N20)NGG	136872852	136872853	1	657	TGATAATGCAATAGCAGGACAGG (SEQ ID NO: 175)	1	4	26
(N20)NGG	136872866	136872867	1	643	CAGGACAGGATGACAATACCAGG (SEQ ID NO: 176)	1	1	15
(N20)NGG	136872870	136872871	1	639	ACAGGATGACAATACCAGGCAGG (SEQ ID NO: 177)	1	3	16
(N20)NGG	136872876	136872877	1	633	TGACAATACCAGGCAGGATAAGG (SEQ ID NO: 178)	1	2	17
(N20)NGG	136872900	136872901	1	609	CAACCATGATGTGCTGAAACTGG (SEQ ID NO: 179)	1	1	13
(N20)NGG	136872925	136872926	1	584	CACAACCACCCACAAGTCATTGG (SEQ ID NO: 180)	1	2	23
(N20)NGG	136872926	136872927	1	583	ACAACCACCCACAAGTCATTGGG (SEQ ID NO: 181)	1	2	14

FIG. 2 cont.

site_type	site_start	site_end	site_strand	relative_start	site_sequence	genome wide hits with 1 mismatches	genome wide hits with 2 mismatches	genome wide hits with 3 mismatches
(N20)NGG	136872927	136872928	1	582	CAACCACCCACAAGTCATTGGGG (SEQ ID NO: 182)	1	1	6
(N20)NGG	136872936	136872937	1	573	ACAAGTCATTGGGGTAGAAGCGG (SEQ ID NO: 183)	1	2	60
(N20)NGG	136872973	136872974	1	536	GTCATCTGCCTCACTGACGTTGG (SEQ ID NO: 184)	1	1	24
(N20)NGG	136872988	136872989	1	521	GACGTTGGCAAAGATGAAGTCGG (SEQ ID NO: 185)	1	1	18
(N20)NGG	136872989	136872990	1	520	ACGTTGGCAAAGATGAAGTCGGG (SEQ ID NO: 186)	1	1	13
(N20)NGG	136873002	136873003	1	507	TGAAGTCGGGAATAGTCAGCAGG (SEQ ID NO: 187)	1	1	9
(N20)NGG	136873005	136873006	1	504	AGTCGGGAATAGTCAGCAGGAGG (SEQ ID NO: 188)	1	2	12
(N20)NGG	136873006	136873007	1	503	GTCGGGAATAGTCAGCAGGAGGG (SEQ ID NO: 189)	1	2	11
(N20)NGG	136873010	136873011	1	499	GGAATAGTCAGCAGGAGGGCAGG (SEQ ID NO: 190)	1	2	23
(N20)NGG	136873011	136873012	1	498	GAATAGTCAGCAGGAGGGCAGGG (SEQ ID NO: 191)	1	3	26
(N20)NGG	136873058	136873059	1	451	TTTTCAGCCAACAGCTTCCTTGG (SEQ ID NO: 192)	1	2	34
(N20)NGG	136873072	136873073	1	437	CTTCCTTGGCCTCTGACTGTTGG (SEQ ID NO: 193)	1	3	62
(N20)NGG	136873075	136873076	1	434	CCTTGGCCTCTGACTGTTGGTGG (SEQ ID NO: 194)	1	2	28
(N20)NGG	136873080	136873081	1	429	GCCTCTGACTGTTGGTGGCGTGG (SEQ ID NO: 195)	1	1	16

FIG. 2 cont.

site_type	site_start	site_end	site_strand	relative_start	site_sequence	genome wide hits with **1** mismatches	genome wide hits with 2 mismatches	genome wide hits with 3 mismatches
(N20)NGG	136873087	136873088	1	422	ACTGTTGGTGGCGTGGACGATGG (SEQ ID NO: 196)	1	1	12
(N20)NGG	136873092	136873093	1	417	TGGTGGCGTGGACGATGGCCAGG (SEQ ID NO: 197)	1	1	14
(N20)NGG	136873098	136873099	1	411	CGTGGACGATGGCCAGGTAGCGG (SEQ ID NO: 198)	2	5	19
(N20)NGG	136873114	136873115	1	395	GTAGCGGTCCAGACTGATGAAGG (SEQ ID NO: 199)	1	2	10
(N20)NGG	136873119	136873120	1	390	GGTCCAGACTGATGAAGGCCAGG (SEQ ID NO: 200)	2	2	12
(N20)NGG	136873125	136873126	1	384	GACTGATGAAGGCCAGGATGAGG (SEQ ID NO: 201)	1	2	39
(N20)NGG	136873140	136873141	1	369	GGATGAGGACACTGCTGTAGAGG (SEQ ID NO: 202)	1	4	35
(N20)NGG	136873161	136873162	1	348	GGTTGACTGTGTAGATGACATGG (SEQ ID NO: 203)	2	2	17
(N20)NGG	136873176	136873177	1	333	TGACATGGACTGCCTTGCATAGG (SEQ ID NO: 204)	1	2	14
(N20)NGG	136873204	136873205	1	305	CCCAAAGTACCAGTTTGCCACGG (SEQ ID NO: 205)	1	1	17
(N20)NGG	136873222	136873223	1	287	CACGGCATCAACTGCCCAGAAGG (SEQ ID NO: 206)	1	1	14
(N20)NGG	136873223	136873224	1	286	ACGGCATCAACTGCCCAGAAGGG (SEQ ID NO: 207)	1	1	10
(N20)NGG	136873242	136873243	1	267	AGGGAAGCGTGATGACAAAGAGG (SEQ ID NO: 208)	1	2	23
(N20)NGG	136873245	136873246	1	264	GAAGCGTGATGACAAAGAGGAGG (SEQ ID NO: 209)	1	1	13

FIG. 2 cont.

site_type	site_start	site_end	site_strand	relative_start	site_sequence	genome wide hits with 1 mismatches	genome wide hits with 2 mismatches	genome wide hits with 3 mismatches
(N20)NGG	136873249	136873250	1	260	CGTGATGACAAAGAGGAGGTCGG (SEQ ID NO: 210)	1	2	23
(N20)NGG	136873260	136873261	1	249	AGAGGAGGTCGGCCACTGACAGG (SEQ ID NO: 211)	1	1	17
(N20)NGG	136873302	136873303	1	207	TCATGCTTCTCAGTTTCTTCTGG (SEQ ID NO: 212)	1	3	72
(N20)NGG	136873317	136873318	1	192	TCTTCTGGTAACCCATGACCAGG (SEQ ID NO: 213)	1	1	9
(N20)NGG	136873360	136873361	1	149	AATGCCAGTTAAGAAGATGATGG (SEQ ID NO: 214)	1	2	34
(N20)NGG	136873369	136873370	1	140	TAAGAAGATGATGGAGTAGATGG (SEQ ID NO: 215)	1	8	62
(N20)NGG	136873372	136873373	1	137	GAAGATGATGGAGTAGATGGTGG (SEQ ID NO: 216)	1	5	81
(N20)NGG	136873373	136873374	1	136	AAGATGATGGAGTAGATGGTGGG (SEQ ID NO: 217)	1	1	34
(N20)NGG	136873377	136873378	1	132	TGATGGAGTAGATGGTGGGCAGG (SEQ ID NO: 218)	1	6	34
(N20)NGG	136873410	136873411	1	99	TGAAATTAGCATTTTCTTCACGG (SEQ ID NO: 219)	1	7	109
(N20)NGG	136873417	136873418	1	92	AGCATTTTCTTCACGGAAACAGG (SEQ ID NO: 220)	1	2	16
(N20)NGG	136873418	136873419	1	91	GCATTTTCTTCACGGAAACAGGG (SEQ ID NO: 221)	1	3	29
(N20)NGG	136873429	136873430	1	80	ACGGAAACAGGGTTCCTTCATGG (SEQ ID NO: 222)	1	3	14
(N20)NGG	136873459	136873460	1	50	GTCCCCTGAGCCCATTTCCTCGG (SEQ ID NO: 223)	1	4	38

FIG. 2 cont.

site_type	site_start	site_end	site_strand	relative_start	site_sequence	genome wide hits with 1 mismatches	genome wide hits with 2 mismatches	genome wide hits with 3 mismatches
(N20)NGG	136873493	136873494	1	16	GAAGTGTATATCTGCAAAAGAGG (SEQ ID NO: 224)	1	2	24
(N20)NGG	136873499	136873500	1	10	TATATCTGCAAAAGAGGCAAAGG (SEQ ID NO: 225)	1	6	36
(N20)NGG	136873504	136873505	1	5	CTGCAAAAGAGGCAAAGGAATGG (SEQ ID NO: 226)	1	7	92
(N20)NGG	136872490	136872491	-1	1019	CTCTCCAAAGGAAAGCGAGGTGG (SEQ ID NO: 227)	1	1	20
(N20)NGG	136872493	136872494	-1	1016	ATCCTCTCCAAAGGAAAGCGAGG (SEQ ID NO: 228)	1	3	13
(N20)NGG	136872502	136872503	-1	1007	AGCCTCAAGATCCTCTCCAAAGG (SEQ ID NO: 229)	1	2	15
(N20)NGG	136872528	136872529	-1	981	CACTCACCTCTGTGAGCAGAGGG (SEQ ID NO: 230)	1	13	91
(N20)NGG	136872529	136872530	-1	980	GCACTCACCTCTGTGAGCAGAGG (SEQ ID NO: 231)	1	7	69
(N20)NGG	136872580	136872581	-1	929	CCCATCCTCTATGCTTTCCTTGG (SEQ ID NO: 232)	1	3	38
(N20)NGG	136872632	136872633	-1	877	CAAGTGGATTTCCATCACCGAGG (SEQ ID NO: 233)	1	3	9
(N20)NGG	136872648	136872649	-1	861	TTGAGAACACTGTGCACAAGTGG (SEQ ID NO: 234)	1	1	15
(N20)NGG	136872678	136872679	-1	831	TCCTGGAAATCATCAAGCAAGGG (SEQ ID NO: 235)	1	2	24
(N20)NGG	136872679	136872680	-1	830	CTCCTGGAAATCATCAAGCAAGG (SEQ ID NO: 236)	1	2	30
(N20)NGG	136872695	136872696	-1	814	CATCGACTCCTTCATCCTCCTGG (SEQ ID NO: 237)	1	2	12

FIG. 2 cont.

site_type	site_start	site_end	site_strand	relative_start	site_sequence	genome wide hits with **1** mismatches	genome wide hits with 2 mismatches	genome wide hits with 3 mismatches
(N20)NGG	136872723	136872724	-1	786	GTTGGCTGCCTTACTACATTGGG (SEQ ID NO: 238)	1	3	19
(N20)NGG	136872724	136872725	-1	785	TGTTGGCTGCCTTACTACATTGG (SEQ ID NO: 239)	1	3	12
(N20)NGG	136872741	136872742	-1	768	TCCTGGCTTTCTTCGCCTGTTGG (SEQ ID NO: 240)	1	2	15
(N20)NGG	136872758	136872759	-1	751	GACCACAGTCATCCTCATCCTGG (SEQ ID NO: 241)	1	2	24
(N20)NGG	136872788	136872789	-1	721	CAAGGGCCACCAGAAGCGCAAGG (SEQ ID NO: 242)	1	1	16
(N20)NGG	136872805	136872806	-1	704	TCCAAGCTGTCACACTCCAAGGG (SEQ ID NO: 243)	1	3	15
(N20)NGG	136872806	136872807	-1	703	CTCCAAGCTGTCACACTCCAAGG (SEQ ID NO: 244)	1	1	15
(N20)NGG	136872862	136872863	-1	647	ATGGTTGGCCTTATCCTGCCTGG (SEQ ID NO: 245)	1	1	13
(N20)NGG	136872877	136872878	-1	632	CAGTTTCAGCACATCATGGTTGG (SEQ ID NO: 246)	1	3	9
(N20)NGG	136872881	136872882	-1	628	GTTCCAGTTTCAGCACATCATGG (SEQ ID NO: 247)	1	4	46
(N20)NGG	136872908	136872909	-1	601	CTACCCCAATGACTTGTGGGTGG (SEQ ID NO: 248)	1	1	7
(N20)NGG	136872911	136872912	-1	598	CTTCTACCCCAATGACTTGTGGG (SEQ ID NO: 249)	1	1	12
(N20)NGG	136872912	136872913	-1	597	GCTTCTACCCCAATGACTTGTGG (SEQ ID NO: 250)	1	1	8
(N20)NGG	136872959	136872960	-1	550	CATCTTTGCCAACGTCAGTGAGG (SEQ ID NO: 251)	1	1	12

FIG. 2 cont.

site_type	site_start	site_end	site_strand	relative_start	site_sequence	genome wide hits with **1** mismatches	genome wide hits with 2 mismatches	genome wide hits with 3 mismatches
(N20)NGG	136873014	136873015	-1	495	AGGTGGTCTATGTTGGCGTCTGG (SEQ ID NO: 252)	1	1	3
(N20)NGG	136873021	136873022	-1	488	GCTGAAAAGGTGGTCTATGTTGG (SEQ ID NO: 253)	1	1	16
(N20)NGG	136873031	136873032	-1	478	GAAGCTGTTGGCTGAAAAGGTGG (SEQ ID NO: 254)	1	3	52
(N20)NGG	136873034	136873035	-1	475	AAGGAAGCTGTTGGCTGAAAAGG (SEQ ID NO: 255)	2	6	42
(N20)NGG	136873043	136873044	-1	466	TCAGAGGCCAAGGAAGCTGTTGG (SEQ ID NO: 256)	1	4	56
(N20)NGG	136873053	136873054	-1	456	CCACCAACAGTCAGAGGCCAAGG (SEQ ID NO: 257)	1	2	34
(N20)NGG	136873059	136873060	-1	450	TCCACGCCACCAACAGTCAGAGG (SEQ ID NO: 258)	1	1	15
(N20)NGG	136873088	136873089	-1	421	CATCAGTCTGGACCGCTACCTGG (SEQ ID NO: 259)	1	5	26
(N20)NGG	136873100	136873101	-1	409	CATCCTGGCCTTCATCAGTCTGG (SEQ ID NO: 260)	1	2	19
(N20)NGG	136873115	136873116	-1	394	CTACAGCAGTGTCCTCATCCTGG (SEQ ID NO: 261)	1	2	30
(N20)NGG	136873166	136873167	-1	343	CTTTGGGAACTTCCTATGCAAGG (SEQ ID NO: 262)	1	4	22
(N20)NGG	136873182	136873183	-1	327	CCGTGGCAAACTGGTACTTTGGG (SEQ ID NO: 263)	1	1	6
(N20)NGG	136873183	136873184	-1	326	GCCGTGGCAAACTGGTACTTTGG (SEQ ID NO: 264)	1	1	6
(N20)NGG	136873191	136873192	-1	318	CAGTTGATGCCGTGGCAAACTGG (SEQ ID NO: 265)	1	1	6

FIG. 2 cont.

site_type	site_start	site_end	site_strand	relative_start	site_sequence	genome wide hits with **1** mismatches	genome wide hits with 2 mismatches	genome wide hits with 3 mismatches
(N20)NGG	136873199	136873200	-1	310	CTTCTGGGCAGTTGATGCCGTGG (SEQ ID NO: 266)	1	1	8
(N20)NGG	136873214	136873215	-1	295	TGTCATCACGCTTCCCTTCTGGG (SEQ ID NO: 267)	1	1	11
(N20)NGG	136873215	136873216	-1	294	TTGTCATCACGCTTCCCTTCTGG (SEQ ID NO: 268)	1	1	7
(N20)NGG	136873250	136873251	-1	259	GTACAGGCTGCACCTGTCAGTGG (SEQ ID NO: 269)	1	2	12
(N20)NGG	136873266	136873267	-1	243	GAAGCATGACGGACAAGTACAGG (SEQ ID NO: 270)	1	1	6
(N20)NGG	136873277	136873278	-1	232	GAAGAAACTGAGAAGCATGACGG (SEQ ID NO: 271)	1	5	90
(N20)NGG	136873306	136873307	-1	203	GGATTGGTCATCCTGGTCATGGG (SEQ ID NO: 272)	1	1	20
(N20)NGG	136873307	136873308	-1	202	TGGATTGGTCATCCTGGTCATGG (SEQ ID NO: 273)	1	1	13
(N20)NGG	136873313	136873314	-1	196	GGGCAATGGATTGGTCATCCTGG (SEQ ID NO: 274)	1	1	8
(N20)NGG	136873322	136873323	-1	187	TGGCATTGTGGGCAATGGATTGG (SEQ ID NO: 275)	1	1	23
(N20)NGG	136873327	136873328	-1	182	TTAACTGGCATTGTGGGCAATGG (SEQ ID NO: 276)	1	2	14
(N20)NGG	136873333	136873334	-1	176	ATCTTCTTAACTGGCATTGTGGG (SEQ ID NO: 277)	1	1	18
(N20)NGG	136873334	136873335	-1	175	CATCTTCTTAACTGGCATTGTGG (SEQ ID NO: 278)	1	3	15
(N20)NGG	136873342	136873343	-1	167	TACTCCATCATCTTCTTAACTGG (SEQ ID NO: 279)	1	2	22

FIG. 2 cont.

site_type	site_start	site_end	site_strand	relative_start	site_sequence	genome wide hits with **1** mismatches	genome wide hits with 2 mismatches	genome wide hits with 3 mismatches
(N20)NGG	136873421	136873422	-1	88	AGGGGACTATGACTCCATGAAGG (SEQ ID NO: 280)	1	2	10
(N20)NGG	136873439	136873440	-1	70	CACCGAGGAAATGGGCTCAGGGG (SEQ ID NO: 281)	2	2	19
(N20)NGG	136873440	136873441	-1	69	ACACCGAGGAAATGGGCTCAGGG (SEQ ID NO: 282)	1	1	16
(N20)NGG	136873441	136873442	-1	68	TACACCGAGGAAATGGGCTCAGG (SEQ ID NO: 283)	1	1	11
(N20)NGG	136873447	136873448	-1	62	GATAACTACACCGAGGAAATGGG (SEQ ID NO: 284)	1	2	6
(N20)NGG	136873448	136873449	-1	61	AGATAACTACACCGAGGAAATGG (SEQ ID NO: 285)	1	2	11
(N20)NGG	136873454	136873455	-1	55	CACTTCAGATAACTACACCGAGG (SEQ ID NO: 286)	1	1	6
(N21)NNAGAAW	136872624	136872625	1	885	GATGGGGTTCAGACAACAGTGGAAGAA A (SEQ ID NO: 287)	1	1	2
(N21)NNAGAAW	136872756	136872757	1	753	GTAGTAAGGCAGCCAACAGGCGAAGAA A (SEQ ID NO: 288)	1	1	1
(N21)NNAGAAW	136872933	136872934	1	576	AACCACCCACAAGTCATTGGGGTAGAA G (SEQ ID NO: 289)	1	1	1
(N21)NNAGAAW	136873221	136873222	1	288	GTTTGCCACGGCATCAACTGCCCAGAAG (SEQ ID NO: 290)	1	1	2
(N21)NNAGAAW	136873353	136873354	1	156	TCCATTGCCCACAATGCCAGTTAAGAAG (SEQ ID NO: 291)	1	1	1
BTTCTNN(N21)	136872663	136872664	-1	846	CATCAAGCAAGGGTGTGAGTTTGAGAA C (SEQ ID NO: 292)	1	1	2
BTTCTNN(N21)	136872795	136872796	-1	714	GCTGTCACACTCCAAGGGCCACCAGAAG (SEQ ID NO: 293)	1	1	2

FIG. 2 cont.

site_type	site_start	site_end	site_strand	relative_start	site_sequence	genome wide hits with **1** mismatches	genome wide hits with 2 mismatches	genome wide hits with 3 mismatches
BTTCTNN(N21)	136873285	136873286	-1	224	TCATGGGTTACCAGAAGAAACTGAGAA G (SEQ ID NO: 294)	1	1	2
BTTCTNN(N21)	136873293	136873294	-1	216	CATCCTGGTCATGGGTTACCAGAAGAAA (SEQ ID NO: 295)	1	1	1
BTTCTNN(N21)	136873296	136873297	-1	213	GGTCATCCTGGTCATGGGTTACCAGAAG (SEQ ID NO: 296)	1	1	1
BTTCTNN(N21)	136873400	136873401	-1	109	ATGAAGGAACCCTGTTTCCGTGAAGAAA (SEQ ID NO: 297)	1	1	1

FIG. 2 cont.

1 MDKKYSIGLD IGTNSVGWAV ITDEYKVPSK KFKVLGNTDR HSIKKNLIGA LLFDSGETAE

61 ATRLKRTARR RYTRRKNRIC YLQEIFSNEM AKVDDSFFHR LEESFLVEED KKHERHPIFG

121 NIVDEVAYHE KYPTIYHLRK KLVDSTDKAD LRLIYLALAH MIKFRGHFLI EGDLNPDNSD

181 VDKLFIQLVQ TYNQLFEENP INASGVDAKA ILSARLSKSR RLENLIAQLP GEKKNGLFGN

241 LIALSLGLTP NFKSNFDLAE DAKLQLSKDT YDDDLDNLLA QIGDQYADLF LAAKNLSDAI

301 LLSDILRVNT EITKAPLSAS MIKRYDEHHQ DLTLLKALVR QQLPEKYKEI FFDQSKNGYA

361 GYIDGGASQE EFYKFIKPIL EKMDGTEELL VKLNREDLLR KQRTFDNGSI PHQIHLGELH

421 AILRRQEDFY PFLKDNREKI EKILTFRIPY YVGPLARGNS RFAWMTRKSE ETITPWNFEE

481 VVDKGASAQS FIERMTNFDK NLPNEKVLPK HSLLYEYFTV YNELTKVKYV TEGMRKPAFL

541 SGEQKKAIVD LLFKTNRKVT VKQLKEDYFK KIECFDSVEI SGVEDRFNAS LGTYHDLLKI

601 IKDKDFLDNE ENEDILEDIV LTLTLFEDRE MIEERLKTYA HLFDDKVMKQ LKRRRYTGWG

661 RLSRKLINGI RDKQSGKTIL DFLKSDGFAN RNFMQLIHDD SLTFKEDIQK AQVSGQGDSL

721 HEHIANLAGS PAIKKGILQT VKVVDELVKV MGRHKPENIV IEMARENQTT QKGQKNSRER

781 MKRIEEGIKE LGSQILKEHP VENTQLQNEK LYLYYLQNGR DMYVDQELDI NRLSDYDVDH

841 IVPQSFLKDD SIDNKVLTRS DKNRGKSDNV PSEEVVKKMK NYWRQLLNAK LITQRKFDNL

901 TKAERGGLSE LDKAGFIKRQ LVETRQITKH VAQILDSRMN TKYDENDKLI REVKVITLKS

961 KLVSDFRKDF QFYKVREINN YHHAHDAYLN AVVGTALIKK YPKLESEFVY GDYKVYDVRK

1021 MIAKSEQEIG KATAKYFFYS NIMNFFKTEI TLANGEIRKR PLIETNGETG EIVWDKGRDF

1081 ATVRKVLSMP QVNIVKKTEV QTGGFSKESI LPKRNSDKLI ARKKDWDPKK YGGFDSPTVA

1141 YSVLVVAKVE KGKSKKLKSV KELLGITIME RSSFEKNPID FLEAKGYKEV KKDLIIKLPK

1201 YSLFELENGR KRMLASAGEL QKGNELALPS KYVNFLYLAS HYEKLKGSPE DNEQKQLFVE

1261 QHKHYLDEII EQISEFSKRV ILADANLDKV LSAYNKHRDK PIREQAENII HLFTLTNLGA

1321 PAAFKYFDTT IDRKRYTSTK EVLDATLIHQ SITGLYETRI DLSQLGGD (SEQ ID NO: 298)

FIG. 3

Guide ID	Target Site Sequence With NGG	Score
crCCR5_F	GTAGAGCGGAGGCAGGAGGCGGG (SEQ ID NO: 304)	16
crCCR5_G	GTGAGTAGAGCGGAGGCAGGAGG (SEQ ID NO: 305)	32
crCCR5_H	GGTGTTCATCTTTGGTTTTGTGG (SEQ ID NO: 306)	26
crCCR5_I	GTGTTCATCTTTGGTTTTGTGGG (SEQ ID NO: 307)	26
crCCR5_J	GGACAGTAAGAAGGAAAAACAGG (SEQ ID NO: 308)	16
crCCR5_A	GCTGCCGCCCAGTGGGACTTTGG (SEQ ID NO: 309)	64
crCCR5_K	GCAGCATAGTGAGCCCAGAAGGG (SEQ ID NO: 310)	41
crCCR5_L	GGCAGCATAGTGAGCCCAGAAGG (SEQ ID NO: 311)	38
crCCR5_M	GGTACCTATCGATTGTCAGGAGG (SEQ ID NO: 312)	45
crCCR5_N	GTTTGCTTTAAAAGCCAGGACGG (SEQ ID NO: 313)	21
crCCR5_O	GGTGACAAGTGTGATCACTTGGG (SEQ ID NO: 314)	56
crCCR5_P	GACAAGTGTGATCACTTGGGTGG (SEQ ID NO: 315)	61
crCCR5_Q	GCTGTGTTTGCGTCTCTCCCAGG (SEQ ID NO: 316)	53
crCCR5_B	GATCTGGTAAAGATGATTCCTGG (SEQ ID NO: 317)	55
crCCR5_R	GTATGGAAAATGAGAGCTGCAGG (SEQ ID NO: 318)	43
crCCR5_S	GACATTAAAGATAGTCATCTTGG (SEQ ID NO: 319)	50
crCCR5_T	GGTCCTGCCGCTGCTTGTCATGG (SEQ ID NO: 320)	55
crCCR5_U	GTCATGGTCATCTGCTACTCGGG (SEQ ID NO: 321)	38
crCCR5_V	GAATCCTAAAAACTCTGCTTCGG (SEQ ID NO: 322)	43
crCCR5_W	GGTGTCGAAATGAGAAGAAGAGG (SEQ ID NO: 323)	32
crCCR5_X	GACACCGAAGCAGAGTTTTTAGG (SEQ ID NO: 324)	59
crCCR5_Y	GAAATGAGAAGAAGAGGCACAGG (SEQ ID NO: 325)	23
crCCR5_1	GATTGTTTATTTTCTCTTCTGGG (SEQ ID NO: 326)	14
crCCR5_Z	GAGAAAATAAACAATCATGATGG (SEQ ID NO: 327)	14
crCCR5_2	GCTTTTGGAAGAAGACTAAGAGG (SEQ ID NO: 328)	34
crCCR5_3	GTAAACTGAGCTTGCTCGCTCGG (SEQ ID NO: 329)	80
crCCR5_4	GGGGAGCAGGAAATATCTGTGGG (SEQ ID NO: 330)	45
crCCR5_C	ACAATGTGTCAACTCTTGACAGG (SEQ ID NO: 331)	67
crCCR5_D	TCACTATGCTGCCGCCCAGTGGG (SEQ ID NO: 332)	81
crCCR5_E	GGTACCTATCGATTGTCAGGAGG (SEQ ID NO: 333)	45

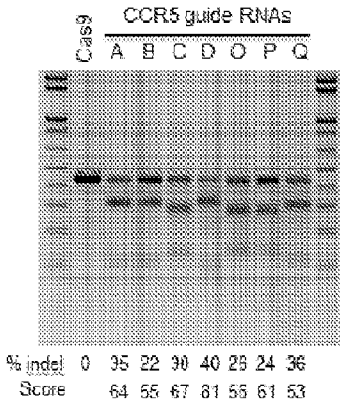
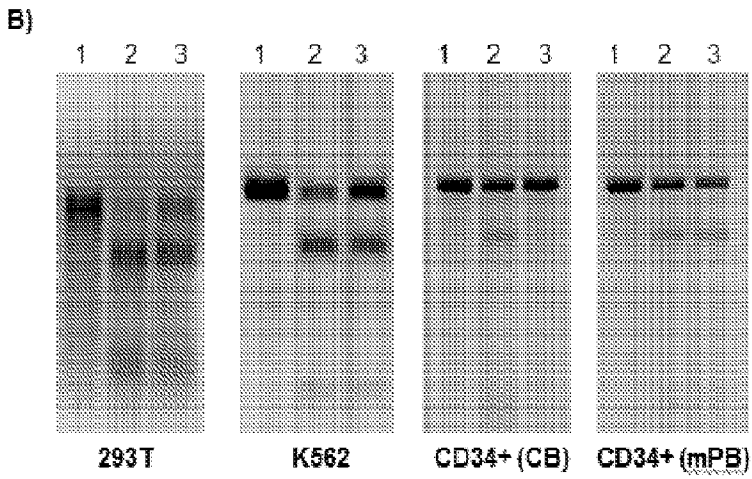
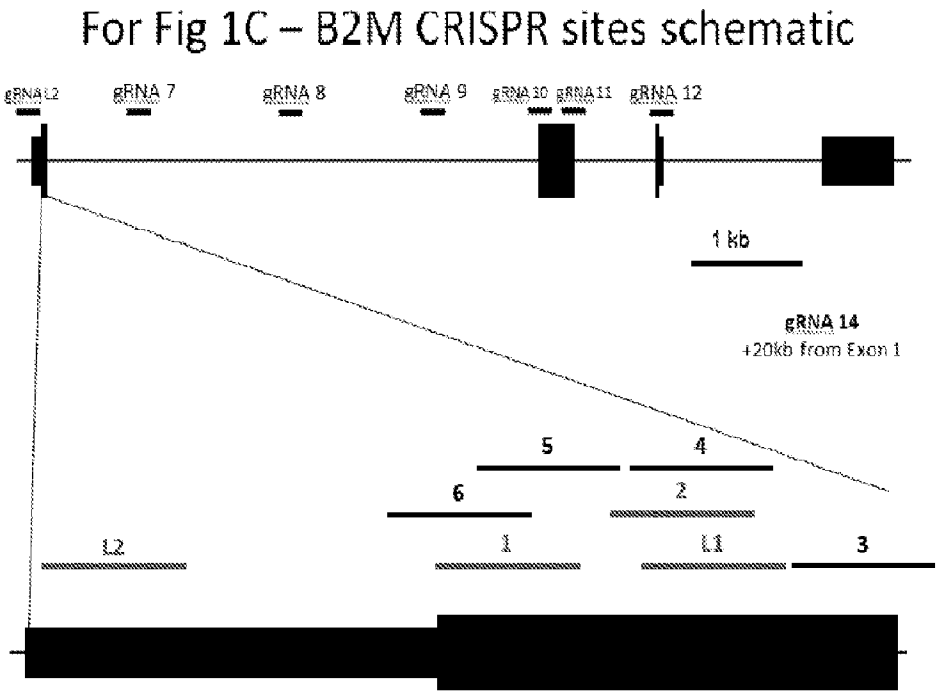
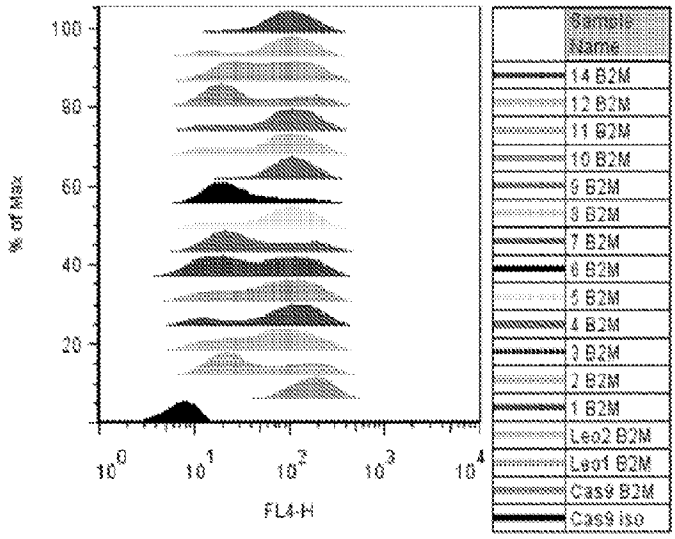


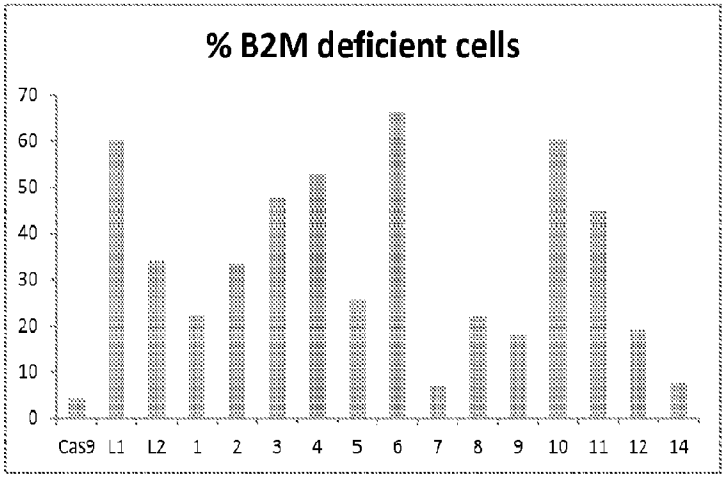
FIG. 4A


B)
1 2 3
1 2 3
1 2 3
1 2 3
293T
K562
CD34+ (CB)
CD34+ (mPB)


For Fig 1C – B2M CRISPR sites schematic
gRNA L2
gRNA 7
gRNA 8
gRNA 9
gRNA 10
gRNA 11
gRNA 12
1 kb
gRNA 14
+20kb from Exon 1
5
4
6
2
L2
1
L1
3

FIGS. 4B-4C





	% B2M deficient cells
Cas9	4.25
L1	60.12
L2	33.9
1	22.27
2	33.52
3	47.72
4	52.88
5	25.61
6	66.33
7	6.91
8	22.05
9	17.97
10	60.38
11	44.98
12	19.09
14	7.54

FIG. 4D

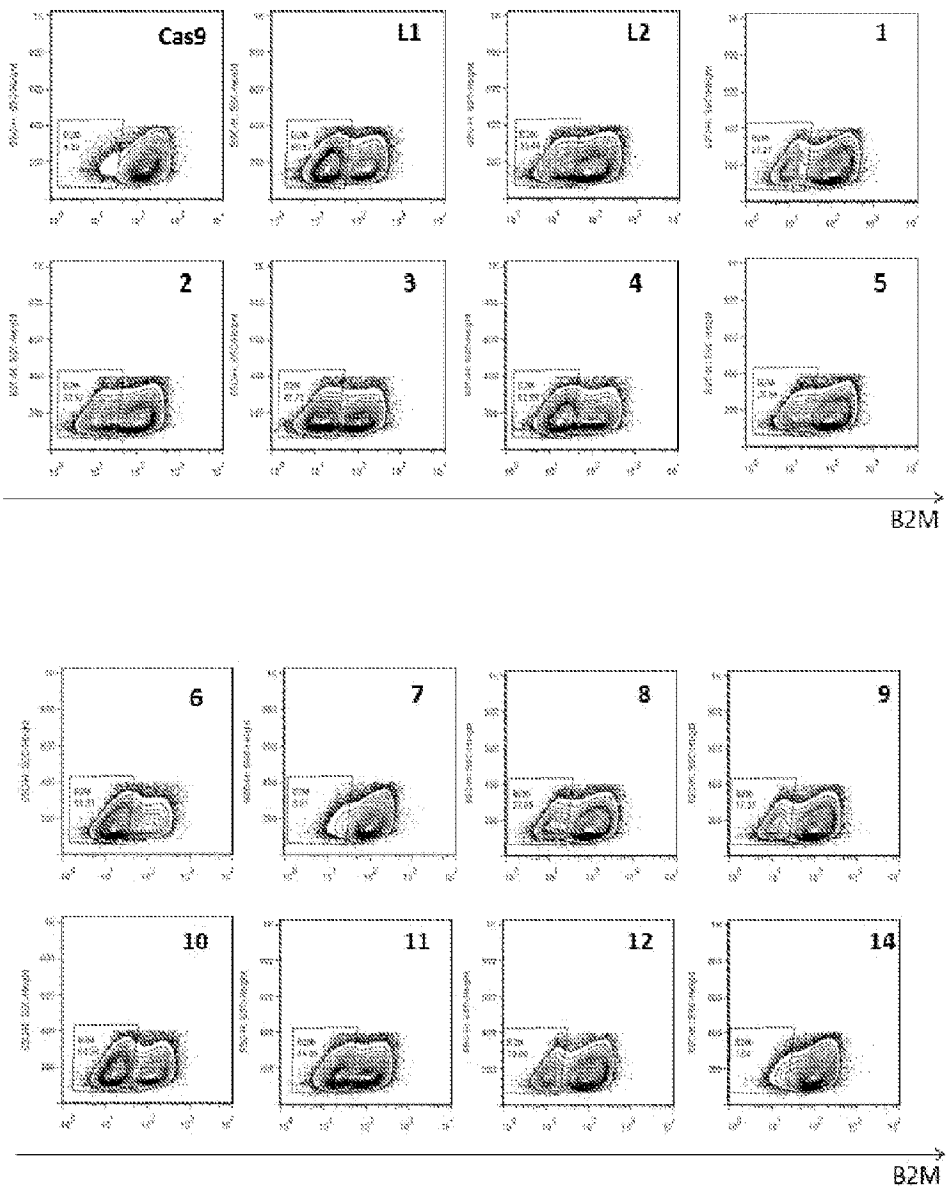
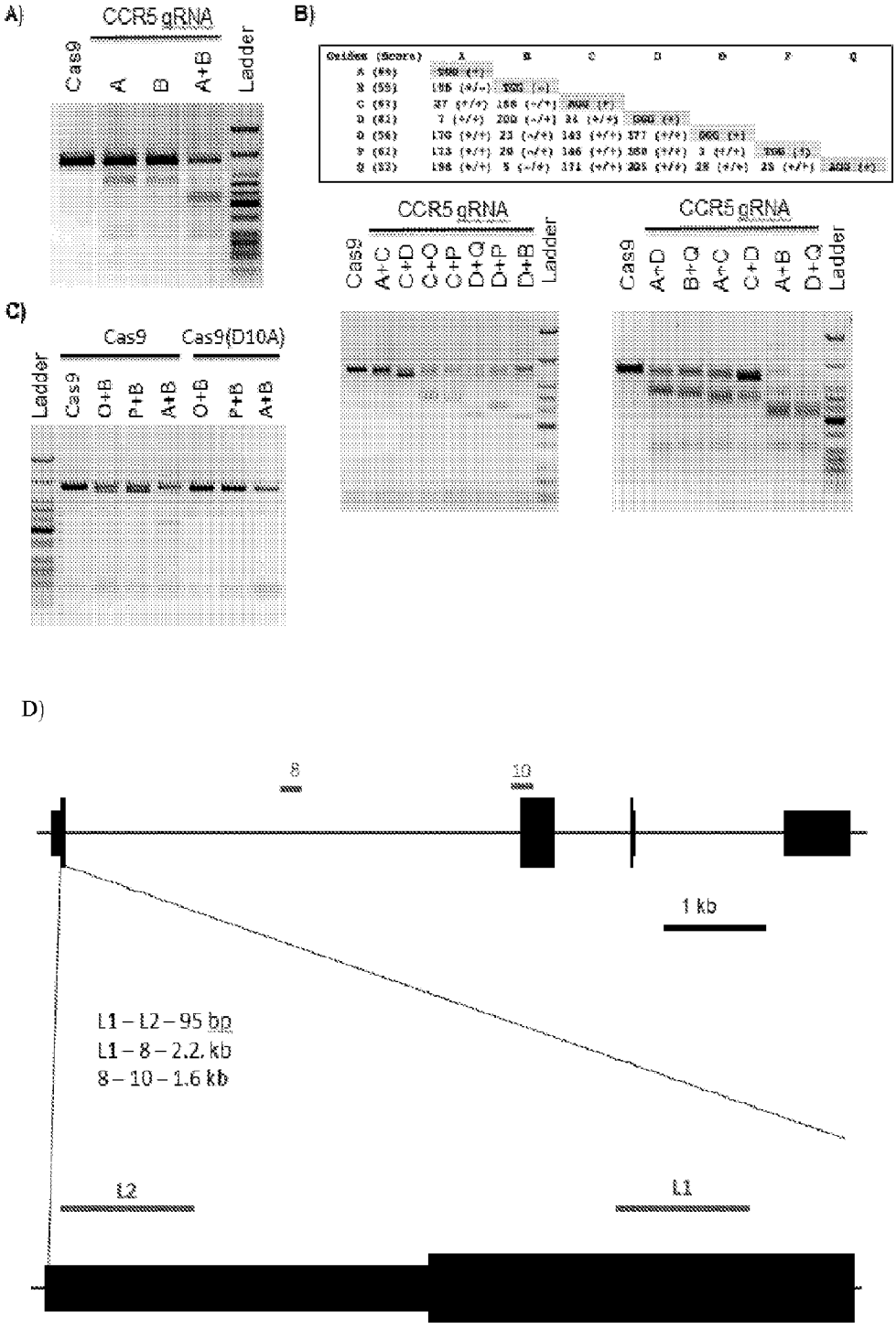
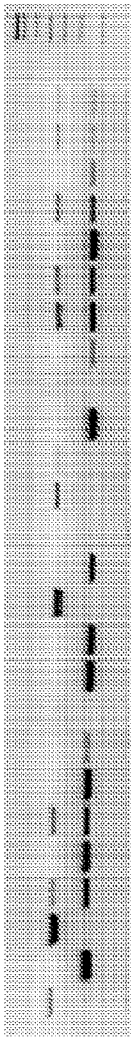


FIG. 4E


A)
CCR5 gRNA
Cas9
A
B
A+B
Ladder
B)
CCR5 gRNA
Cas9
A+C
C+D
C+Q
C+P
D+Q
D+P
D+B
Ladder
CCR5 gRNA
Cas9
A+D
B+Q
A+C
C+D
A+B
D+Q
Ladder
C)
Cas9
Cas9(D10A)
Ladder
Cas9
O+B
P+B
A+B
O+B
P+B
A+B
D)
8
10
1 kb
L1 – L2 – 95 bp
L1 – 8 – 2.2 kb
8 – 10 – 1.6 kb
L2
L1

FIGS. 5A-5D

A)

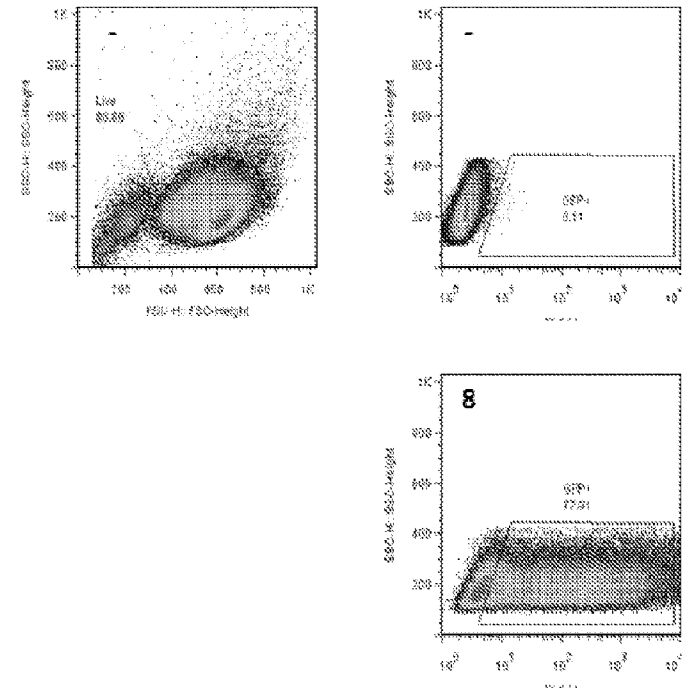


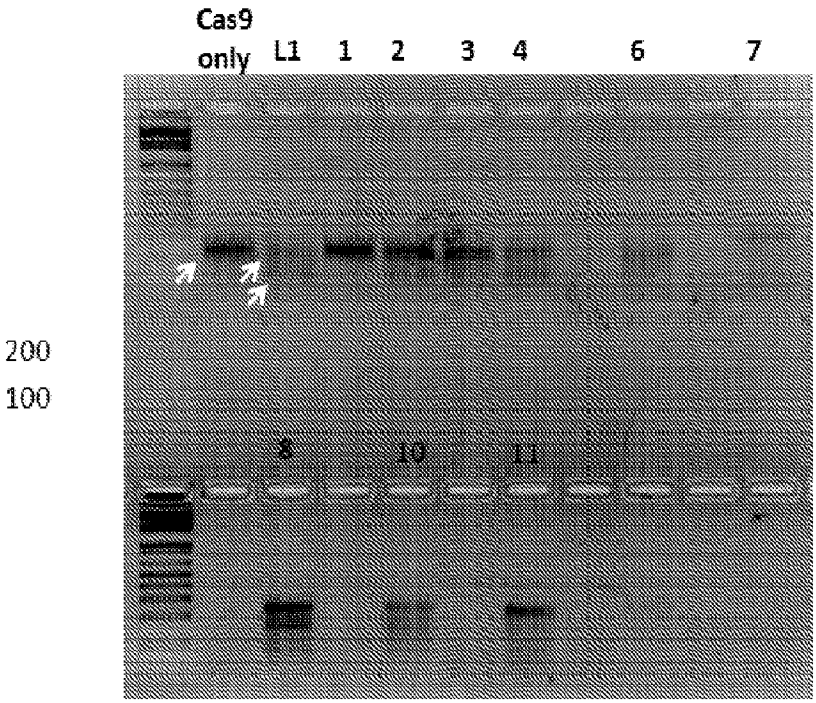
B)

Guide combination	Experiment # 1			Experiment # 2		
	Screened	Null (%)	Hets (%)	Screened	Null (%)	Hets (%)
A+B	87	8 (9)	10 (11.5)	96	8 (8)	26 (27)
D+Q	88	8 (9)	6 (6.8)	84	36 (42)	21 (25)
C+D	87	4 (4.6)	11 (12.6)	NA	-	-

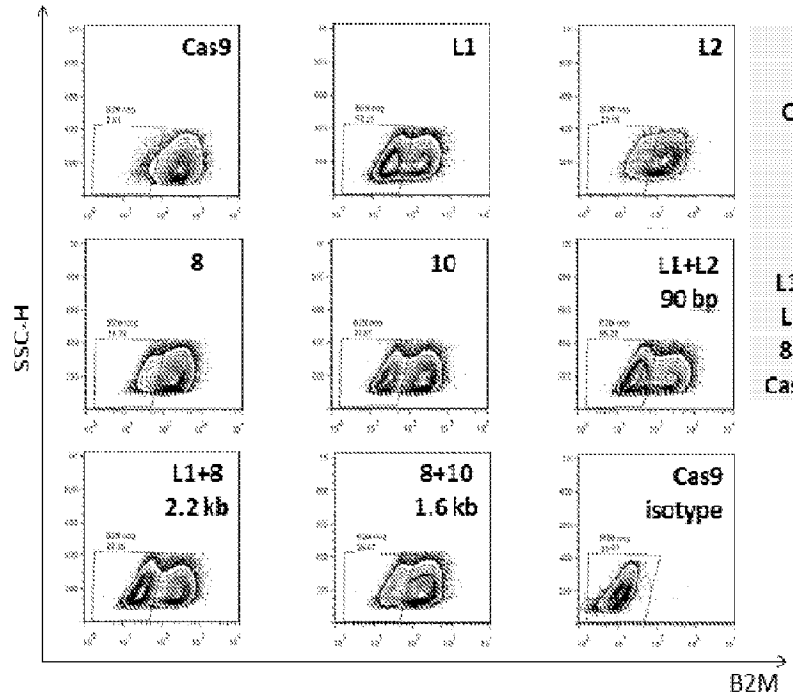
FIGS. 6A-6B

A





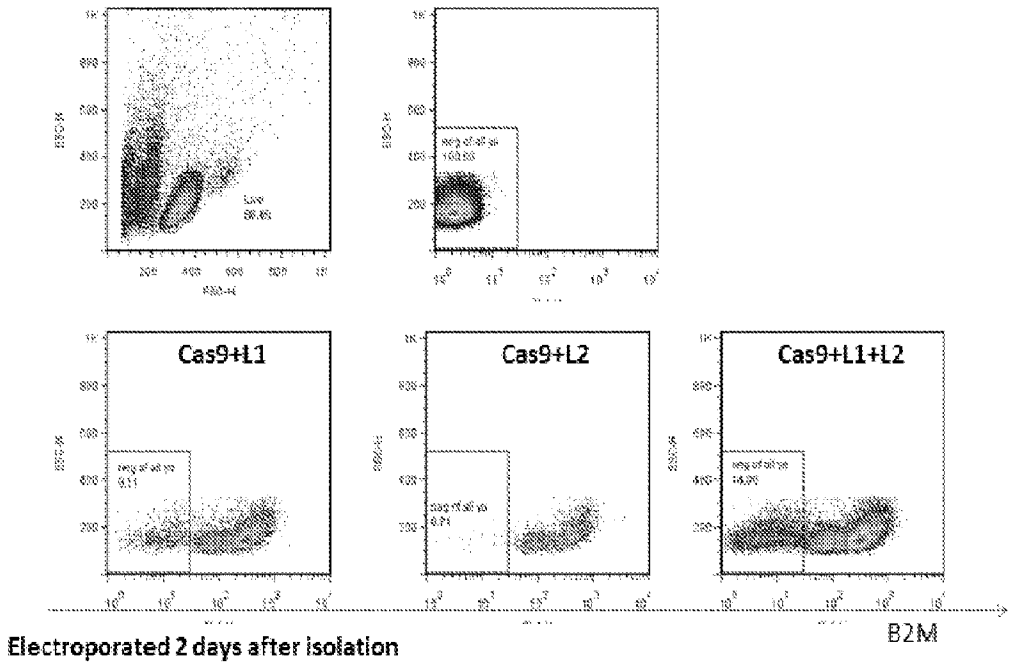
FIGS. 7A-7B



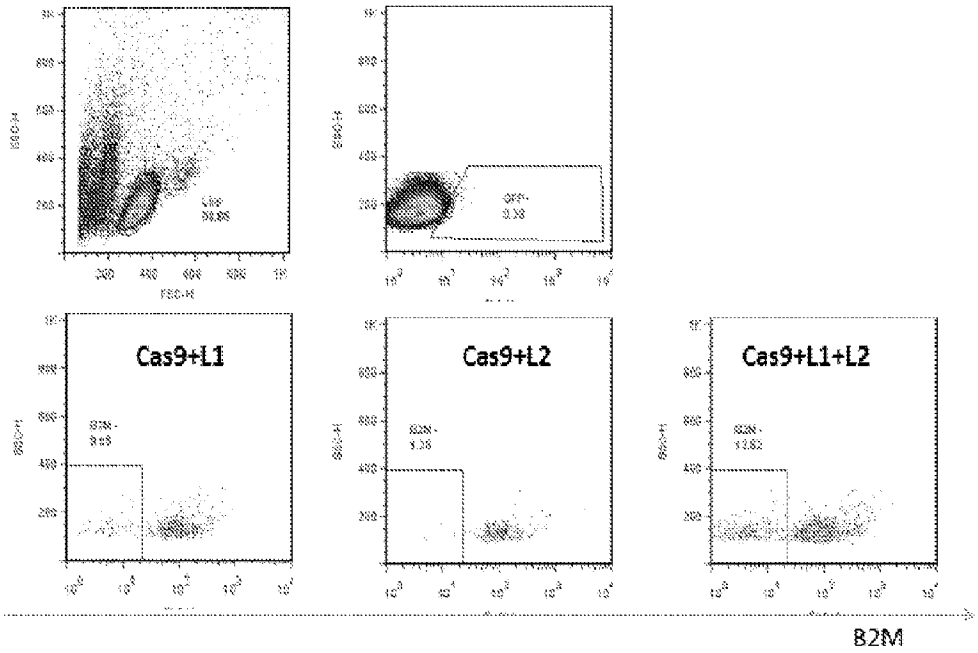
	% B2M neg
-	99.07
Cas9	2.63
L1	52.21
L2	29.56
8	14.34
10	33.87
L1+L2	59.21
L1+8	49.36
8+10	25.67
Cas9 Iso	99.87

FIG. 7C

A

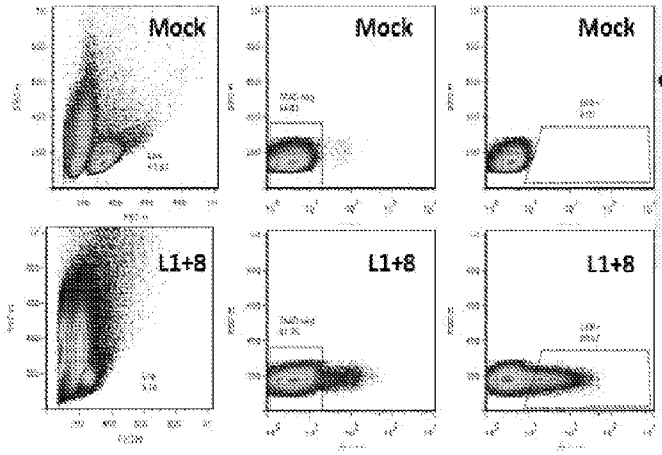

Cas9+L1
Cas9+L2
Cas9+L1+L2
B2M
Electroporated 2 days after isolation

B


Cas9+L1
Cas9+L2
Cas9+L1+L2
B2M

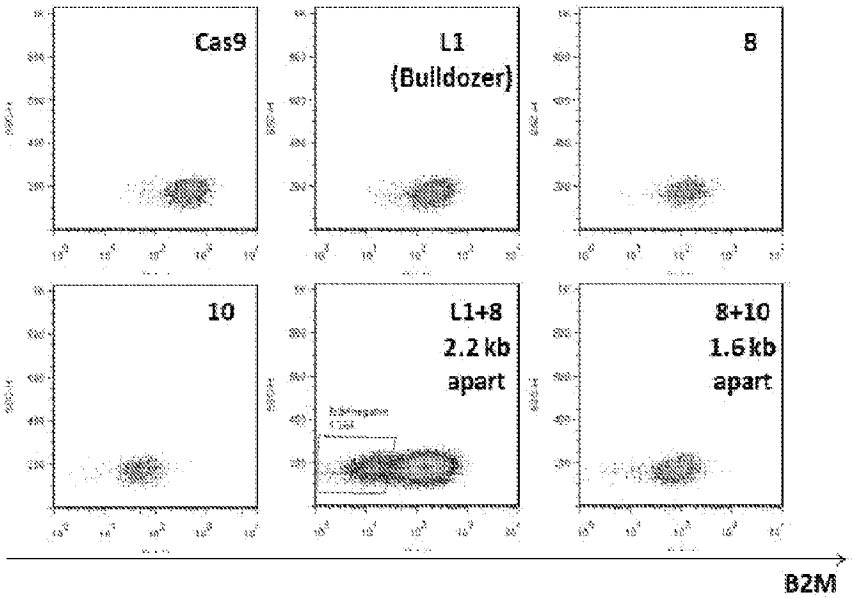
FIGS. 8A-8B

C



	% Live	% GFP+	# GFP+
Cas9	5.33	15.47	3110
Cas9 iso	1.1	26.4	1633
L1	1.67	25.41	4821
L1+8	9.1	20.87	31107
Mock	63.83	-	-
8	1.12	13.93	1902
8+10	1.17	23.85	3888
10	2.95	31.79	1610

D



Gated on Live/7AADneg/GFP+cells

FIGS. 8C-8D