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(54) **ENGINEERING AND OPTIMIZATION OF SYSTEMS, METHODS AND COMPOSITIONS FOR SEQUENCE MANIPULATION WITH FUNCTIONAL DOMAINS**

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(21) Appl. No.: **19/025,722**

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C12N 15/10	(2006.01)
C12N 15/85	(2006.01)
C12N 15/86	(2006.01)

(52) **U.S. Cl.**

CPC ***C12N 15/63*** (2013.01); ***C12N 7/00*** (2013.01); ***C12N 9/22*** (2013.01); ***C12N 15/01*** (2013.01); ***C12N 15/102*** (2013.01); ***C12N 15/85*** (2013.01); ***C12N 15/86*** (2013.01); *C12N 15/1082* (2013.01); *C12N 2320/30* (2013.01); *C12N 2740/15043* (2013.01)

(57) **ABSTRACT**

The invention provides for engineering and optimization of systems, methods, and compositions for manipulation of sequences and/or activities of target sequences. Provided are vectors and vector systems, some of which encode one or more components of a CRISPR complex, as well as methods for the design and use of such vectors with additional functional domains. Also provided are methods of directing CRISPIR complex formation in prokaryotic and eukaryotic cells to ensure enhanced specificity for target recognition and avoidance of toxicity.

Specification includes a Sequence Listing.

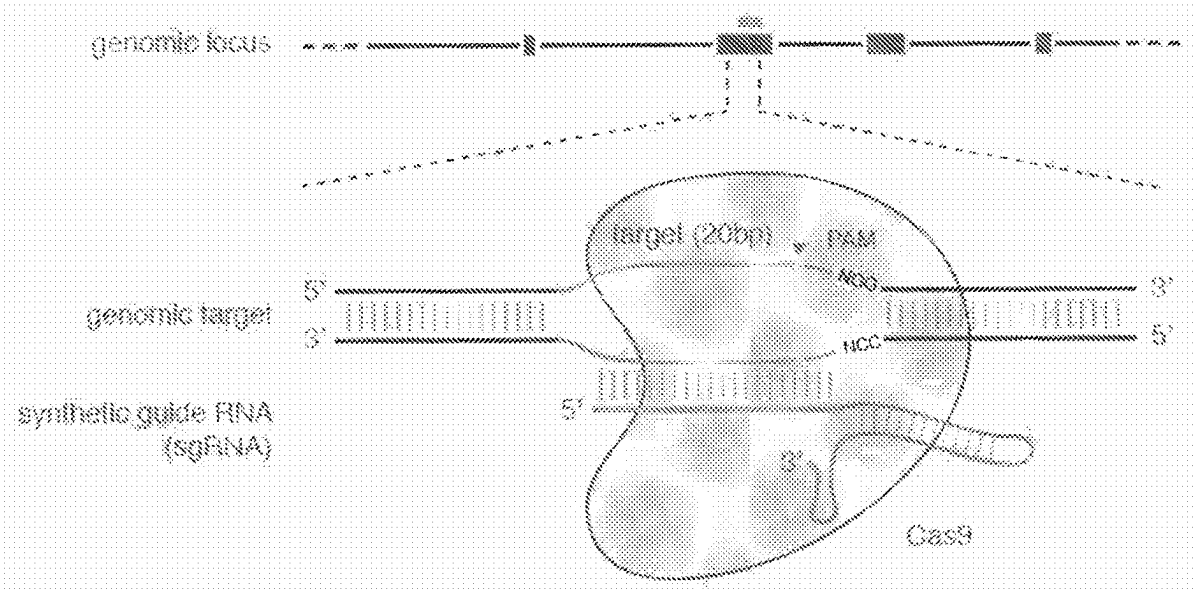

genomic locus
target (20bp)
PAM
NGG
NCC
genomic target
5'
3'
3'
5'
synthetic guide RNA
(sgRNA)
5'
3'
Cas9

FIG. 1

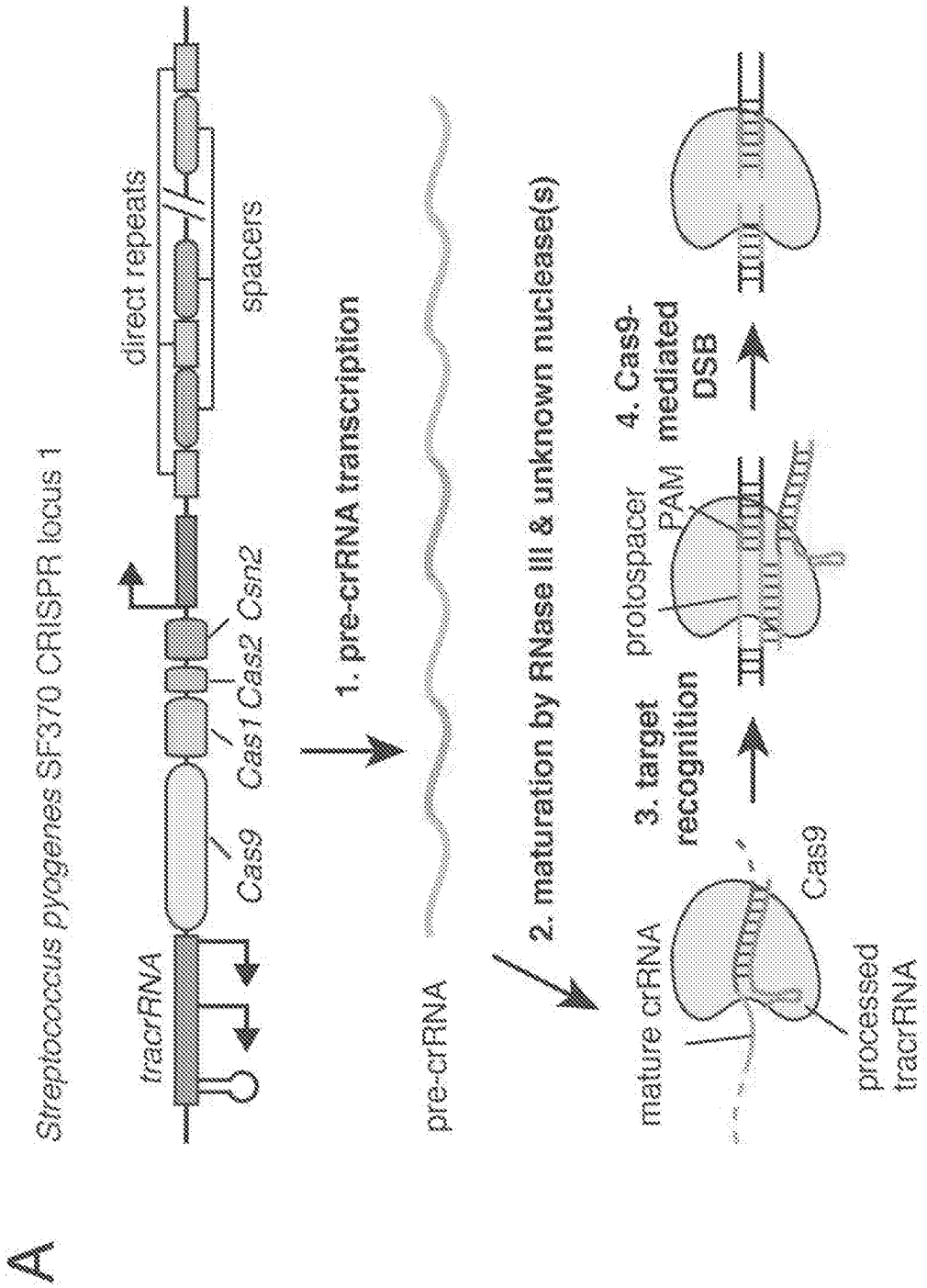

A
Streptococcus pyogenes SF370 CRISPR locus 1
tracrRNA
Cas9
Cas1
Cas2
Csn2
direct repeats
spacers
1. pre-crRNA transcription
pre-crRNA
2. maturation by RNase III & unknown nuclease(s)
mature crRNA
Cas9
processed tracrRNA
3. target recognition
protospacer
PAM
4. Cas9-mediated DSB

FIG. 2A

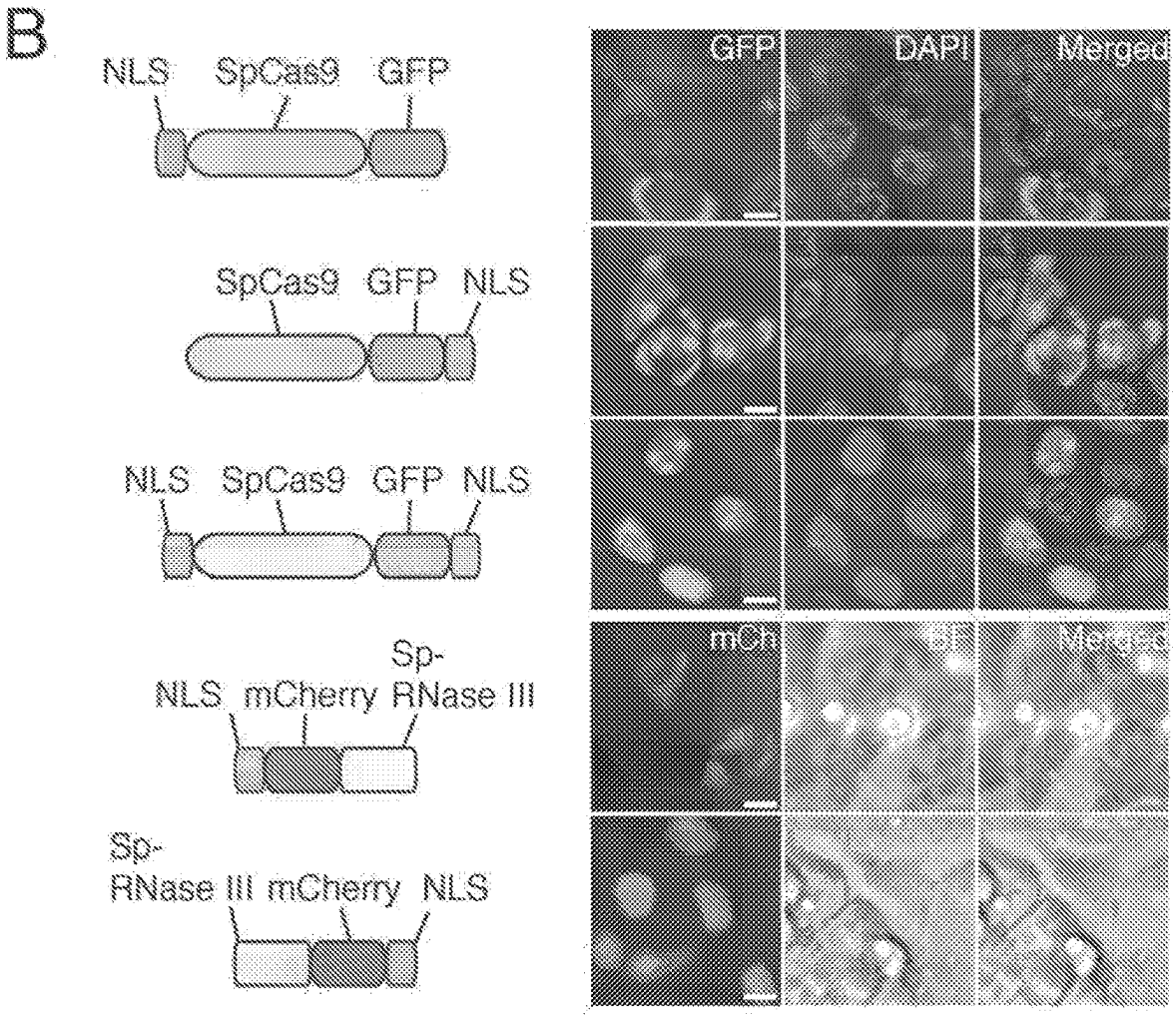

B
NLS SpCas9 GFP
SpCas9 GFP NLS
NLS SpCas9 GFP NLS
NLS mCherry Sp-RNase III
Sp-RNase III mCherry NLS
GFP
DAPI
Merged
mCh
BF
Merged

FIG. 2B

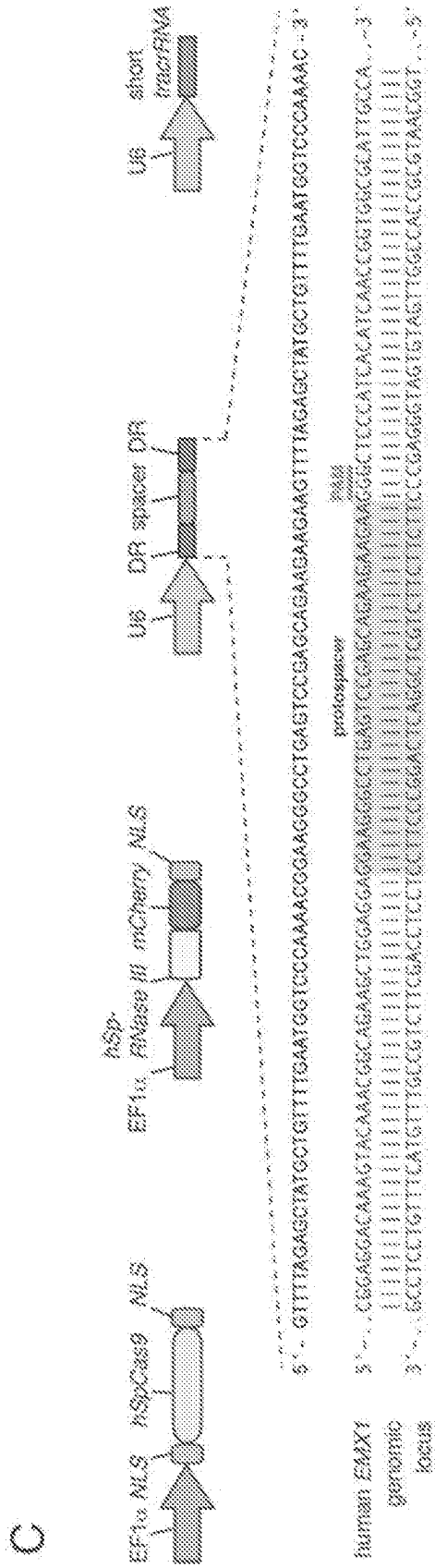

C
EF1α
NLS
hSpCas9
NLS
EF1α
hSp-RNase III
mCherry
NLS
U6
DR
spacer
DR
U6
short tracrRNA
5'- GTTTTAGAGCTATGCTGTTTTGAATGGTCCCAAAACGGAAGGGCCTGAGTCCGAGCAGAAGAAGAAGTTTTAGAGCTATGCTGTTTTGAATGGTCCCAAAAC-3'
protospacer
PAM
human EMX1 genomic locus
3'-...CGGAGGACAAAGTACAAACGGCAGAACTGGAGGAGGGAAGGGCTTAGTCCGAGCAGAAGAAGAAGGGCTCCCATCACATCAACCGGTGGCGCATTGCCA...-3'
5'-...GCCTCCTGTTTCATGTTTGCCGTCTTGACCTCCTCCCTTCCCGAACTCAGGCTCGTCTTCTTCTTCTTCCCGAAGGGATAGTGTAGTTGGCCACCGGCGTAACGGT...-5'

FIG.2C

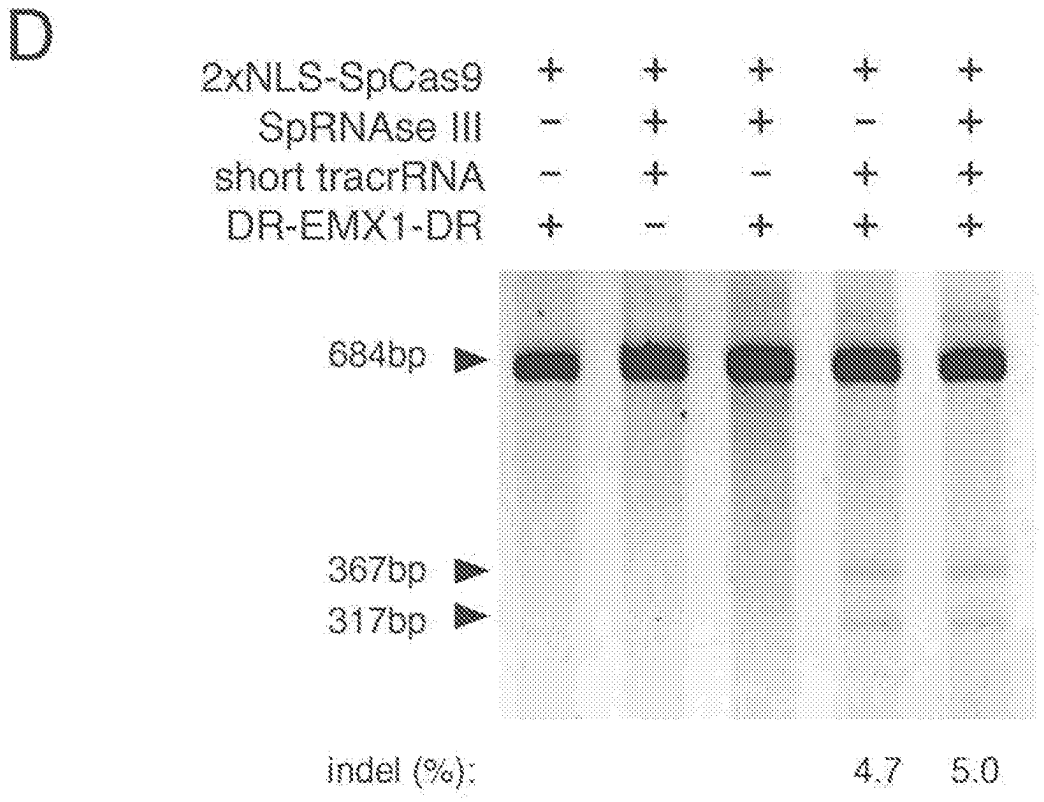
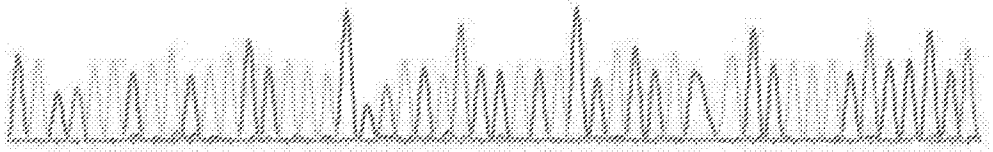

D
2xNLS-SpCas9 + + + + +
SpRNAse III − + + − +
short tracrRNA − + − + +
DR-EMX1-DR + − + + +
684bp
367bp
317bp
indel (%): 4.7 5.0

FIG. 2D

E

Target locus 5'-..AGCTGGAGGAGGAAGGGCCTGAGTCCGAGCAGAAGAAGAAGGGCTCCCAC..-3'
PAM
3'-..TCGACCTCCTCCTTCCCGGACTCAGGCTCGTCTTCTTCTTCCCGAGGGTG..-5'
crRNA 5'- GAGUCCGAGCAGAAGAAGAAGUUUUAGAGC..-3'



indel AGCTGGAGGAGGAAGGGCCTGAGTCCGAGCAGAAGA-GAAGGGCTCCCAT

F

human *EMX1* protospacer target (mutation in 5 of 43 sequenced clones = 11.6%)

WT 5'-..CTGGAGGAGGAAGGGCCTGAGTCCGAGCAGAAG AAGAAGGGCTCCCATCACAT..-3'
Δ1 CTGGAGGAGGAAGGGCCTGAGTCCGAGCAGAAG--AGAAGGGCTCCCATCACAT
+1 CTGGAGGAGGAAGGGCCTGAGTCCGAGCAGAAGAAAGAAGGGCTCCCATCACAT
Δ3 CTGGAGGAGGAAGGGCCTGAGTCCGAGCAGAAG----AAGGGCTCCCATCACAT
m1, Δ6 CTGGAGGAGGAAGGGCCTGAGCCCGAGCAGAAG------GGCTCCCATCACAT

FIG. 2E-F

hypothetical protein 8Bu_3912 [Bradyrhizobium sp. BTAi1]
CRISPR-associated protein, Cas1 family [Candidatus Pseudomonas mursenae HAL007]
CRISPR-associated protein, Cas1 family [Bacillus cereus group] bacterium polyxena RA-2011]
CRISPR-associated endonuclease Cas1 family protein [Bacillus cereus group] bacterium ATCC 11178]
Cas1-like CRISPR-associated protein, Cas1 family [Bacillus cereus group] bacterium ATCC 11178]
CRISPR-associated protein, Cas1 family protein [Bacillus cereus group] bacterium ATCC 11178]
CRISPR-associated protein, Cas1 family protein [Bacillus cereus group] bacterium ATCC 11178]
CRISPR-associated endonuclease Cas1 family protein [Bacillus cereus group] bacterium ATCC 11178]
CRISPR-associated endonuclease Cas1 family protein [Bacillus cereus group] bacterium ATCC 11178]
CRISPR-associated protein, Cas1 family [Candidatus Pseudomonas mursenae HAL007]
hypothetical protein BACCEL_0113 [Bacillus cereus group] bacterium ATCC 11178]
hypothetical protein BACCEL_0113 [Bacillus cereus group] bacterium ATCC 11178]
CRISPR-associated protein, Cas1 family [Candidatus Pseudomonas mursenae HAL007

FIG. 3B

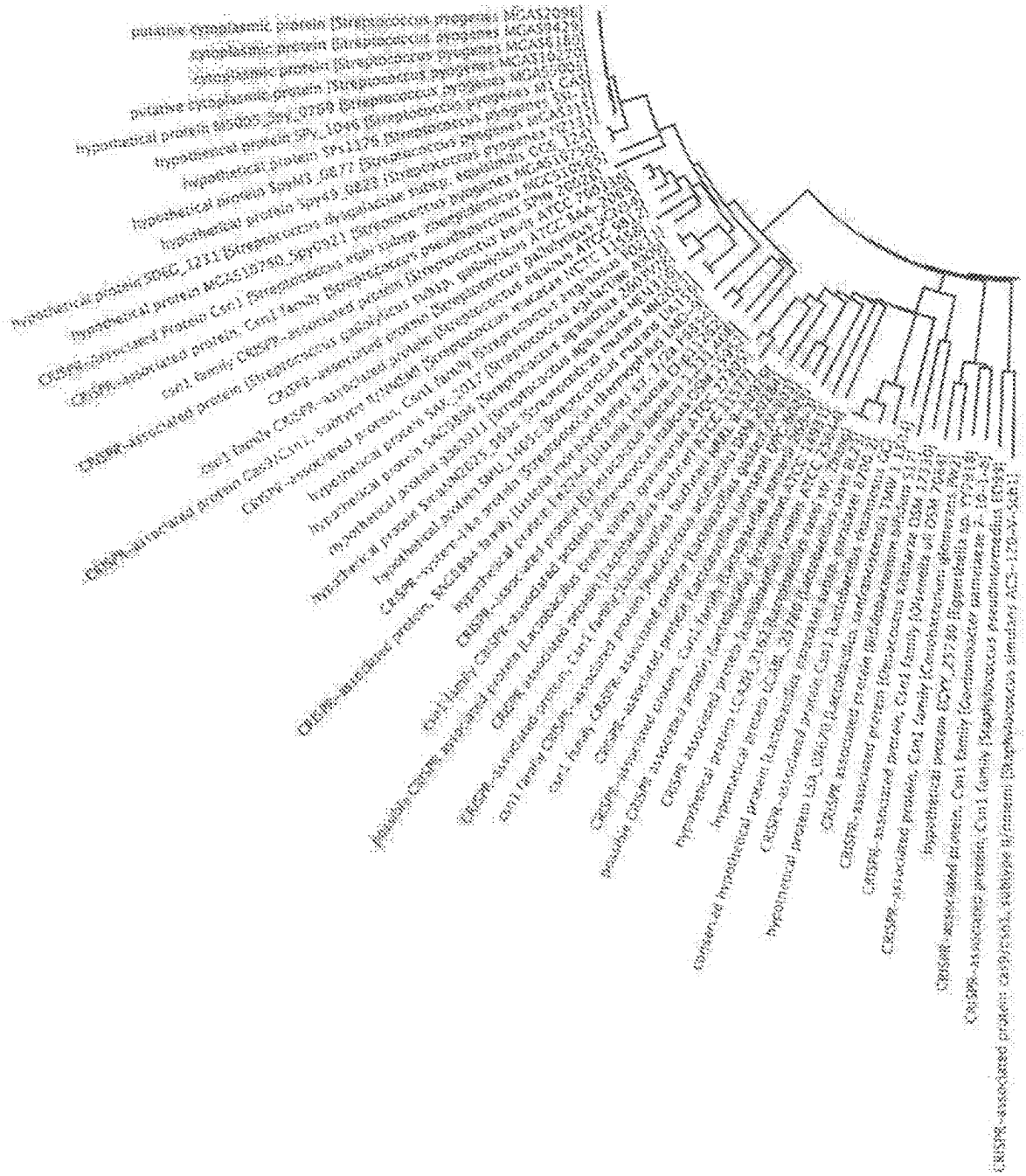
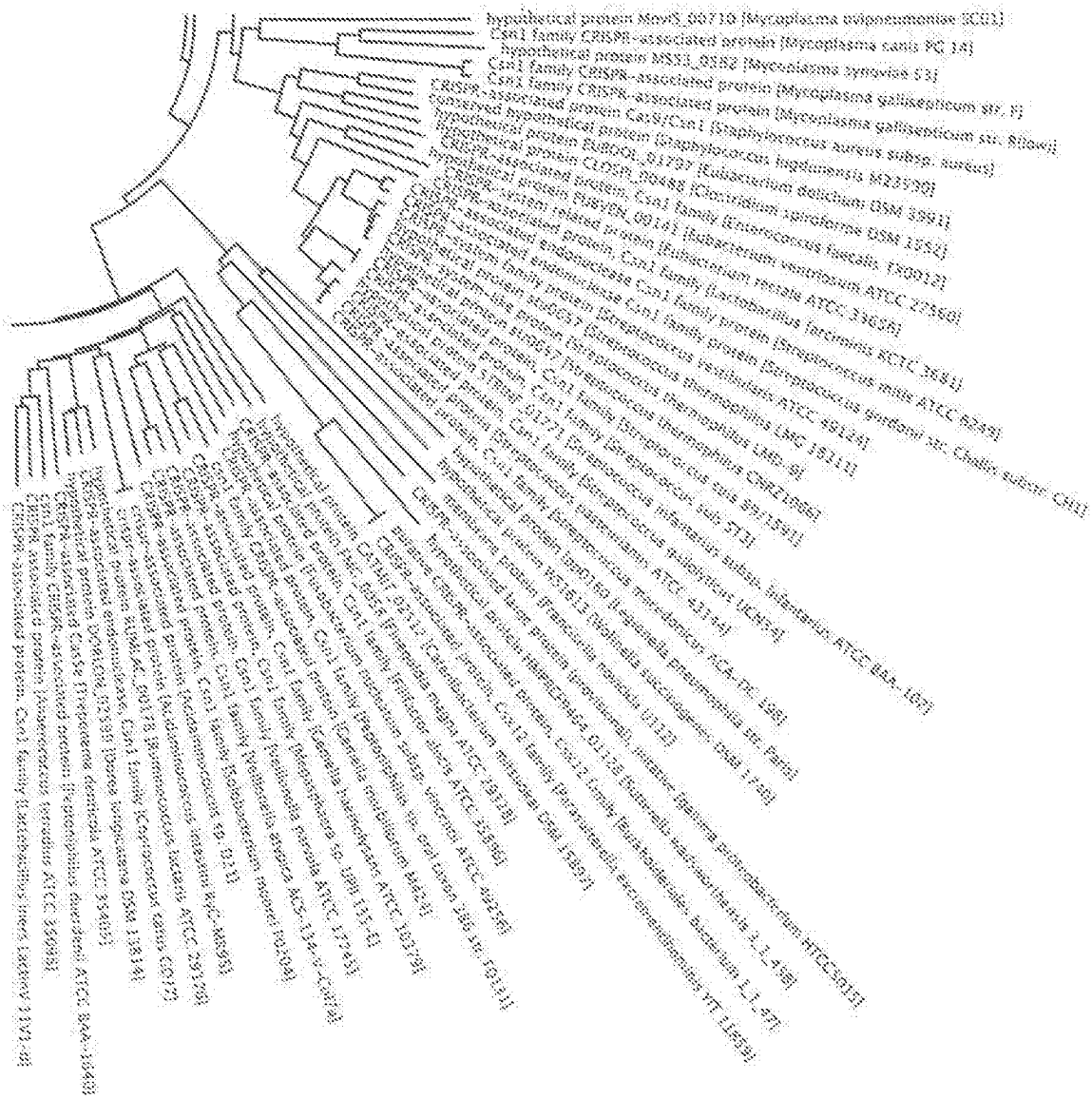


FIG. 3C


hypothetical protein MovS_00710 [Mycoplasma ovipneumoniae SC01]
Cas1 family CRISPR-associated protein [Mycoplasma canis PG 14]
hypothetical protein MSS3_0582 [Mycoplasma synoviae 53]
Cas1 family CRISPR-associated protein [Mycoplasma gallisepticum str. F]
CRISPR-associated protein [Mycoplasma gallisepticum str. Rlow]
Conserved hypothetical protein Cas9/Cas1 [Staphylococcus aureus subsp. aureus]
hypothetical protein EubDOL_01797 [Eubacterium dolichum DSM 3991]
hypothetical protein CLOSH_00488 [Clostridium sporosporum DSM 1552]
CRISPR-associated protein, Cas1 family [Enterococcus faecalis TX0012]
CRISPR-associated protein [Eubacterium ventriosum ATCC 27560]
CRISPR-associated endonuclease Cas1 family protein [Streptococcus pneumoniae ATCC 33400]
CRISPR-associated endonuclease Cas1 family protein [Streptococcus thermophilus LMG 18111]
CRISPR-associated protein, Cas1 family [Streptococcus thermophilus CNRZ1066]
CRISPR-associated protein, Cas1 family [Streptococcus thermophilus LMD-9]
CRISPR-associated protein, Cas1 family [Streptococcus thermophilus ATCC BAA-167]
hypothetical protein [Streptococcus thermophilus ATCC 4314]
hypothetical protein [Streptococcus thermophilus ATCC 4314]
hypothetical protein [Streptococcus thermophilus ATCC 4314

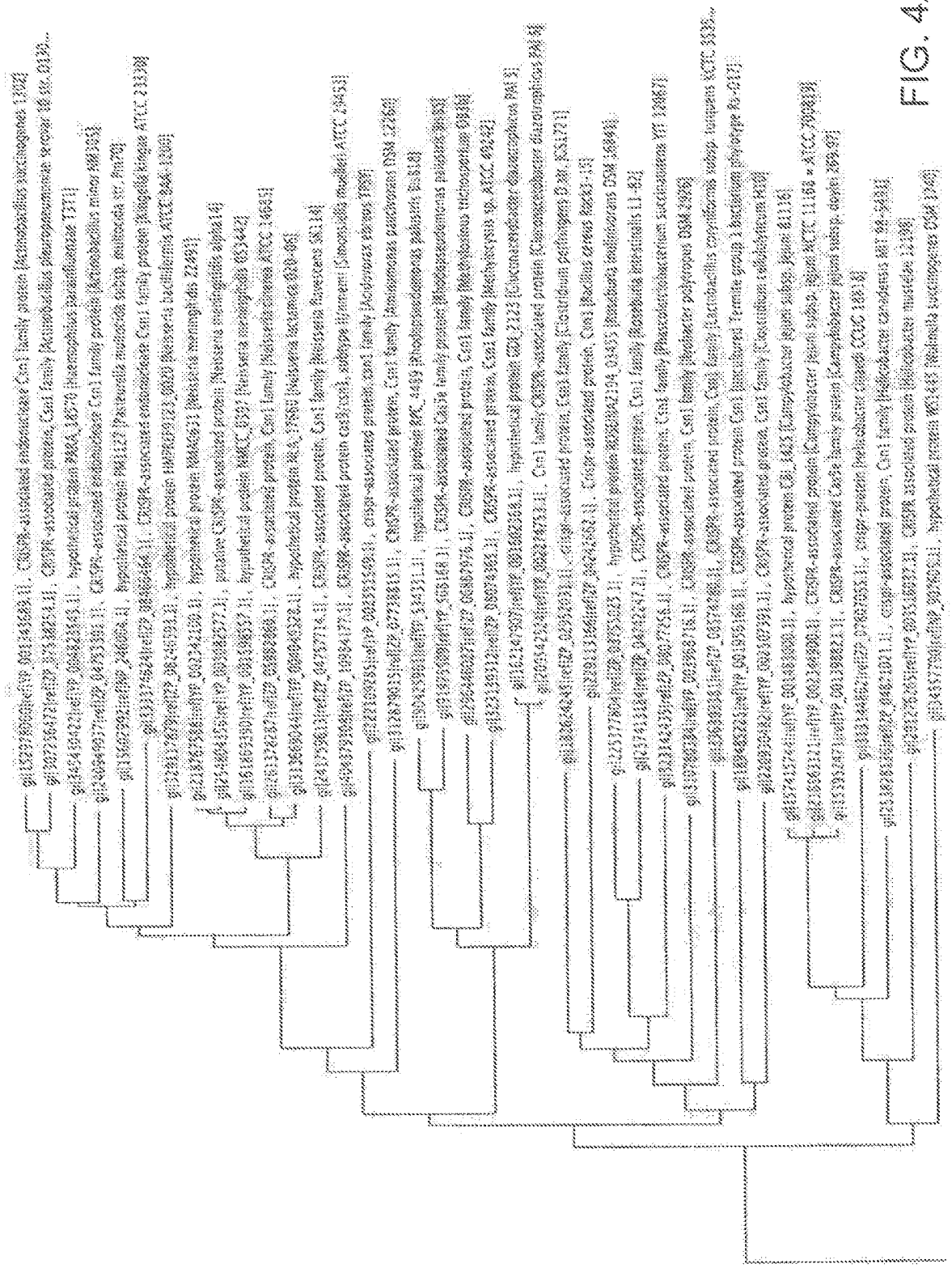


FIG. 4A

FIG. 4B

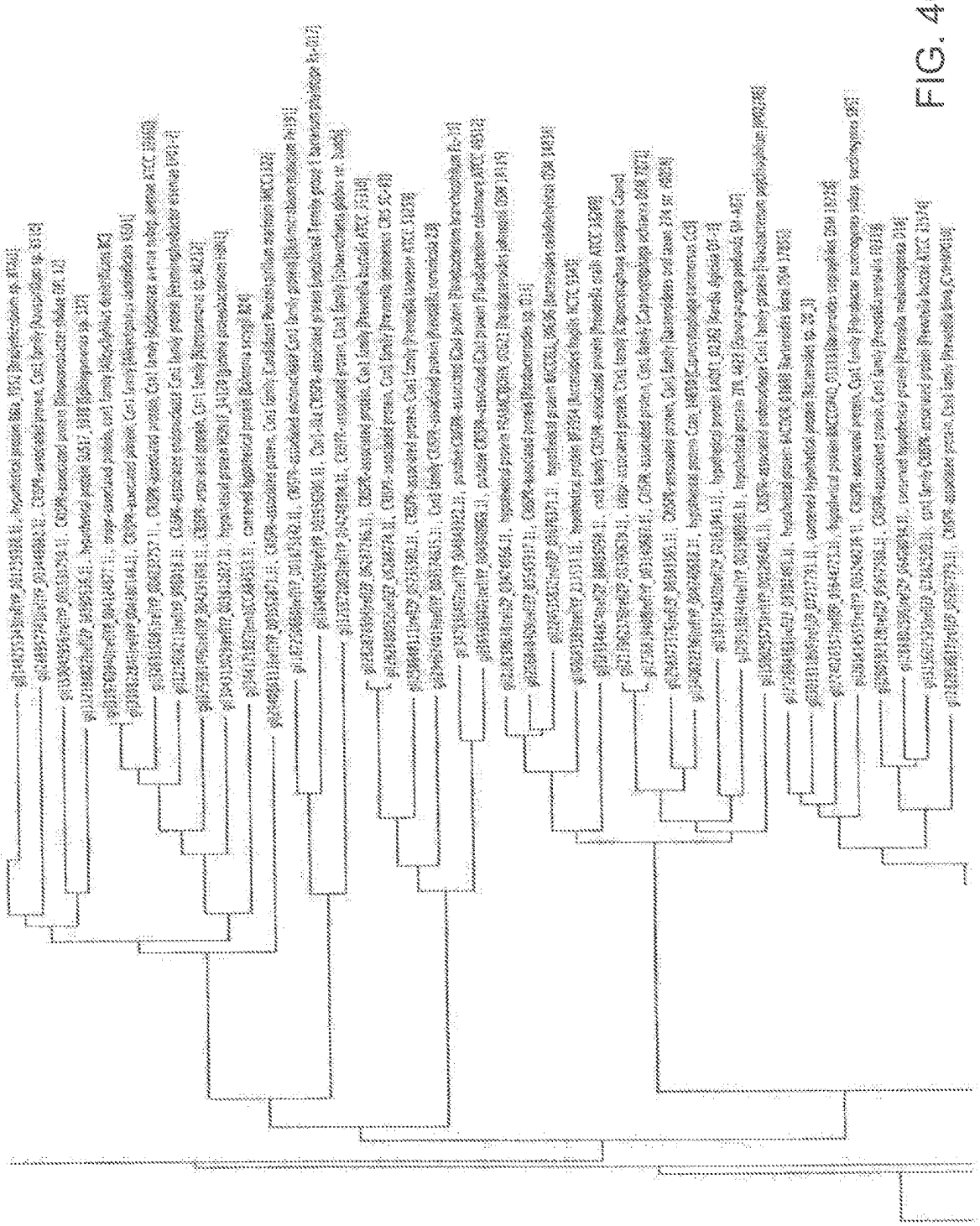


FIG. 4C

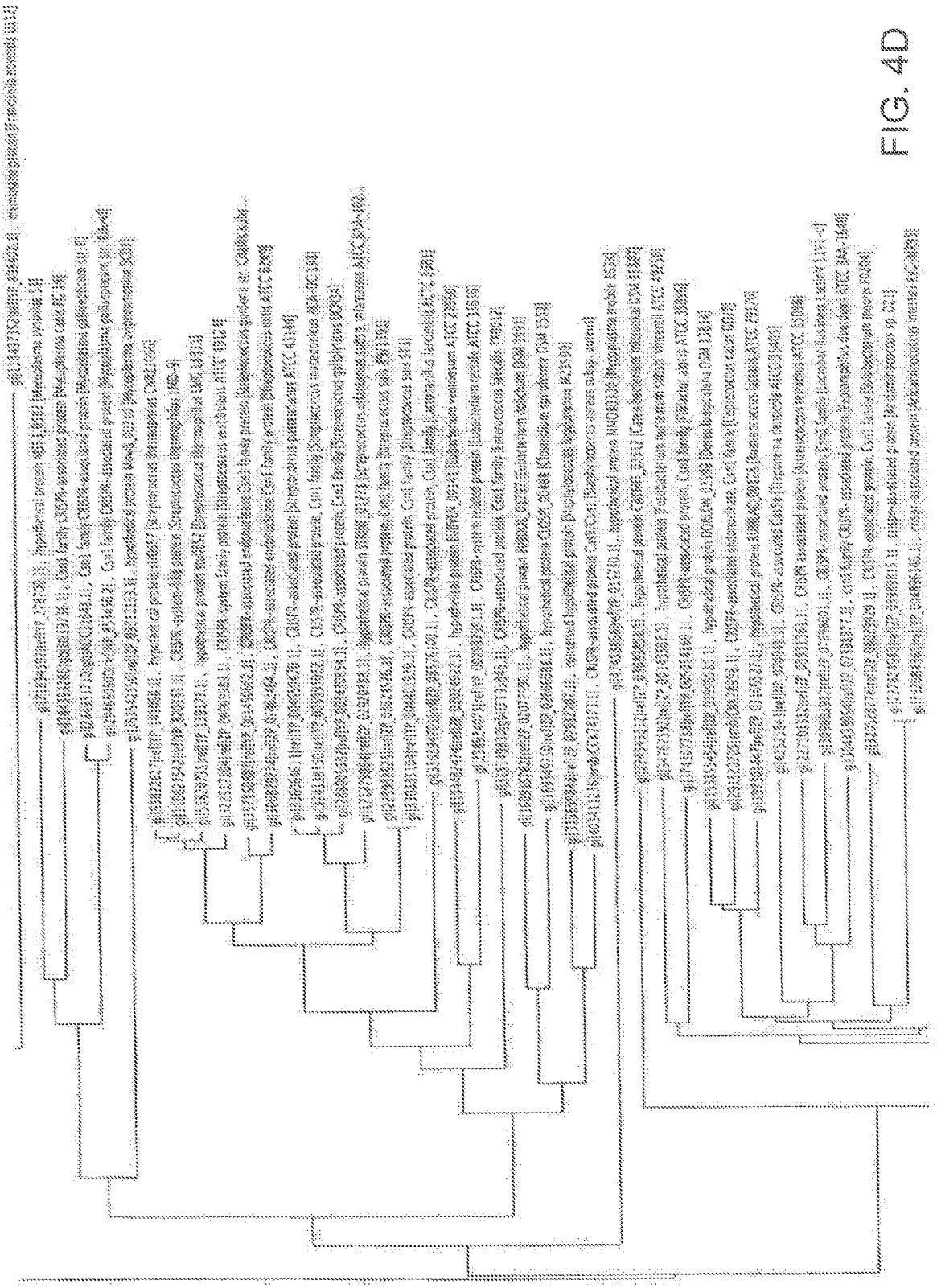


FIG. 4D

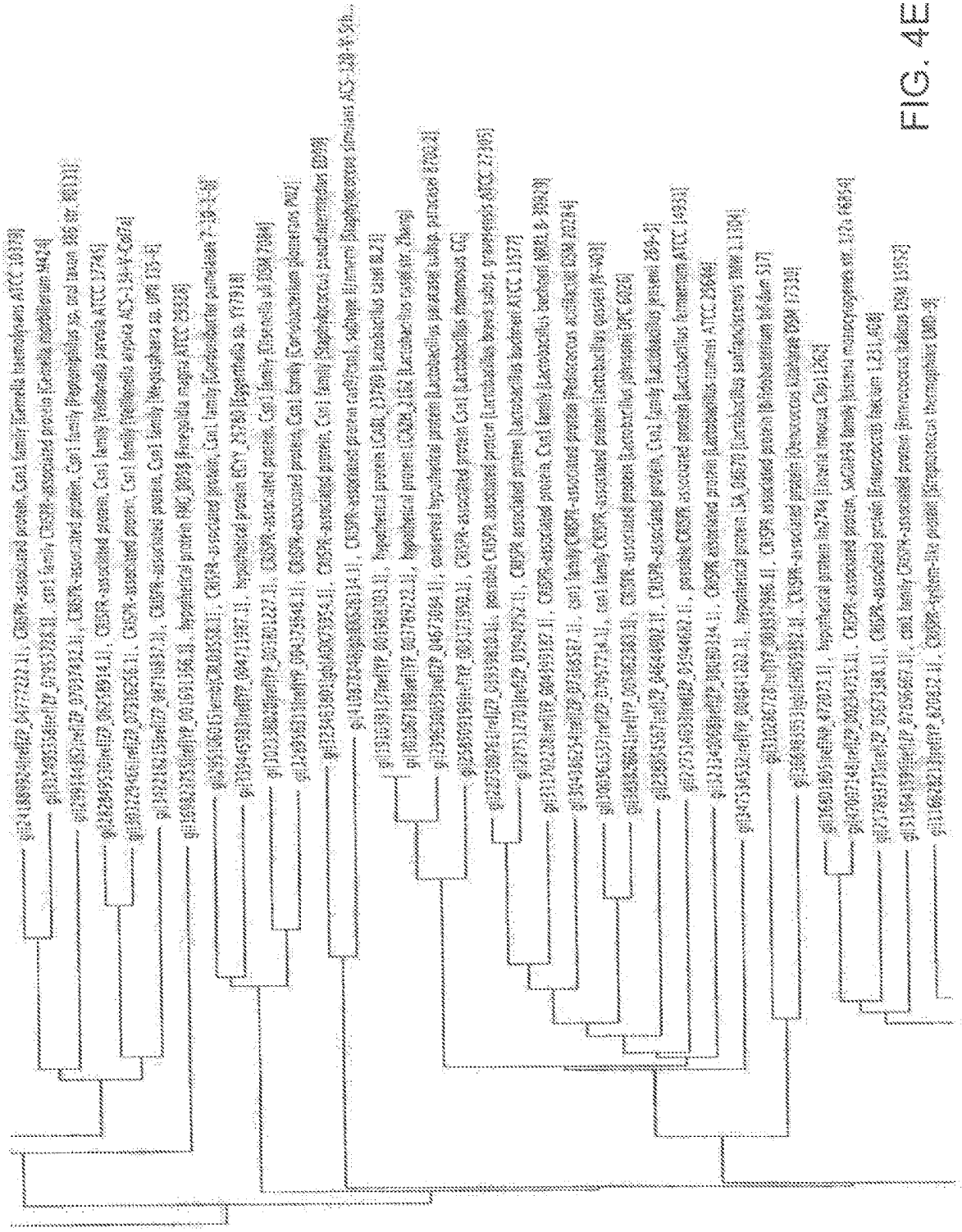


FIG. 4E

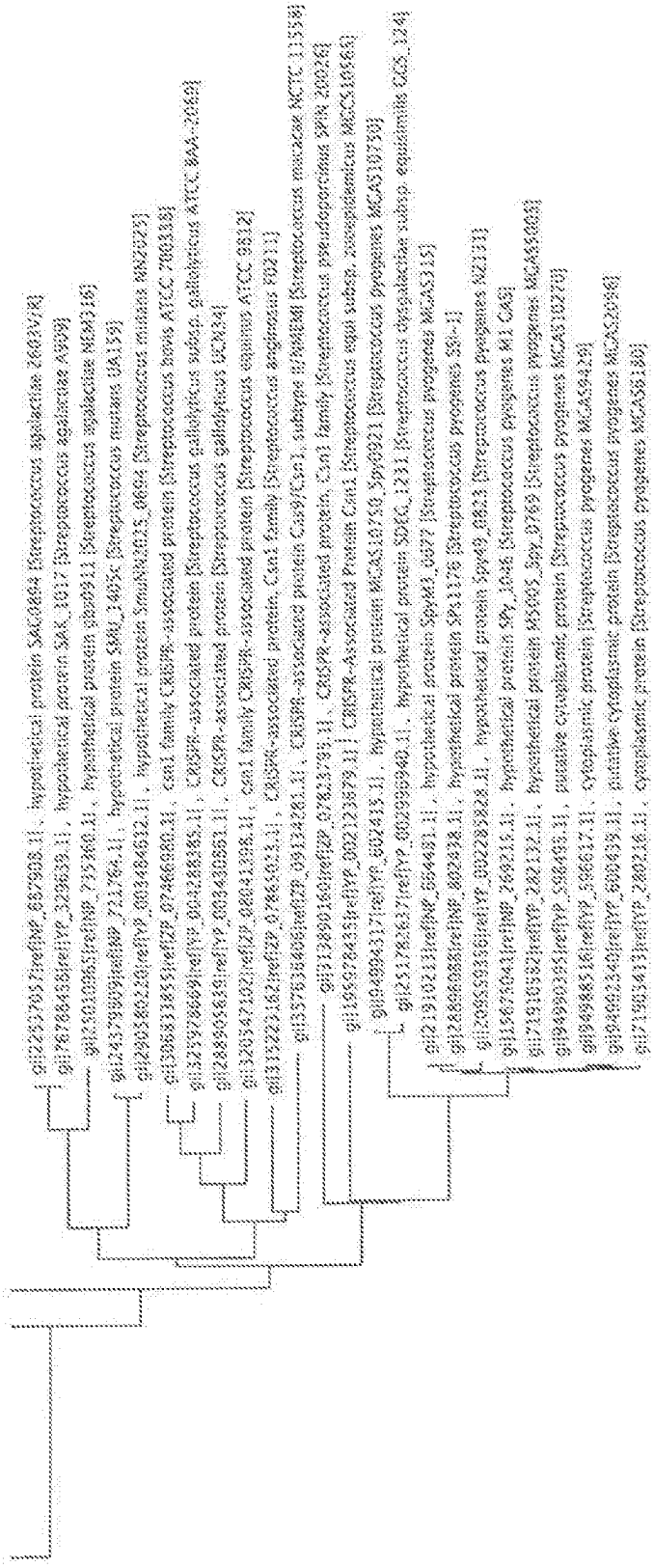

gi|22551795|ref|NP_857908.1| hypothetical protein SAC0884 [Streptococcus agalactiae 2603V/R]
gi|76788456|ref|YP_329639.1| hypothetical protein SAK_1017 [Streptococcus agalactiae A909]
gi|23310086|ref|NP_755860.1| hypothetical protein gbs0911 [Streptococcus agalactiae NEM316]
gi|24379099|ref|NP_721764.1| hypothetical protein SMU_1403c [Streptococcus mutans UA159]
gi|29058220|ref|YP_003486632.1| hypothetical protein SmuNN2035_0694 [Streptococcus mutans NN2025]
gi|336813855|ref|ZP_07466920.1| cas1 family CRISPR-associated protein [Streptococcus bovis ATCC 700338]
gi|237786669|ref|YP_002428385.1| CRISPR-associated protein [Streptococcus gallolyticus subsp. gallolyticus ATCC BAA-2069]
gi|288905635|ref|YP_003430861.1| CRISPR-associated protein [Streptococcus gallolyticus UCN34]
gi|320347102|ref|ZP_08041398.1| cas1 family CRISPR-associated protein [Streptococcus equinus ATCC 9812]
gi|315222182|ref|ZP_07465023.1| CRISPR-associated protein, Cas1 family [Streptococcus anginosus F0211]
gi|357634068|ref|ZP_09134281.1| CRISPR-associated protein Cas97/Csn1, subtype II-A/DM3 [Streptococcus macacae NCTC 11558]
gi|312899180|ref|ZP_07623739.1| CRISPR-associated protein, Csn1 family [Streptococcus pseudporcinus SPIN 20026]
gi|169597843|ref|YP_002123679.1| CRISPR-Associated Protein Csn1 [Streptococcus equi subsp. zooepidemicus MGCS10565]
gi|94499417|ref|YP_602415.1| hypothetical protein MGAS10750_Spy0921 [Streptococcus pyogenes MGAS10750]
gi|251778437|ref|YP_002999940.1| hypothetical protein SDEG_1231 [Streptococcus dysgalactiae subsp. equisimilis GGS_124]
gi|21910211|ref|NP_664481.1| hypothetical protein Spy0d1_0627 [Streptococcus pyogenes MGAS315]
gi|28895895|ref|NP_802278.1| hypothetical protein SPs1178 [Streptococcus pyogenes SSI-1]
gi|20555556|ref|YP_002289828.1| hypothetical protein Spy49_0823 [Streptococcus pyogenes M1 GAS]
gi|15675641|ref|NP_269216.1| hypothetical protein SPy_1046 [Streptococcus pyogenes M1 GAS]
gi|71916582|ref|YP_282132.1| hypothetical protein MS005_Spy_0769 [Streptococcus pyogenes MGAS5005]
gi|94990295|ref|YP_598493.1| putative cytoplasmic protein [Streptococcus pyogenes MGAS10270]
gi|94988516|ref|YP_596637.1| cytoplasmic protein [Streptococcus pyogenes MGAS9429]
gi|94991340|ref|YP_600438.1| putative cytoplasmic protein [Streptococcus pyogenes MGAS2096]
gi|71983413|ref|YP_280216.1| cytoplasmic protein [Streptococcus pyogenes MGAS6180]

FIG. 4F

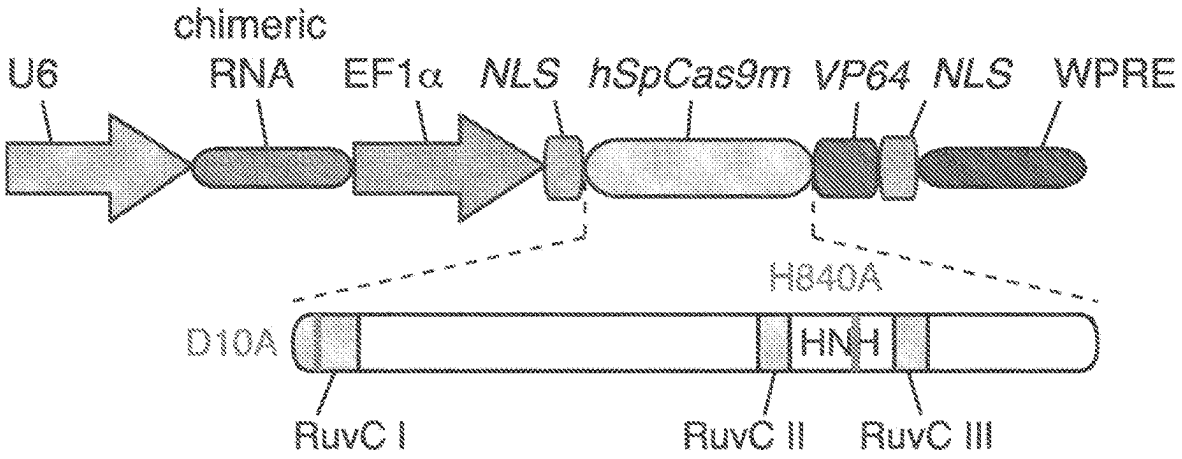

chimeric
U6
RNA
EF1α
NLS
hSpCas9m
VP64
NLS
WPRE
H840A
D10A
HN
H
RuvC I
RuvC II
RuvC III

FIG. 5

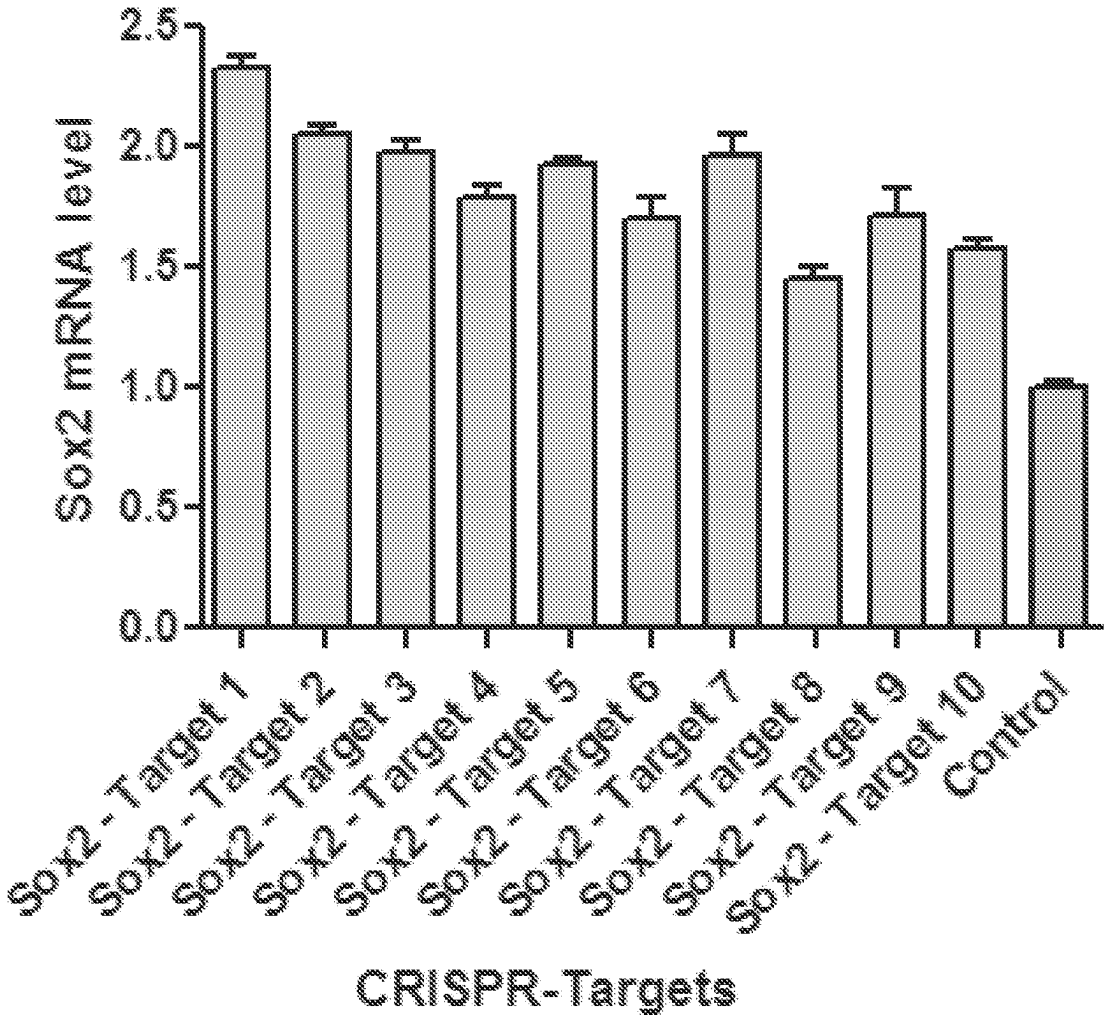

Sox2 mRNA level
2.5
2.0
1.5
1.0
0.5
0.0
Sox2 - Target 1
Sox2 - Target 2
Sox2 - Target 3
Sox2 - Target 4
Sox2 - Target 5
Sox2 - Target 6
Sox2 - Target 7
Sox2 - Target 8
Sox2 - Target 9
Sox2 - Target 10
Control
CRISPR-Targets

FIG. 6

Figure 1

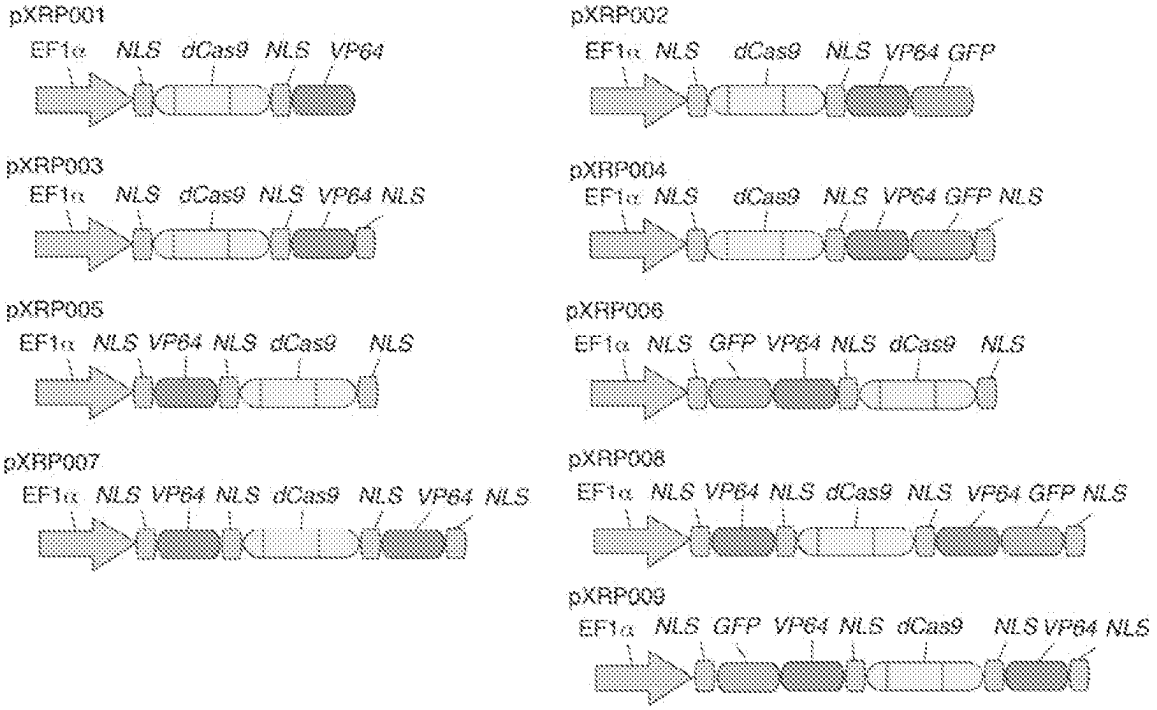
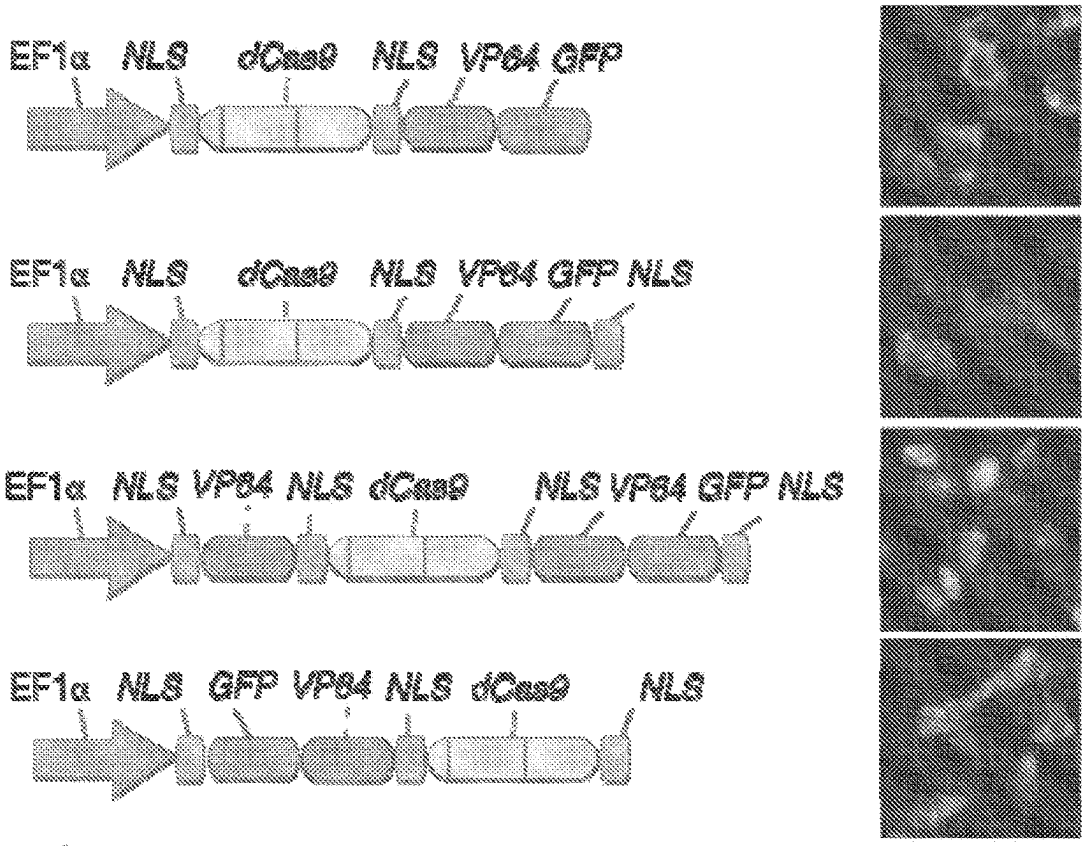
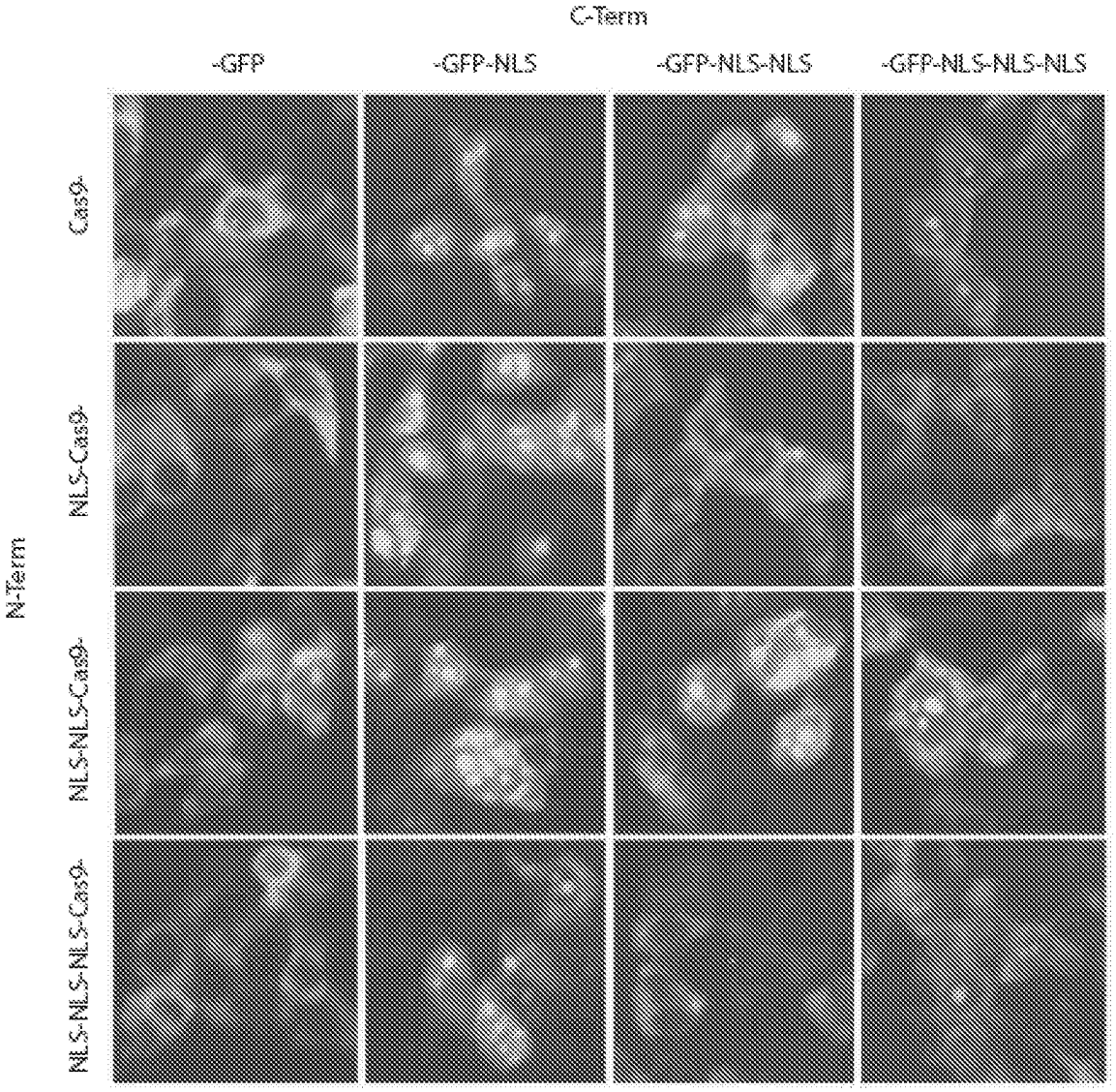


FIG. 7



From top to bottom:
pXRP02- pLenti2-EF1a-NLS-hSpCsn1(10A,840A)-NLS-VP64-EGFP
pXRP04- pLenti2-EF1a-NLS-hSpCsn1(10A,840A)-NLS-VP64-2A-EGFP-NLS
pXRP06- pLenti2-EF1a-NLS-EGFP-VP64-NLS-hSpCsn1(10A,840A)-NLS
pXRP08- pLenti2-EF1a-NLS-VP64-NLS-hSpCsn1(10A,840A)-NLS-VP64-EGFP-NLS

FIG. 8



Constructs have the following names:

pXRPNLS001	pXRPNLS005	pXRPNLS009	pXRPNLS013
pXRPNLS002	pXRPNLS006	pXRPNLS010	pXRPNLS014
pXRPNLS003	pXRPNLS007	pXRPNLS011	pXRPNLS015
pXRPNLS004	pXRPNLS008	pXRPNLS012	pXRPNLS016

Importin alpha NLS sequence:
AAAAGGCCGGCGGCCACGAAAAAGGCCGGCCAGGCAAAAAAGAAAAAG

FIG. 9

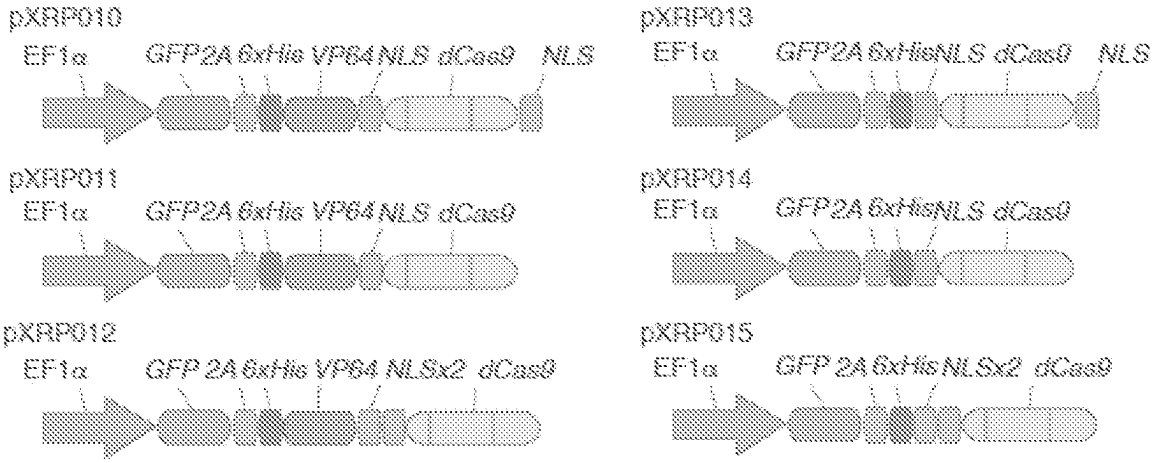
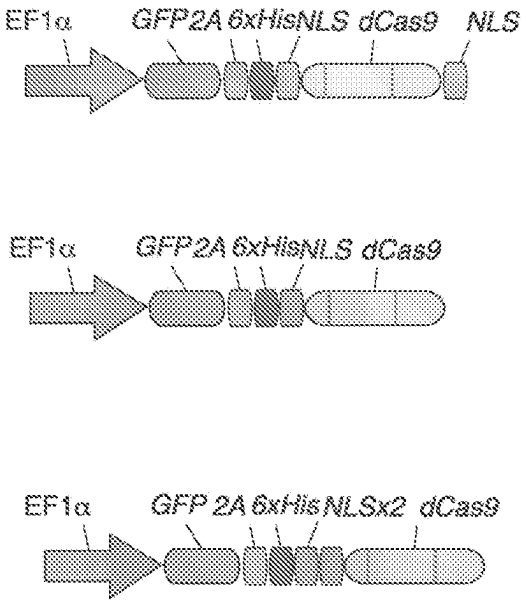
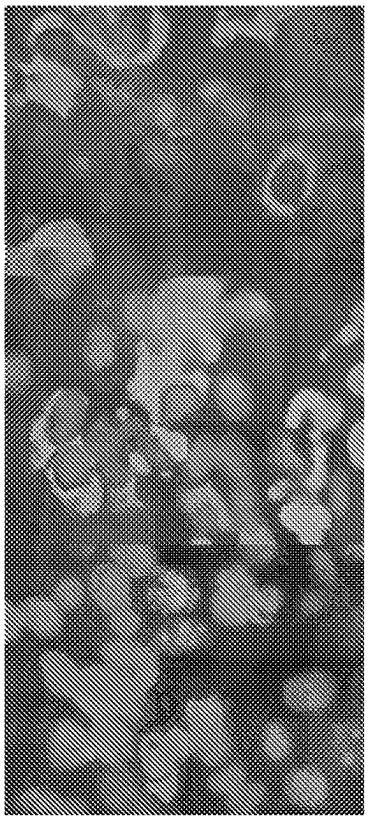

pXRP010
EF1α
GFP2A 6xHis VP64 NLS dCas9
NLS
pXRP011
EF1α
GFP2A 6xHis VP64 NLS dCas9
pXRP012
EF1α
GFP 2A 6xHis VP64 NLSx2 dCas9
pXRP013
EF1α
GFP2A 6xHisNLS dCas9
NLS
pXRP014
EF1α
GFP2A 6xHisNLS dCas9
pXRP015
EF1α
GFP 2A 6xHis NLSx2 dCas9

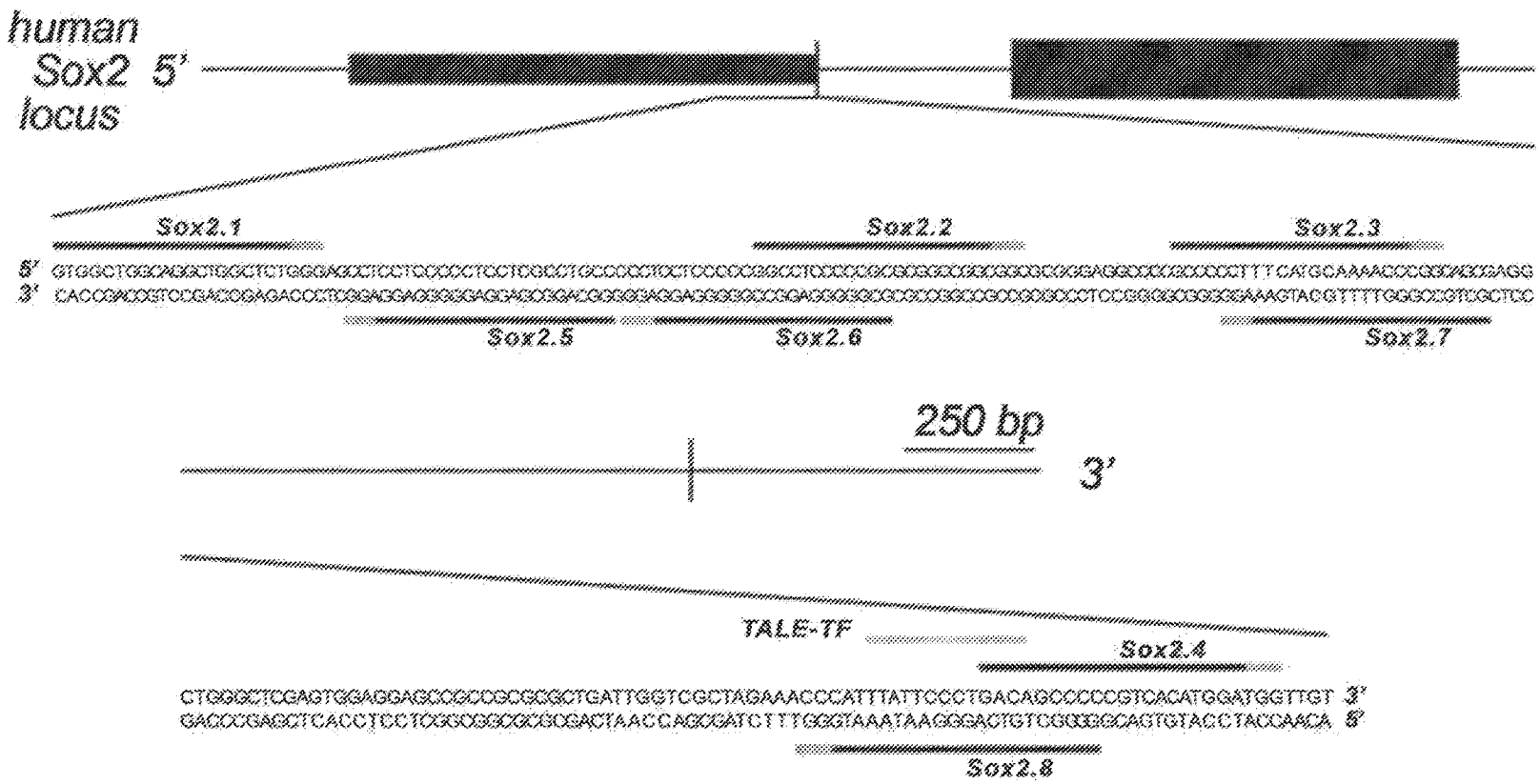
FIG. 10





Red = alexa-fluor 594
Blue = DAPI

FIG. 11



gRNA	gRNA sequence (5'-3')
hSox2.1	GTGGCTGGCAGGCTGGCTCT
hSox2.2	GGCCTCCCCCGCGCGGCCGG
hSox2.3	GCCCCCTTTCATGCAAAACC
hSox2.4	GACAGCCCCCGTCACATGGA
hSox2.5	GGCAGGCGAGGAGGGGGAGG
hSox2.6	GGCGGGGCCTCCCGCGCCGC
hSox2.7	GCTGCCGGGTTTTGCATGAA
hSox2.8	GGGGCTGTCAGGGAATAAAT

FIG. 12

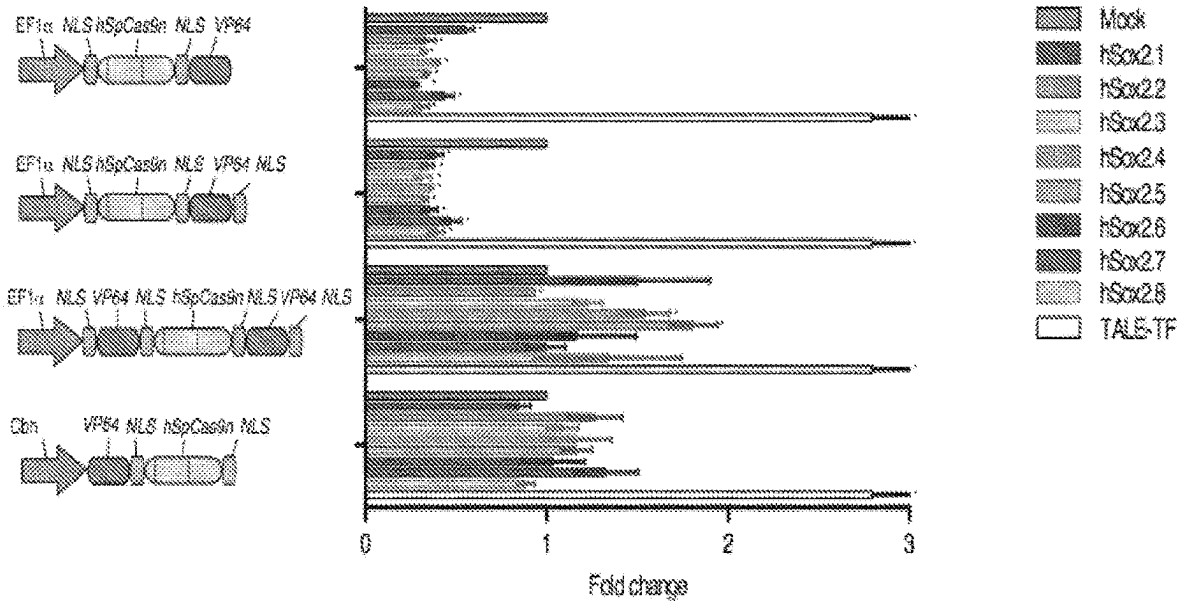
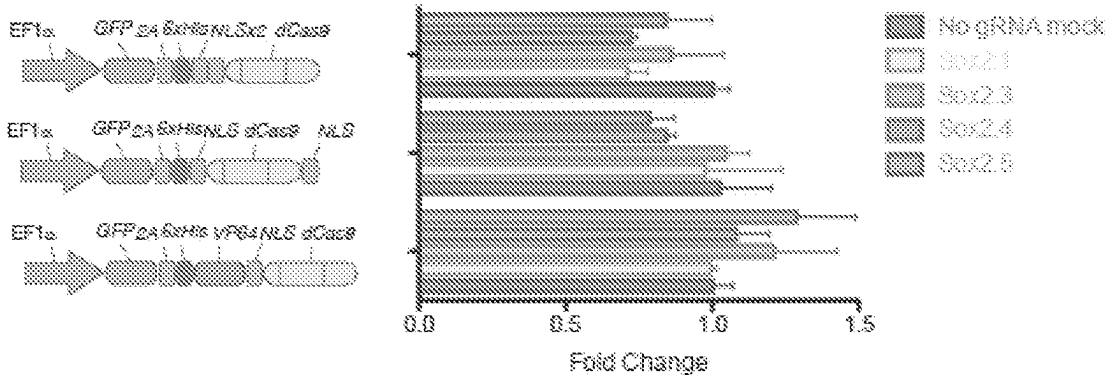

EF1α NLS hSpCas9n NLS VP64
EF1α NLS hSpCas9n NLS VP64 NLS
EF1α NLS VP64 NLS hSpCas9n NLS VP64 NLS
Cbh VP64 NLS hSpCas9n NLS
0
1
2
3
Fold change
Mock
hSox2.1
hSox2.2
hSox2.3
hSox2.4
hSox2.5
hSox2.6
hSox2.7
hSox2.8
TALE-TF

FIG. 13



From top to bottom:
pXRP015
pXRP013
pXRP011

FIG. 14

pXRP024-pLenti2-EF1a-VP64-NLS-FLAG-Linker-dCas9-NLS-gLuc-2A-GFP-WPRE
pXRP025-pLenti2-EF1a-VP64-NLS-GGGGS$_3$Linker-dCas9-NLS-gLuc-2A-GFP-WPRE
pXRP026-pLenti2-EF1a-VP64-NLS-EAAAK$_3$Linker-dCas9-NLS-gLuc-2A-GFP-WPRE
pXRP027-pLenti2-EF1a-NLS-FLAG-Linker-dCas9-NLS-gLuc-2A-GFP-WPRE
pXRP028-pLenti2-EF1a-NLS-GGGGS$_3$Linker-dCas9-NLS-gLuc-2A-GFP-WPRE
pXRP029-pLenti2-EF1a-NLS-EAAAK$_3$Linker-dCas9-NLS-gLuc-2A-GFP-WPRE
pXRP030-pLenti2-pSV40-VP64-NLS-FLAG-Linker-dCas9-NLS-gLuc-2A-GFP-WPRE
pXRP031-pLenti2-pPGK-VP64-NLS-FLAG-Linker-dCas9-NLS-gLuc-2A-GFP-WPRE
pXRP032-pLenti2-LTR-VP64-NLS-FLAG-Linker-dCas9-NLS-gLuc-2A-GFP-WPRE
pXRP033-pLenti2-pSV40-VP64-NLS-GGGGS$_3$Linker-dCas9-NLS-gLuc-2A-GFP-WPRE
pXRP034-pLenti2-pPGK-VP64-NLS-GGGGS$_3$Linker-dCas9-NLS-gLuc-2A-GFP-WPRE
pXRP035-pLenti2-LTR-VP64-NLS-GGGGS$_3$Linker-dCas9-NLS-gLuc-2A-GFP-WPRE
pXRP036-pLenti2-pSV40-VP64-NLS-EAAAK$_3$Linker-dCas9-NLS-gLuc-2A-GFP-WPRE
pXRP037-pLenti2-pPGK-VP64-NLS-EAAAK$_3$Linker-dCas9-NLS-gLuc-2A-GFP-WPRE
pXRP038-pLenti2-LTR-VP64-NLS-EAAAK$_3$Linker-dCas9-NLS-gLuc-2A-GFP-WPRE
pXRP048-pLenti2-EF1a-SID4x-NLS-FLAG-Linker-dCas9-NLS-gLuc-2A-GFP-WPRE
pXRP049-pLenti2-EF1a-SID4X-NLS-GGGGS$_3$Linker-dCas9-NLS-gLuc-2A-GFP-WPRE
pXRP050-pLenti2-EF1a-SID4X-NLS-EAAAK$_3$Linker-dCas9-NLS-gLuc-2A-GFP-WPRE
pXRP051-pLenti2-EF1a-KRAB-NLS-FLAG-Linker-dCas9-NLS-gLuc-2A-GFP-WPRE
pXRP052-pLenti2-EF1a-KRAB-NLS-GGGGS$_3$Linker-dCas9-NLS-gLuc-2A-GFP-WPRE
pXRP053-pLenti2-EF1a-KRAB-NLS-EAAAK$_3$Linker-dCas9-NLS-gLuc-2A-GFP-WPRE
pXRP054-pLenti2-EF1a-dCas9-Linker-FLAG-NLS-VP64-gLuc-2A-GFP-WPRE
pXRP055-pLenti2-EF1a-dCas9-Linker-FLAG-NLS-SID4X-gLuc-2A-GFP-WPRE
pXRP056-pLenti2-EF1a-dCas9-Linker-FLAG-NLS-KRAB-gLuc-2A-GFP-WPRE
pXRP057-pLenti2-EF1a-dCas9-GGGGGS$_3$-NLS-VP64-gLuc-2A-GFP-WPRE
pXRP058-pLenti2-EF1a-dCas9-GGGGGS$_3$-NLS-SID4X-gLuc-2A-GFP-WPRE
pXRP059-pLenti2-EF1a-dCas9-GGGGGS$_3$-NLS-KRAB-gLuc-2A-GFP-WPRE
pXRP060-pLenti2-EF1a-dCas9-EAAAK$_3$-NLS-VP64-gLuc-2A-GFP-WPRE
pXRP061-pLenti2-EF1a-dCas9-EAAAK$_3$-NLS-SID4X-gLuc-2A-GFP-WPRE
pXRP062-pLenti2-EF1a-dCas9-EAAAK$_3$-NLS-KRAB-gLuc-2A-GFP-WPRE
pXRP024-pLenti2-EF1a-VP64-NLS-FLAG-Linker-Cas9-NLS-gLuc-2A-GFP-WPRE
pXRP025-pLenti2-EF1a-VP64-NLS-GGGGS$_3$Linker-Cas9-NLS-gLuc-2A-GFP-WPRE
pXRP026-pLenti2-EF1a-VP64-NLS-EAAAK$_3$Linker-Cas9-NLS-gLuc-2A-GFP-WPRE
pXRP027-pLenti2-EF1a-NLS-FLAG-Linker-Cas9-NLS-gLuc-2A-GFP-WPRE
pXRP028-pLenti2-EF1a-NLS-GGGGS$_3$Linker-Cas9-NLS-gLuc-2A-GFP-WPRE
pXRP029-pLenti2-EF1a-NLS-EAAAK$_3$Linker-Cas9-NLS-gLuc-2A-GFP-WPRE

FIG. 15A

pXRP030-pLenti2-pSV40-VP64-NLS-FLAG-Linker-Cas9-NLS-gLuc-2A-GFP-WPRE
pXRP031-pLenti2-pPGK-VP64-NLS-FLAG-Linker-Cas9-NLS-gLuc-2A-GFP-WPRE
pXRP032-pLenti2-LTR-VP64-NLS-FLAG-Linker-Cas9-NLS-gLuc-2A-GFP-WPRE
pXRP033-pLenti2-pSV40-VP64-NLS-$GGGGS_3$Linker-Cas9-NLS-gLuc-2A-GFP-WPRE
pXRP034-pLenti2-pPGK-VP64-NLS-$GGGGS_3$Linker-Cas9-NLS-gLuc-2A-GFP-WPRE
pXRP035-pLenti2-LTR-VP64-NLS-$GGGGS_3$Linker-Cas9-NLS-gLuc-2A-GFP-WPRE
pXRP036-pLenti2-pSV40-VP64-NLS-$EAAAK_3$Linker-Cas9-NLS-gLuc-2A-GFP-WPRE
pXRP037-pLenti2-pPGK-VP64-NLS-$EAAAK_3$Linker-Cas9-NLS-gLuc-2A-GFP-WPRE
pXRP038-pLenti2-LTR-VP64-NLS-$EAAAK_3$Linker-Cas9-NLS-gLuc-2A-GFP-WPRE
pXRP048-pLenti2-EF1a-SID4x-NLS-FLAG-Linker-Cas9-NLS-gLuc-2A-GFP-WPRE
pXRP049-pLenti2-EF1a-SID4X-NLS-$GGGGS_3$Linker-Cas9-NLS-gLuc-2A-GFP-WPRE
pXRP050-pLenti2-EF1a-SID4X-NLS-$EAAAK_3$Linker-Cas9-NLS-gLuc-2A-GFP-WPRE
pXRP051-pLenti2-EF1a-KRAB-NLS-FLAG-Linker-Cas9-NLS-gLuc-2A-GFP-WPRE
pXRP052-pLenti2-EF1a-KRAB-NLS-$GGGGS_3$Linker-Cas9-NLS-gLuc-2A-GFP-WPRE
pXRP053-pLenti2-EF1a-KRAB-NLS-$EAAAK_3$Linker-Cas9-NLS-gLuc-2A-GFP-WPRE
pXRP054-pLenti2-EF1a-Cas9-Linker-FLAG-NLS-VP64-gLuc-2A-GFP-WPRE
pXRP055-pLenti2-EF1a-Cas9-Linker-FLAG-NLS-SID4X-gLuc-2A-GFP-WPRE
pXRP056-pLenti2-EF1a-Cas9-Linker-FLAG-NLS-KRAB-gLuc-2A-GFP-WPRE
pXRP057-pLenti2-EF1a-Cas9-$GGGGGS_3$-NLS-VP64-gLuc-2A-GFP-WPRE
pXRP058-pLenti2-EF1a-Cas9-$GGGGGS_3$-NLS-SID4X-gLuc-2A-GFP-WPRE
pXRP059-pLenti2-EF1a-Cas9-$GGGGGS_3$-NLS-KRAB-gLuc-2A-GFP-WPRE
pXRP060-pLenti2-EF1a-Cas9-$EAAAK_3$-NLS-VP64-gLuc-2A-GFP-WPRE
pXRP061-pLenti2-EF1a-Cas9-$EAAAK_3$-NLS-SID4X-gLuc-2A-GFP-WPRE
pXRP062-pLenti2-EF1a-Cas9-$EAAAK_3$-NLS-KRAB-gLuc-2A-GFP-WPRE

FIG. 15B

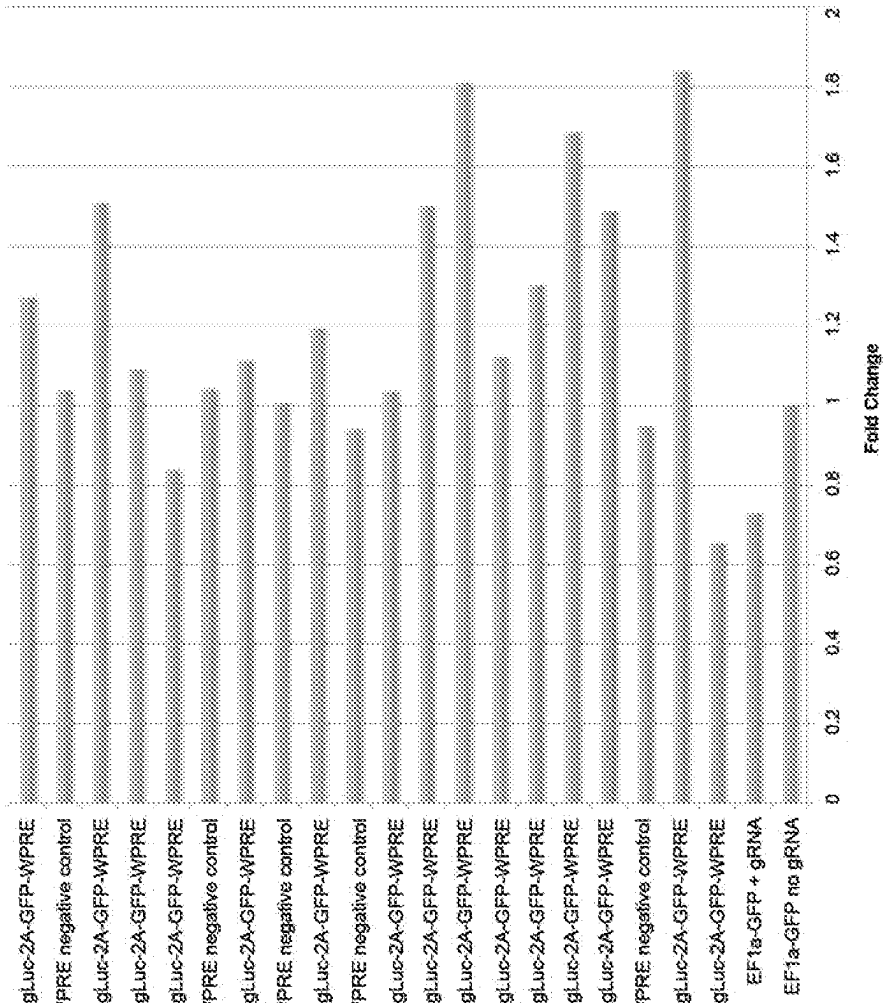
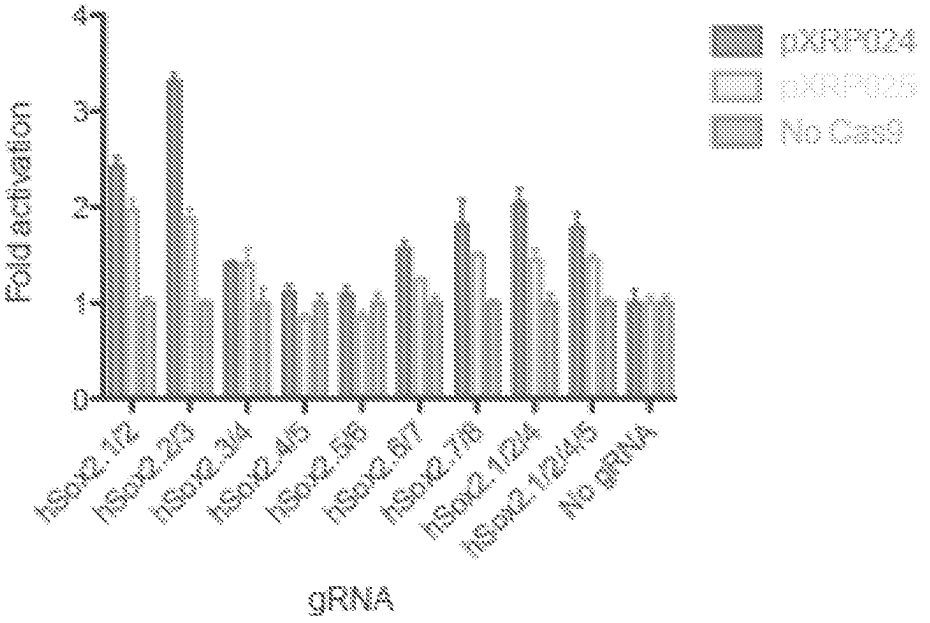

pXRP024-pLenti2-EF1a-VP64-NLS-FLAG-Linker-dCas9-NLS-gLuc-2A-GFP-WPRE
pXRP024-pLenti2-EF1a-VP64-NLS-FLAG-Linker-dCas9-NLS-gLuc-2A-GFP-WPRE negative control
pXRP025-pLenti2-EF1a-VP64-NLS-GGGGS3Linker-dCas9-NLS-gLuc-2A-GFP-WPRE
pXRP026-pLenti2-EF1a-VP64-NLS-EAAAK3Linker-dCas9-NLS-gLuc-2A-GFP-WPRE
pXRP030-pLenti2-pSV40-VP64-NLS-FLAG-Linker-dCas9-NLS-gLuc-2A-GFP-WPRE
pXRP030-pLenti2-pSV40-VP64-NLS-FLAG-Linker-dCas9-NLS-gLuc-2A-GFP-WPRE negative control
pXRP031-pLenti2-pPGK-VP64-NLS-FLAG-Linker-dCas9-NLS-gLuc-2A-GFP-WPRE
pXRP031-pLenti2-pPGK-VP64-NLS-FLAG-Linker-dCas9-NLS-gLuc-2A-GFP-WPRE negative control
pXRP032-pLenti2-LTR-VP64-NLS-FLAG-Linker-dCas9-NLS-gLuc-2A-GFP-WPRE
pXRP032-pLenti2-LTR-VP64-NLS-FLAG-Linker-dCas9-NLS-gLuc-2A-GFP-WPRE negative control
pXRP033-pLenti2-pSV40-VP64-NLS-GGGGS3-Linker-dCas9-NLS-gLuc-2A-GFP-WPRE
pXRP034-pLenti2-pPGK-VP64-NLS-GGGGS3-Linker-dCas9-NLS-gLuc-2A-GFP-WPRE
pXRP035-pLenti2-LTR-VP64-NLS-GGGGS3-Linker-dCas9-NLS-gLuc-2A-GFP-WPRE
pXRP036-pLenti2-pSV40-VP64-NLS-EAAAK3-Linker-dCas9-NLS-gLuc-2A-GFP-WPRE
pXRP037-pLenti2-pPGK-VP64-NLS-EAAAK3-Linker-dCas9-NLS-gLuc-2A-GFP-WPRE
pXRP038-pLenti2-LTR-VP64-NLS-EAAAK3-Linker-dCas9-NLS-gLuc-2A-GFP-WPRE
pXRP054-pLenti2-EF1a-dCas9-Linker-FLAG-NLS-VP64-gLuc-2A-GFP-WPRE
pXRP054-pLenti2-EF1a-dCas9-Linker-FLAG-NLS-VP64-gLuc-2A-GFP-WPRE negative control
pXRP057-pLenti2-EF1a-dCas9-GGGGGGS3-NLS-VP64-gLuc-2A-GFP-WPRE
pXRP060-pLenti2-EF1a-dCas9-EAAAK3-NLS-VP64-gLuc-2A-GFP-WPRE
EF1a-GFP + gRNA
EF1a-GFP no gRNA
0
0.2
0.4
0.6
0.8
1
1.2
1.4
1.6
1.8
2
Fold Change

FIG. 16

FIG. 17


pXRP024
pXRP025
No Cas9
Fold activation
0
1
2
3
4
hSpA2.1/2
hSpA2.2/3
hSpA2.3/4
hSpA2.4/5
hSpA2.5/6
hSpA2.6/7
hSpA2.7/8
hSpA2.1/2/4
hSpA2.1/2/4/5
No gRNA
gRNA

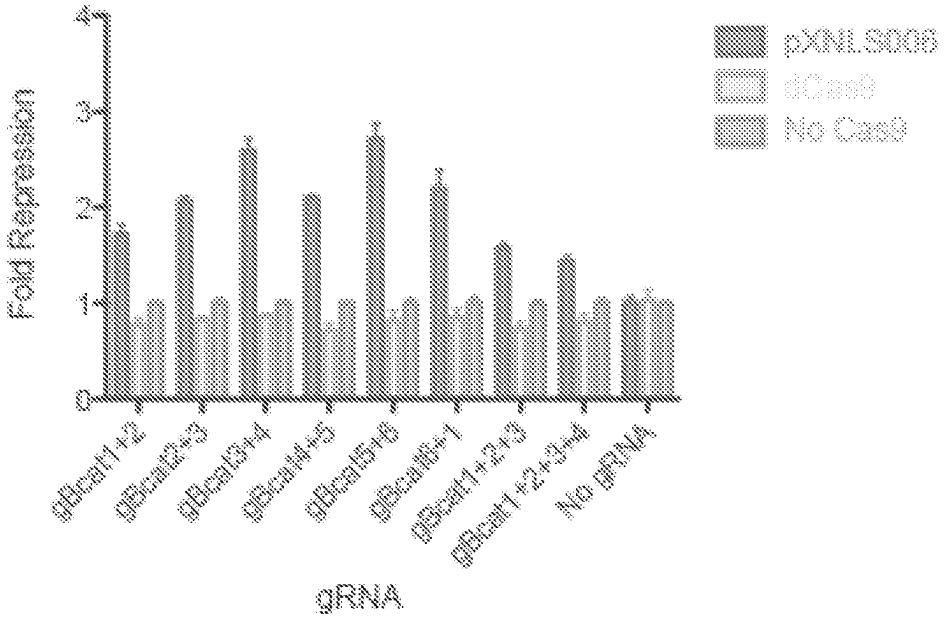

pXNLS008
dCas9
No Cas9
Fold Repression
0
1
2
3
4
gBcat1+2
gBcat2+3
gBcat3+4
gBcat4+5
gBcat5+6
gBcat6+1
gBcat1+2+3
gBcat1+2+3+4
No gRNA
gRNA

FIG. 18

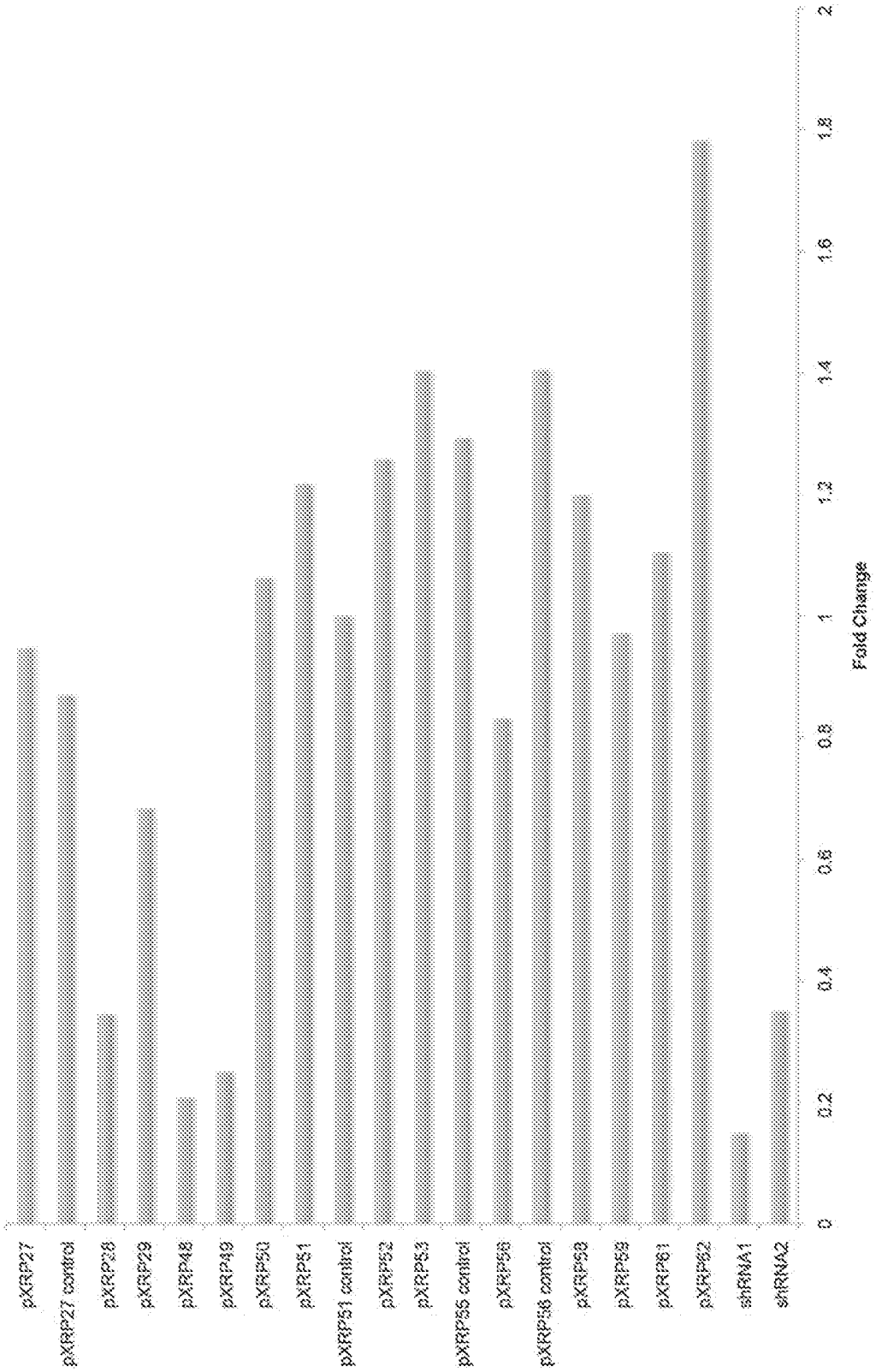

pXRP27
pXRP27 control
pXRP28
pXRP29
pXRP48
pXRP49
pXRP50
pXRP51
pXRP51 control
pXRP52
pXRP53
pXRP55 control
pXRP56
pXRP56 control
pXRP58
pXRP59
pXRP61
pXRP62
shRNA1
shRNA2
0
0.2
0.4
0.6
0.8
1
1.2
1.4
1.6
1.8
2
Fold Change

FIG. 19

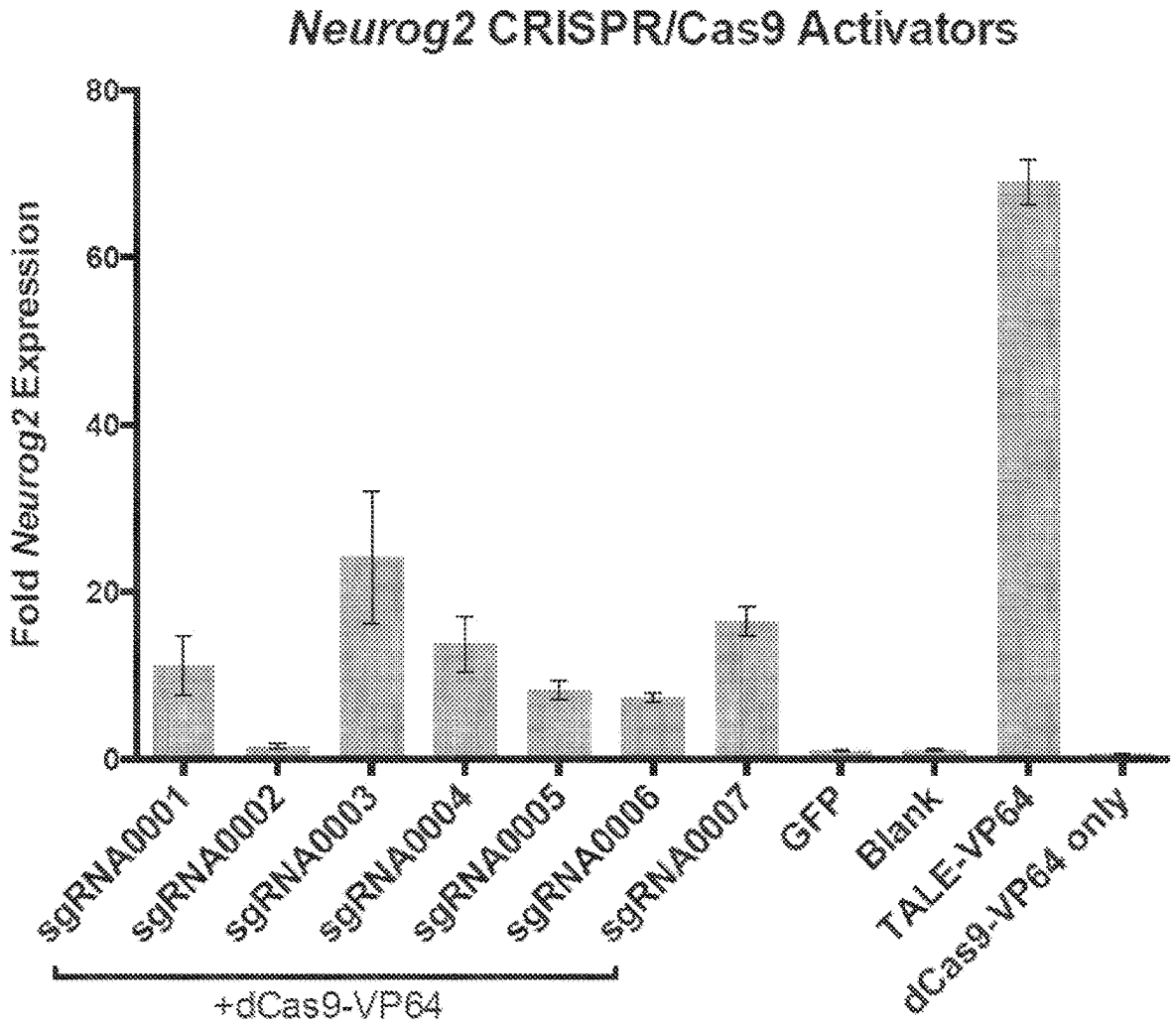

Neurog2 CRISPR/Cas9 Activators
Fold Neurog2 Expression
80
60
40
20
0
sgRNA0001
sgRNA0002
sgRNA0003
sgRNA0004
sgRNA0005
sgRNA0006
sgRNA0007
GFP
Blank
TALE-VP64
dCas9-VP64 only
+dCas9-VP64

FIG. 20

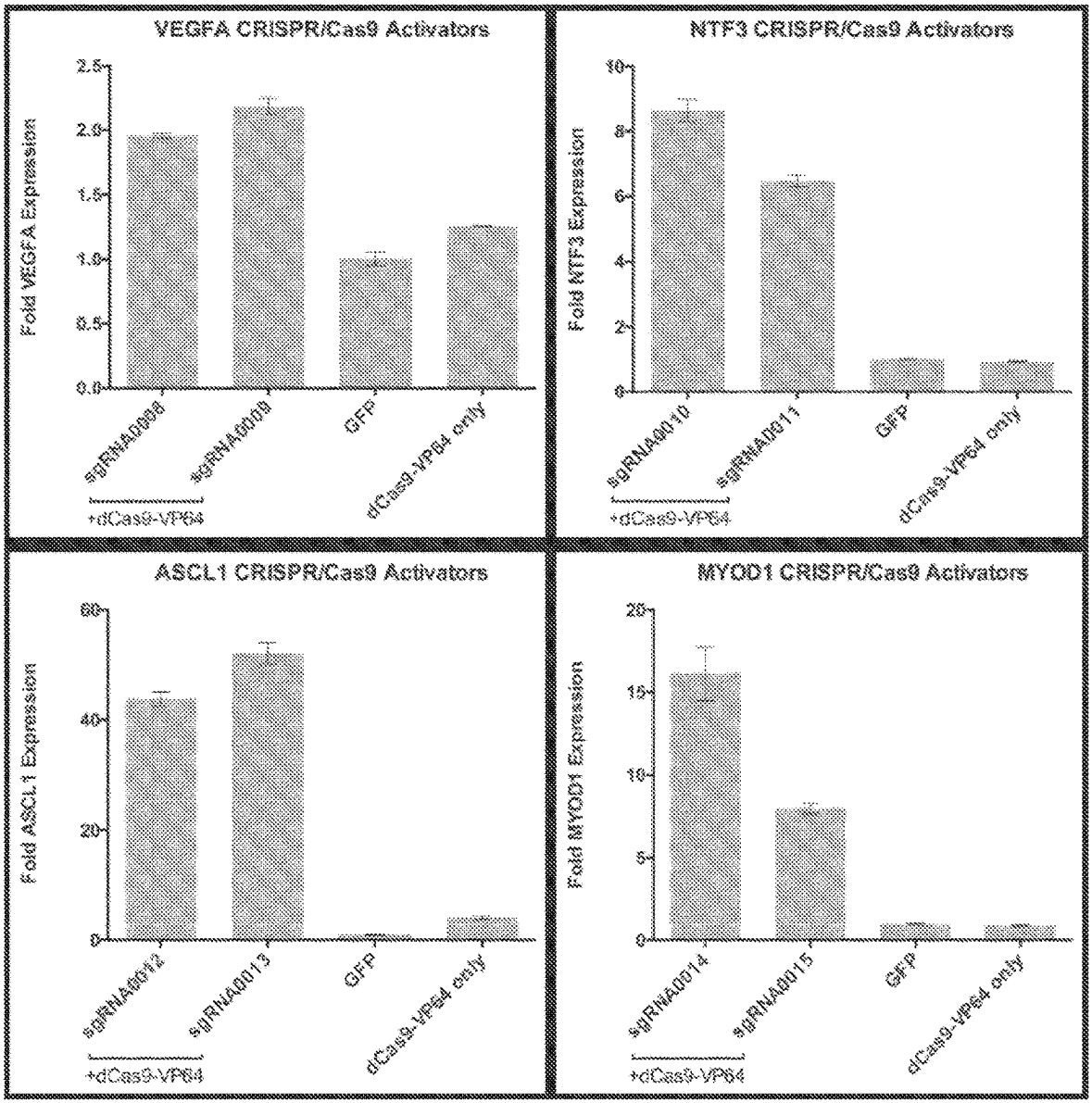

VEGFA CRISPR/Cas9 Activators
Fold VEGFA Expression
2.5
2.0
1.5
1.0
0.5
0.0
sgRNA0008
sgRNA0009
GFP
dCas9-VP64 only
+dCas9-VP64
NTF3 CRISPR/Cas9 Activators
Fold NTF3 Expression
10
8
6
4
2
0
sgRNA0010
sgRNA0011
GFP
dCas9-VP64 only
+dCas9-VP64
ASCL1 CRISPR/Cas9 Activators
Fold ASCL1 Expression
60
40
20
0
sgRNA0012
sgRNA0013
GFP
dCas9-VP64 only
+dCas9-VP64
MYOD1 CRISPR/Cas9 Activators
Fold MYOD1 Expression
20
15
10
5
0
sgRNA0014
sgRNA0015
GFP
dCas9-VP64 only
+dCas9-VP64

FIG. 21

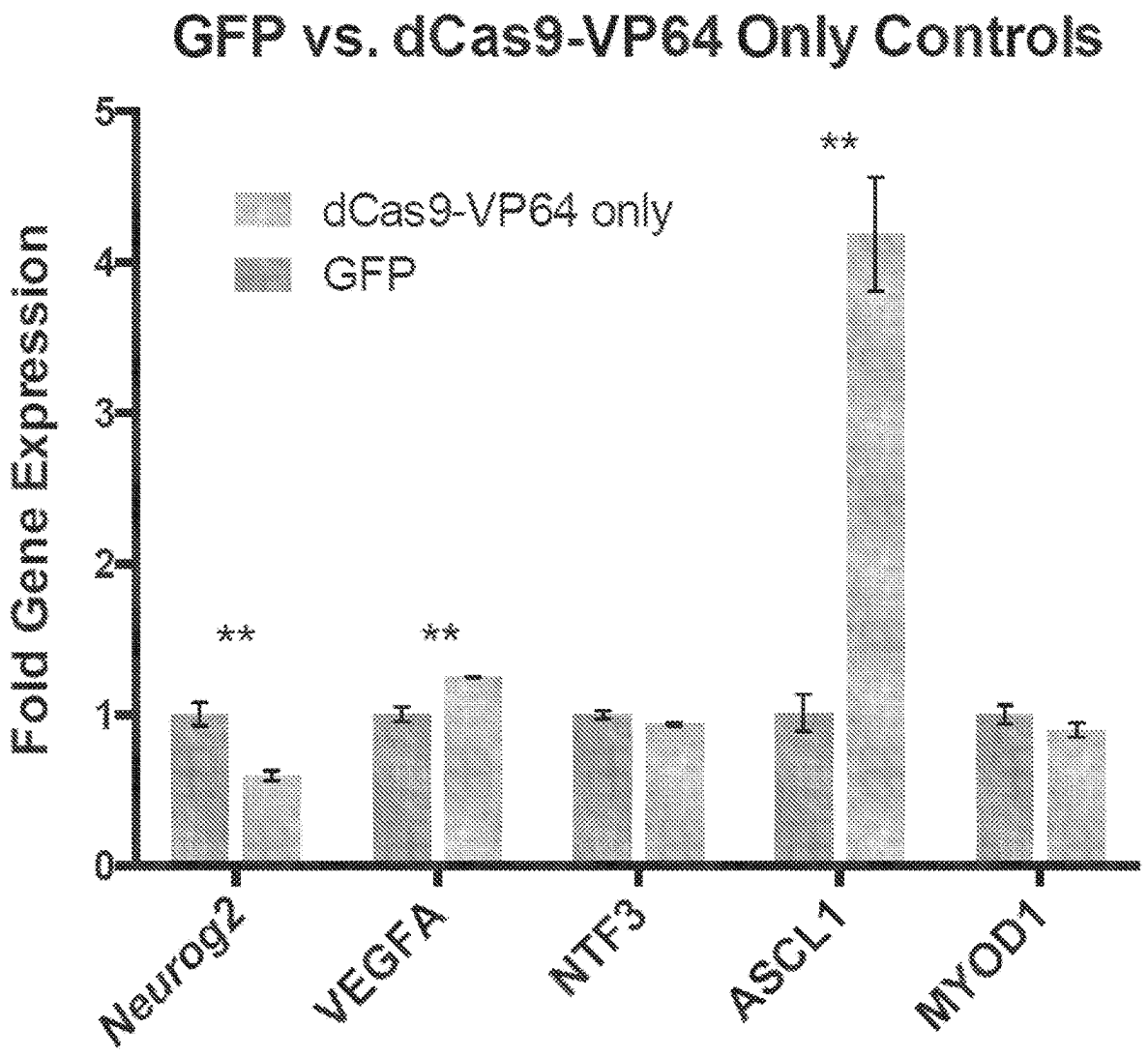

GFP vs. dCas9-VP64 Only Controls
Fold Gene Expression
0
1
2
3
4
5
dCas9-VP64 only
GFP
**
**
**
Neurog2
VEGFA
NTF3
ASCL1
MYOD1

FIG. 22

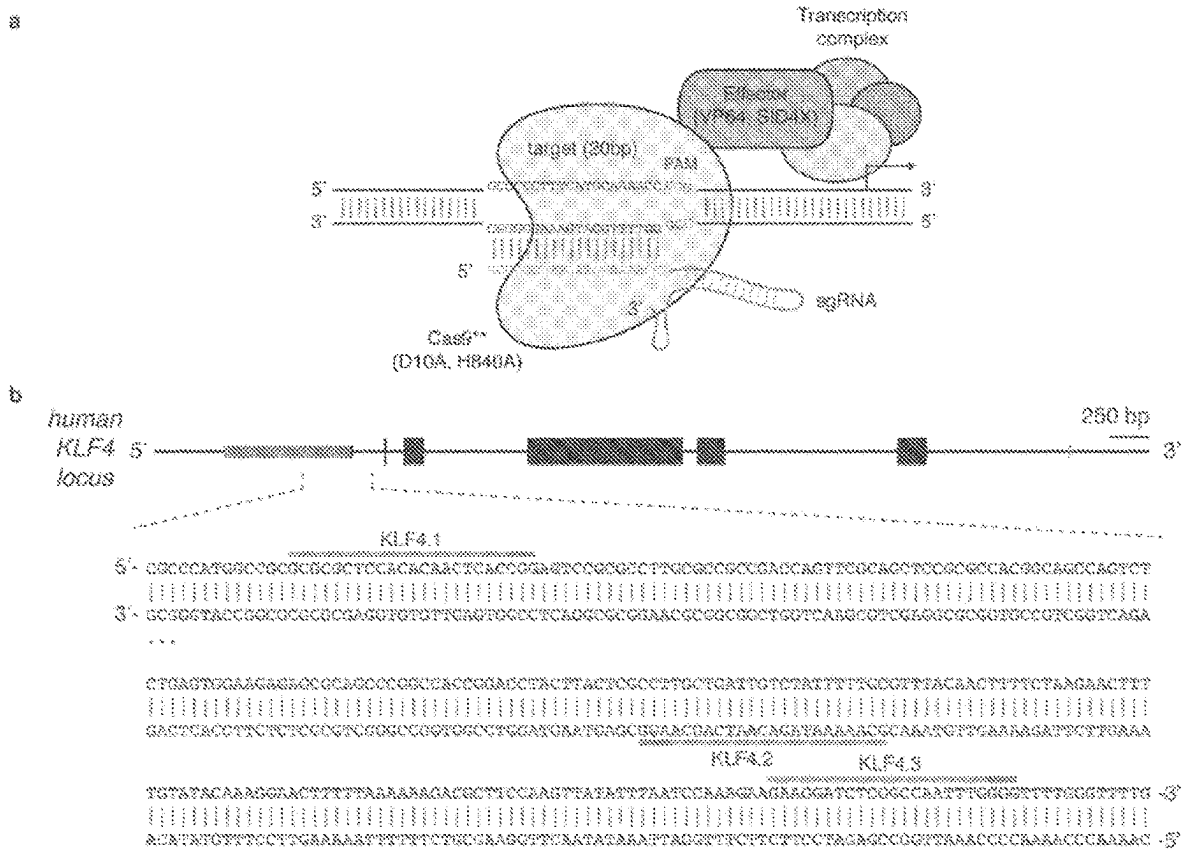

a
Transcription complex
target (20bp)
PAM
5'
3'
sgRNA
Cas9**
(D10A, H840A)
b
human KLF4 locus
250 bp
KLF4.1
KLF4.2
KLF4.3

FIG. 23A-B

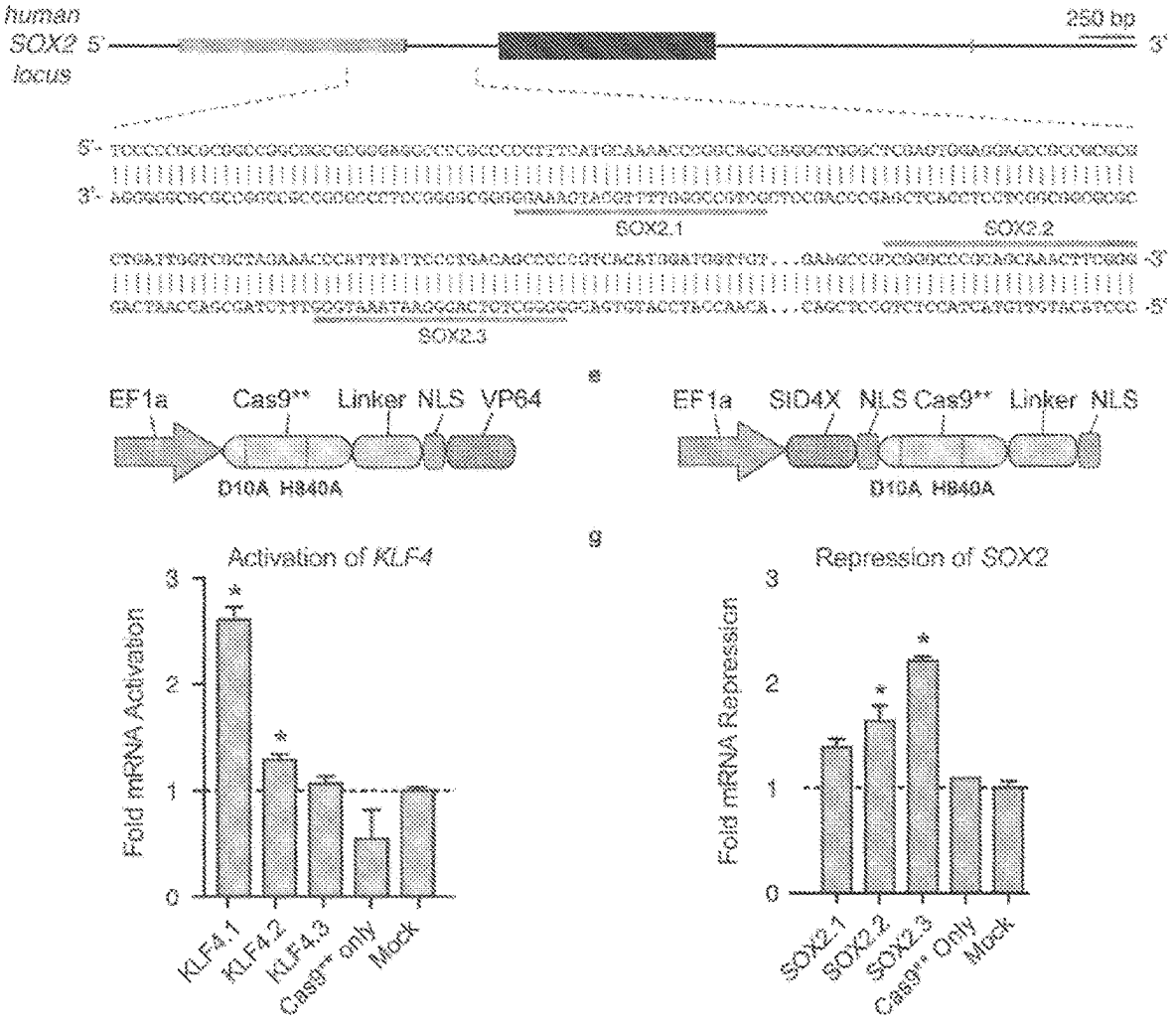

human
SOX2 locus
5'
3'
250 bp
SOX2.1
SOX2.2
SOX2.3
EF1a
Cas9**
Linker
NLS
VP64
D10A H840A
EF1a
SID4X
NLS
Cas9**
Linker
NLS
D10A H840A
Activation of KLF4
Fold mRNA Activation
KLF4.1
KLF4.2
KLF4.3
Cas9** only
Mock
Repression of SOX2
Fold mRNA Repression
SOX2.1
SOX2.2
SOX2.3
Cas9** only
Mock

FIG. 23C-G

hSpCas9(D10A,H840A)-Linker-NLS-VP64

MDKKYSIGLAIGTNSVGWAVITDEYKVPSKKFKVLGNTDRHSIKKNLIGALLFDSGETA
EATRLKRTARRRYTRRKNRICYLQEIFSNEMAKVDDSFFHRLEESFLVEEDKKHERHPIF
GNIVDEVAYHEKYPTIYHLRKKLVDSTDKADLRLIYLALAHMIKFRGHFLIEGDLNPDNS
DVDKLFIQLVQTYNQLFEENPINASGVDAKAILSARLSKSRRLENLIAQLPGEKKNGLFG
NLIALSLGLTPNFKSNFDLAEDAKLQLSKDTYDDDLDNLLAQIGDQYADLFLAAKNLSD
AILLSDILRVNTEITKAPLSASMIKRYDEHHQDLTLLKALVRQQLPEKYKEIFFDQSKNGY
AGYIDGGASQEEFYKFIKPILEKMDGTEELLVKLNREDLLRKQRTFDNGSIPHQIHLGEL
HAILRRQEDFYPFLKDNREKIEKILTFRIPYYVGPLARGNSRFAWMTRKSEETITPWNFEE
VVDKGASAQSFIERMTNFDKNLPNEKVLPKHSLLYEYFTVYNELTKVKYVTEGMRKPA
FLSGEQKKAIVDLLFKTNRKVTVKQLKEDYFKKIECFDSVEISGVEDRFNASLGTYHDLL
KIIKDKDFLDNEENEDILEDIVLTLTLFEDREMIEERLKTYAHLFDDKVMKQLKRRRYTG
WGRLSRKLINGIRDKQSGKTILDFLKSDGFANRNFMQLIHDDSLTFKEDIQKAQVSGQG
DSLHEHIANLAGSPAIKKGILQTVKVVDELVKVMGRHKPENIVIEMARENQTTQKGQKN
SRERMKRIEEGIKELGSQILKEHPVENTQLQNEKLYLYYLQNGRDMYVDQELDINRLSD
YDVDAIVPQSFLKDDSIDNKVLTRSDKNRGKSDNVPSEEVVKKMKNYWRQLLNAKLIT
QRKFDNLTKAERGGLSELDKAGFIKRQLVETRQITKHVAQILDSRMNTKYDENDKLIRE
VKVITLKSKLVSDFRKDFQFYKVREINNYHHAHDAYLNAVVGTALIKKYPKLESEFVYG
DYKVYDVRKMIAKSEQEIGKATAKYFFYSNIMNFFKTEITLANGEIRKRPLIETNGETGEI
VWDKGRDFATVRKVLSMPQVNIVKKTEVQTGGFSKESILPKRNSDKLIARKKDWDPKK
YGGFDSPTVAYSVLVVAKVEKGKSKKLKSVKELLGITIMERSSFEKNPIDFLEAKGYKE
VKKDLIIKLPKYSLFELENGRKRMLASAGELQKGNELALPSKYVNFLYLASHYEKLKGS
PEDNEQKQLFVEQHKHYLDEIIEQISEFSKRVILADANLDKVLSAYNKHRDKPIREQAENI
IHLFTLTNLGAPAAFKYFDTTIDRKRYTSTKEVLDATLIHQSITGLYETRIDLSQLGGDSA
GGGGSGGGGSGGGGSGPKKKRKVAAAGSGRADALDDFDLDMLGSDALDDFDLDMLG
SDALDDFDLDMLGSDALDDFDLDMLIN

FIG. 24

SID4X-NLS-FLAG-Linker-hSpCas9(D10A,H840A)-NLS

MGSGMNIQMLLEAADYLERREREAEHGYASMLPGSGMNIQMLLEAADYLERREREAE
HGYASMLPGSGMNIQMLLEAADYLERREREAEHGYASMLPGSGMNIQMLLEAADYLE
RREREAEHGYASMLPSRSAGPKKKRKVAAADYKDDDDKSRLEPGEKPYKCPECGKSFS
QSGALTRHQRTHTRDKKYSIGLAIGTNSVGWAVITDEYKVPSKKFKVLGNTDRHSIKKN
LIGALLFDSGETAEATRLKRTARRRYTRRKNRICYLQEIFSNEMAKVDDSFFHRLEESFL
VEEDKKHERHPIFGNIVDEVAYHEKYPTIYHLRKKLVDSTDKADLRLIYLALAHMIKFR
GHFLIEGDLNPDNSDVDKLFIQLVQTYNQLFEENPINASGVDAKAILSARLSKSRRLENLI
AQLPGEKKNGLFGNLIALSLGLTPNFKSNFDLAEDAKLQLSKDTYDDDLDNLLAQIGDQ
YADLFLAAKNLSDAILLSDILRVNTEITKAPLSASMIKRYDEHHQDLTLLKALVRQQLPE
KYKEIFFDQSKNGYAGYIDGGASQEEFYKFIKPILEKMDGTEELLVKLNREDLLRKQRTF
DNGSIPHQIHLGELHAILRRQEDFYPFLKDNREKIEKILTFRIPYYVGPLARGNSRFAWMT
RKSEETITPWNFEEVVDKGASAQSFIERMTNFDKNLPNEKVLPKHSLLYEYFTVYNELTK
VKYVTEGMRKPAFLSGEQKKAIVDLLFKTNRKVTVKQLKEDYFKKIECFDSVEISGVED
RFNASLGTYHDLLKIIKDKDFLDNEENEDILEDIVLTLTLFEDREMIEERLKTYAHLFDDK
VMKQLKRRRYTGWGRLSRKLINGIRDKQSGKTILDFLKSDGFANRNFMQLIHDDSLTFK
EDIQKAQVSGQGDSLHEHIANLAGSPAIKKGILQTVKVVDELVKVMGRHKPENIVIEMA
RENQTTQKGQKNSRERMKRIEEGIKELGSQILKEHPVENTQLQNEKLYLYYLQNGRDM
YVDQELDINRLSDYDVDAIVPQSFLKDDSIDNKVLTRSDKNRGKSDNVPSEEVVKKMK
NYWRQLLNAKLITQRKFDNLTKAERGGLSELDKAGFIKRQLVETRQITKHVAQILDSRM
NTKYDENDKLIREVKVITLKSKLVSDFRKDFQFYKVREINNYHHAHDAYLNAVVGTALI
KKYPKLESEFVYGDYKVYDVRKMIAKSEQEIGKATAKYFFYSNIMNFFKTEITLANGEIR
KRPLIETNGETGEIVWDKGRDFATVRKVLSMPQVNIVKKTEVQTGGFSKESILPKRNSDK
LIARKKDWDPKKYGGFDSPTVAYSVLVVAKVEKGKSKKLKSVKELLGITIMERSSFEKN
PIDFLEAKGYKEVKKDLIIKLPKYSLFELENGRKRMLASAGELQKGNELALPSKYVNFLY
LASHYEKLKGSPEDNEQKQLFVEQHKHYLDEIIEQISEFSKRVILADANLDKVLSAYNKH
RDKPIREQAENIIHLFTLTNLGAPAAFKYFDTTIDRKRYTSTKEVLDATLIHQSITGLYETR
IDLSQLGGDKRPAYTKKAGQAKKKK

FIG. 25

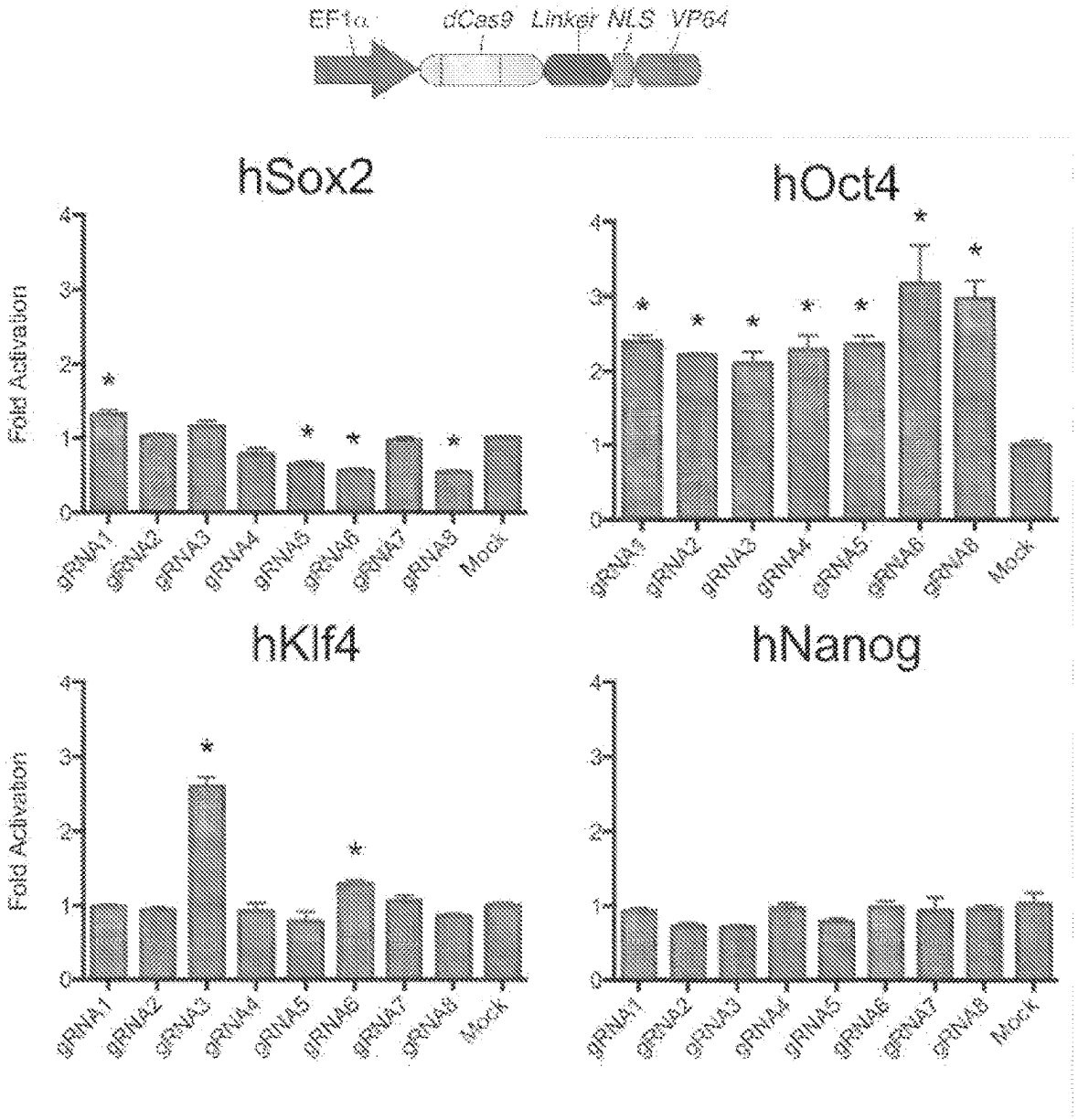

EF1α
dCas9
Linker
NLS
VP64
hSox2
hOct4
hKlf4
hNanog
Fold Activation
gRNA1
gRNA2
gRNA3
gRNA4
gRNA5
gRNA6
gRNA7
gRNA8
Mock

FIG. 26

EF1α
SID4X
NLS
Linker
dCas9
NLS

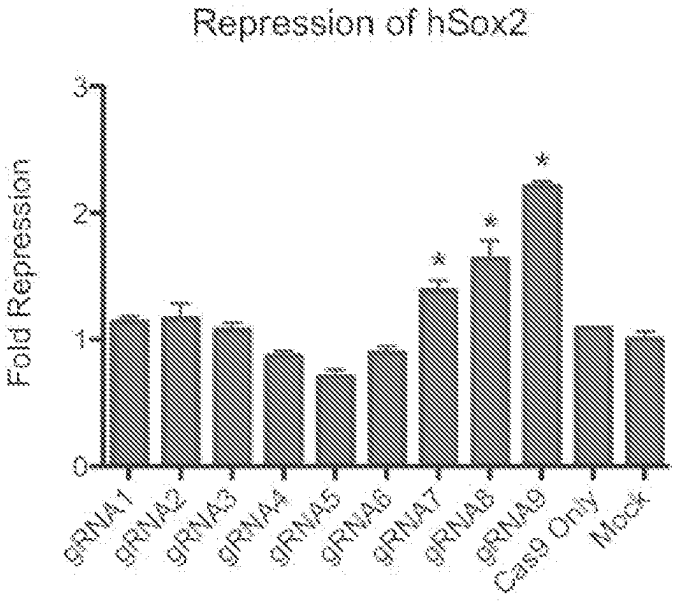

Repression of hSox2
Fold Repression
0
1
2
3
gRNA1
gRNA2
gRNA3
gRNA4
gRNA5
gRNA6
gRNA7
gRNA8
gRNA9
Cas9 Only
Mock

FIG. 27

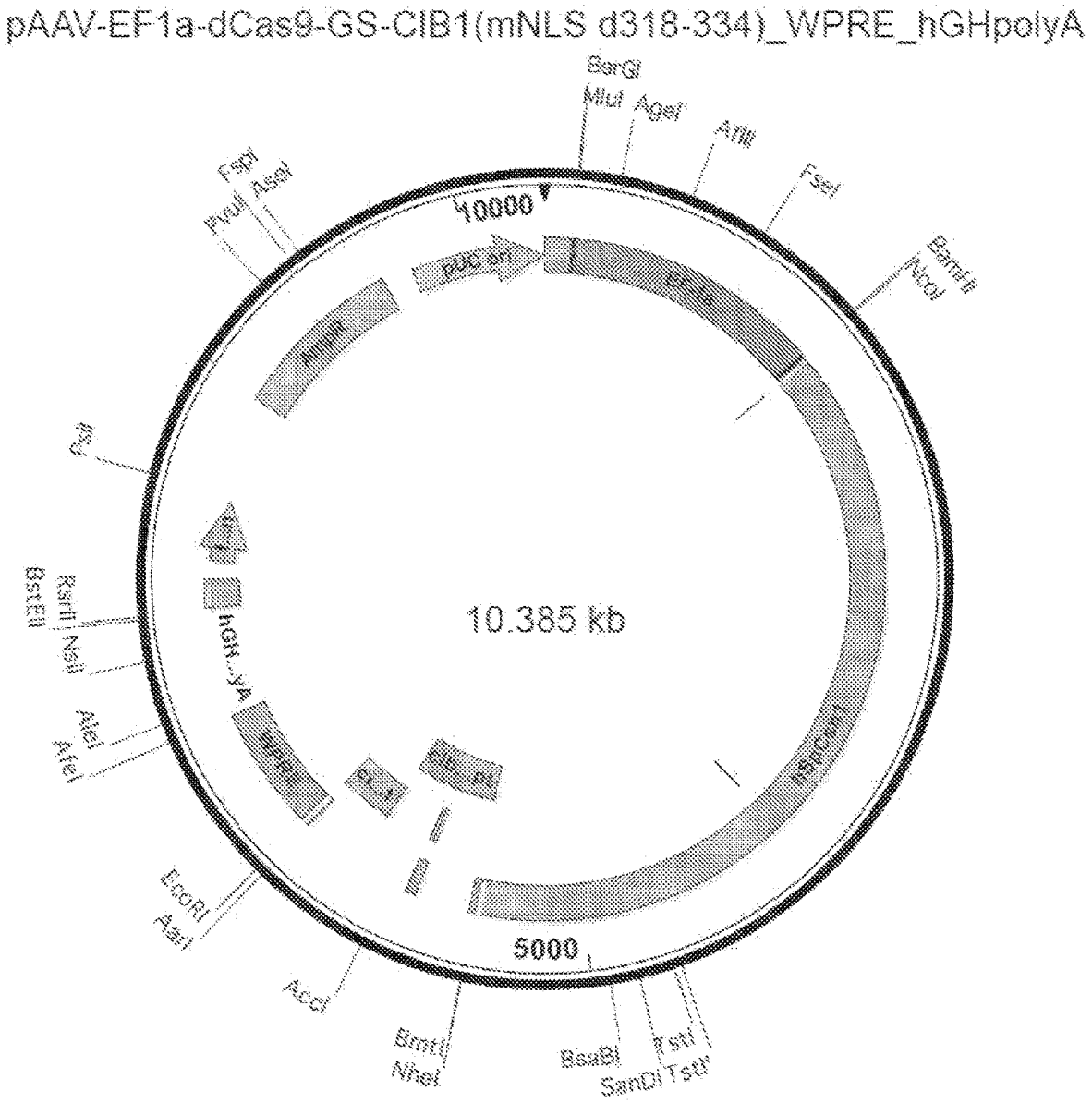

pAAV-EF1a-dCas9-GS-CIB1(mNLS d318-334)_WPRE_hGHpolyA
BsrGI
MluI
AgeI
AflII
FseI
BamHI
NotI
FspI
AseI
PvuII
10000
pUC ori
10.385 kb
AvrII
BstEII
RsrII
NsiI
AleI
AfeI
EcoRI
AsiI
AccI
BmtI
NheI
BsaBI
SanDI
TstI
TstII
5000

FIG. 28

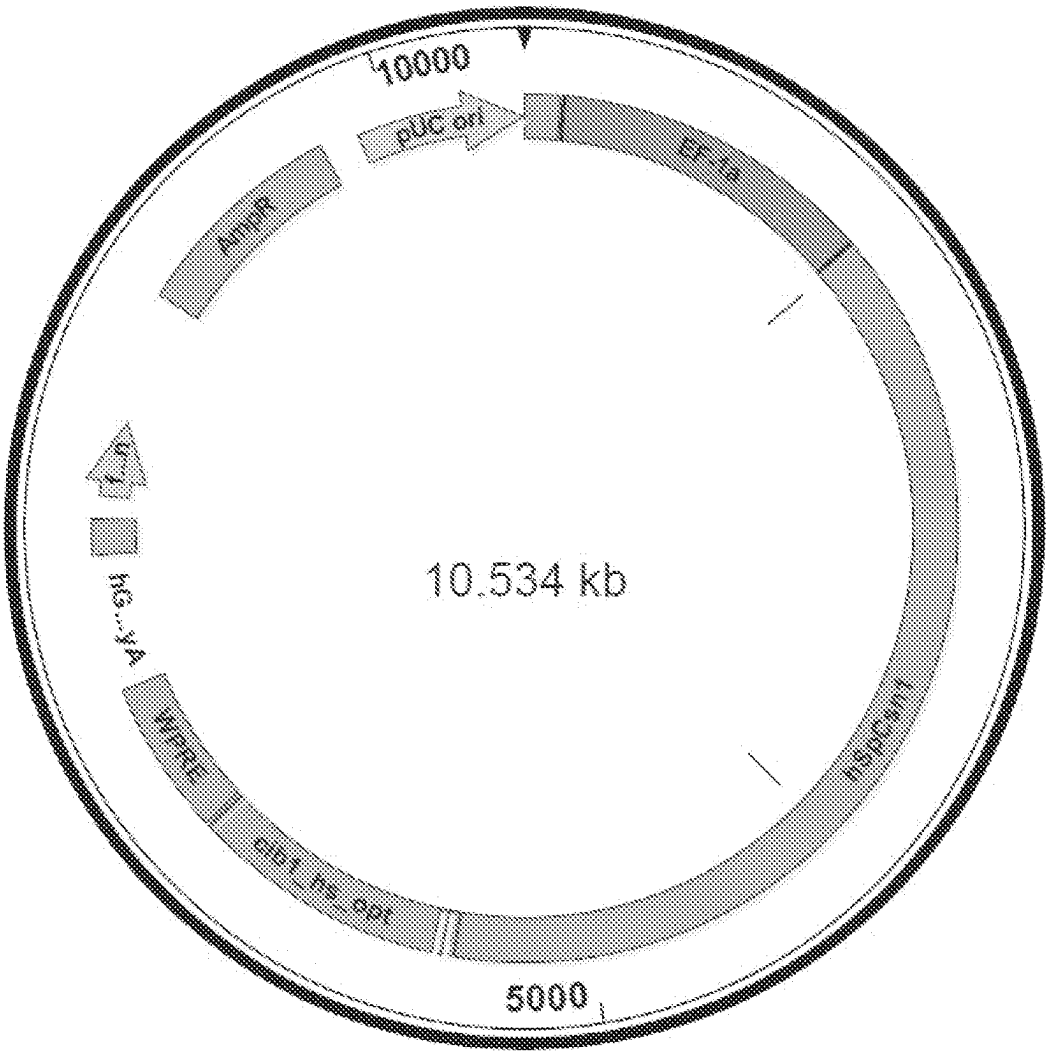


FIG. 29

pAAV-EF1a-dCas9-GS-NLS-NLS-cib1-WPRE-hGHpA

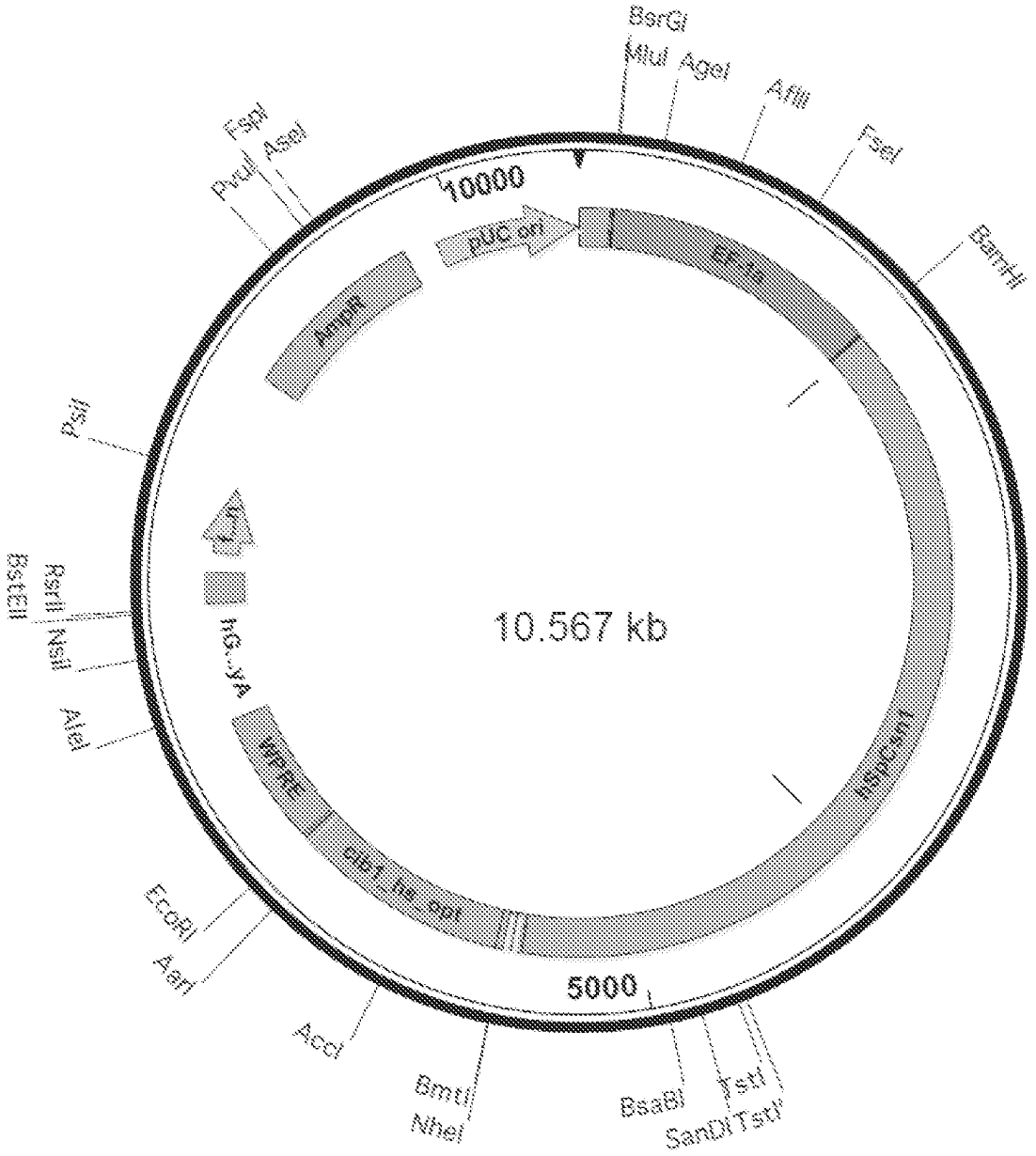


FIG. 30

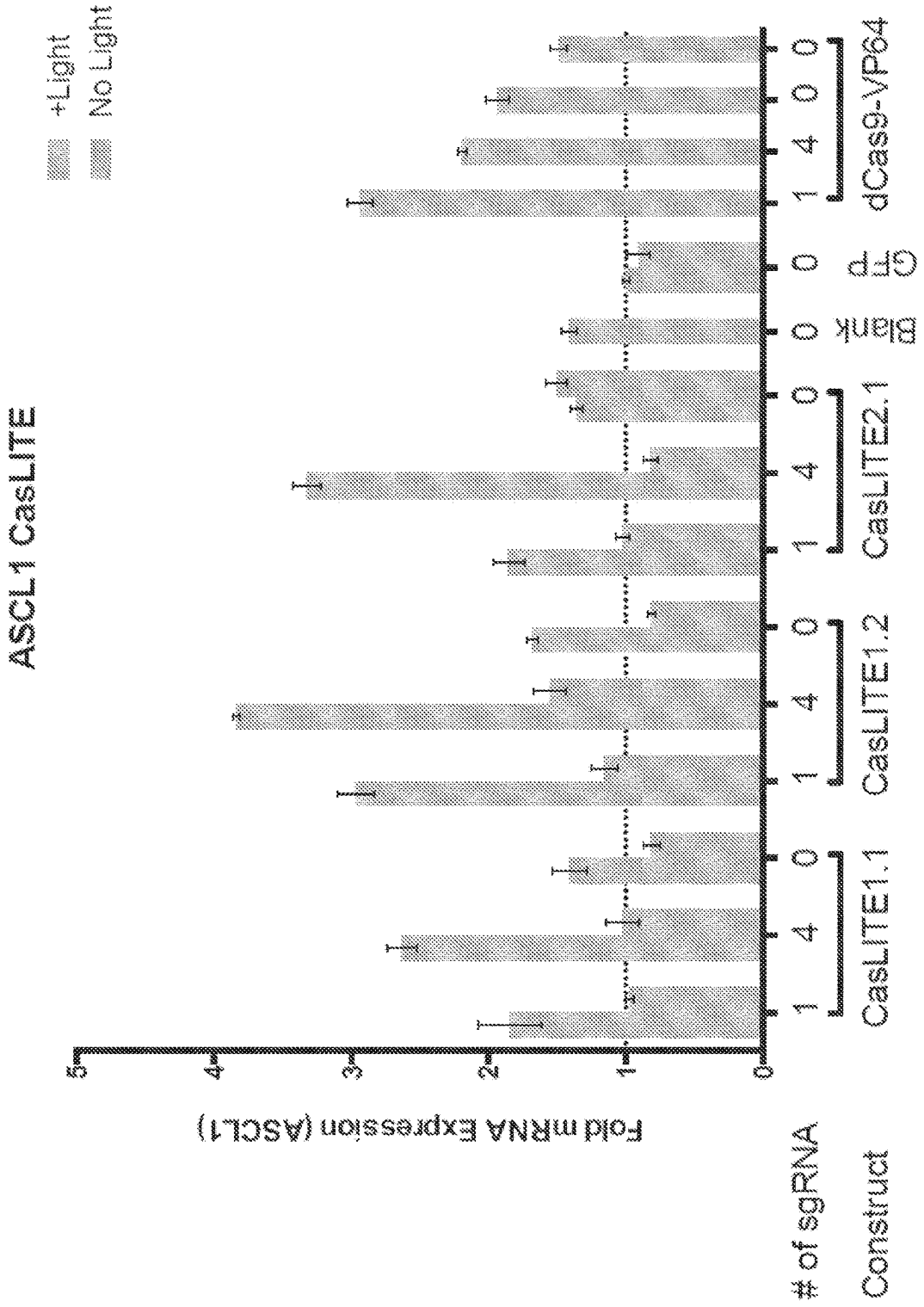
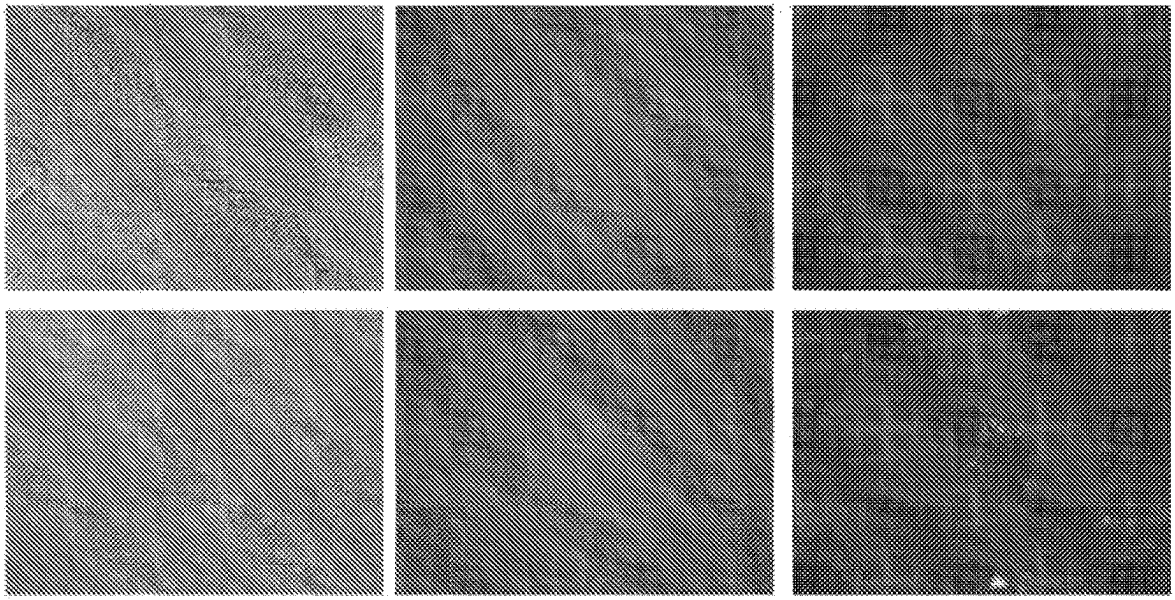
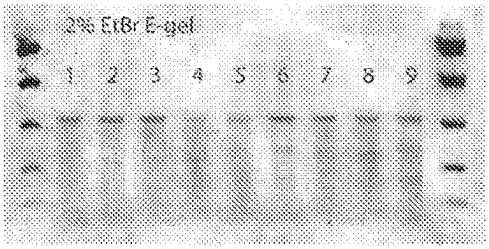

ASCL1 CasLITE
+Light
No Light
Fold mRNA Expression (ASCL1)
0
1
2
3
4
5
# of sgRNA
Construct
1
4
0
CasLITE1.1
1
4
0
CasLITE1.2
1
4
0
CasLITE2.1
0
Blank
0
GFP
1
4
0
0
dCas9-VP64

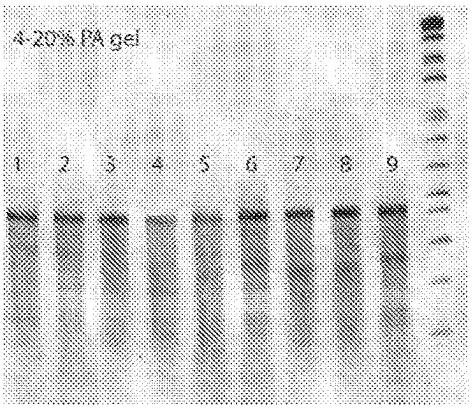
FIG. 31

mESCs (ROSA26-CAG-loxP-polyA-loxP-Cas9)
72 hour post-magnetofection
Fluorescence imaging and Surveyor assay



Top: sgRNA_mShank3.1
Bottom: sgRNA_mShank3.1 + Cre





Lane	% Cutting
1. Neg	
2. GFP	
3. Cre	
4. sgRNA_mShank3.1 + Cre	18
5. sgRNA_mShank3.2 + Cre	18
6. sgRNA_mShank3.3 + Cre	22
7. sgRNA_mShank3.1	32
8. sgRNA_mShank3.2	28
9. sgRNA_mShank3.3	27

FIG. 32

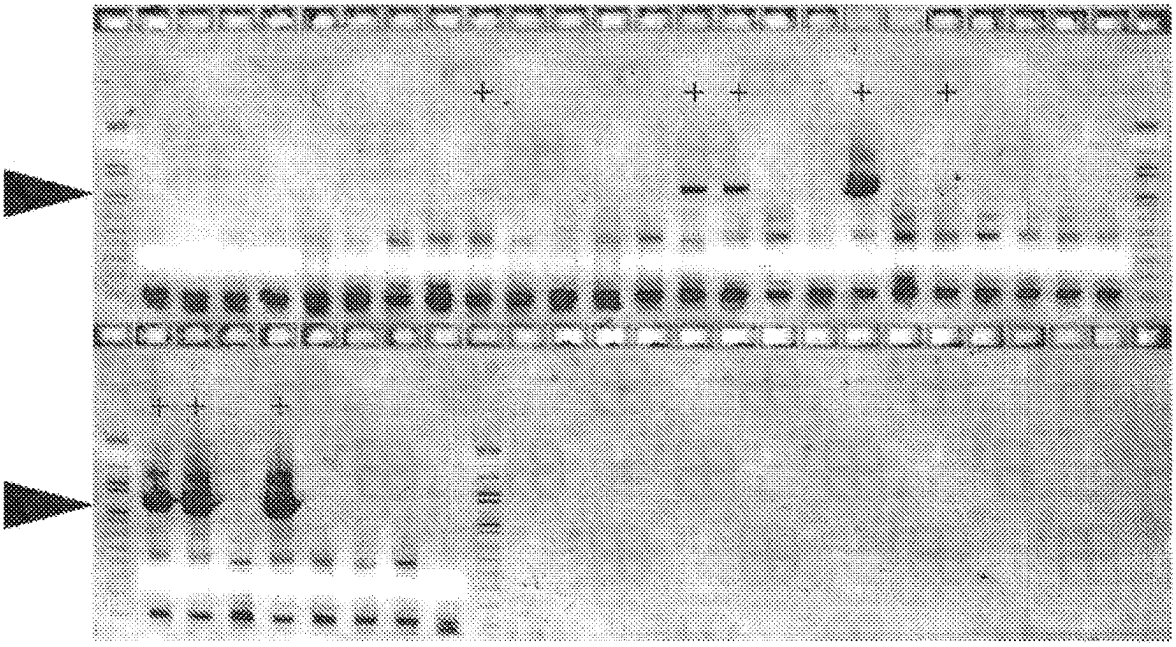


FIG. 33

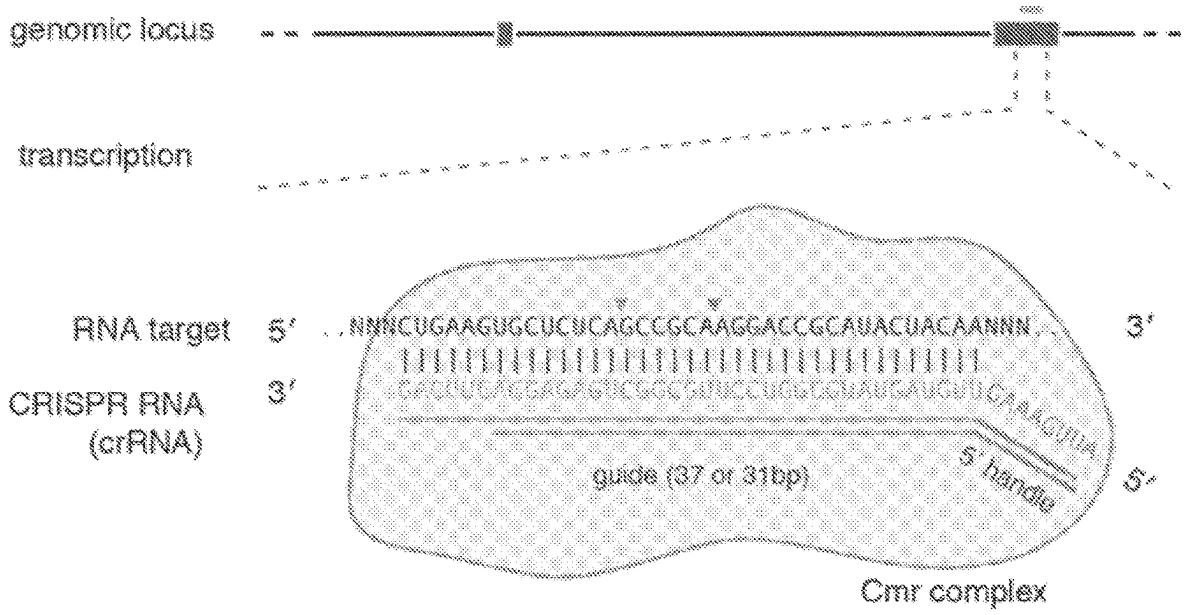
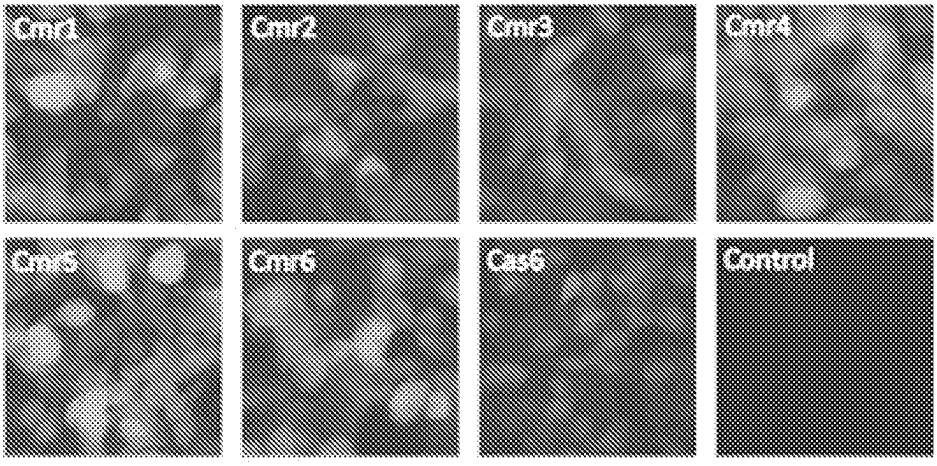

genomic locus
transcription
RNA target
5′
NNNCUGAAGUGCUCUCAGCCGCAAGGACCGCAUACUACAANNN
3′
CRISPR RNA
(crRNA)
3′
guide (37 or 31bp)
5′ handle
5′
Cmr complex

FIG. 34

A


Cmr1
Cmr2
Cmr3
Cmr4
Cmr5
Cmr6
Cas6
Control

B

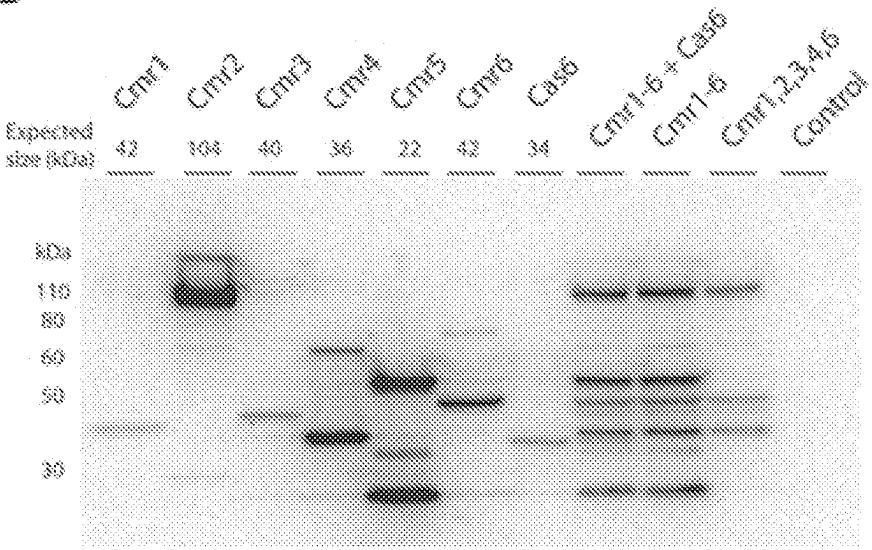

Cmr1
Cmr2
Cmr3
Cmr4
Cmr5
Cmr6
Cas6
Cmr1-6 + Cas6
Cmr1-6
Cmr1,2,3,4,6
Control
Expected size (kDa)
42
104
40
36
22
42
34
kDa
110
80
60
50
30

FIG. 35A-B

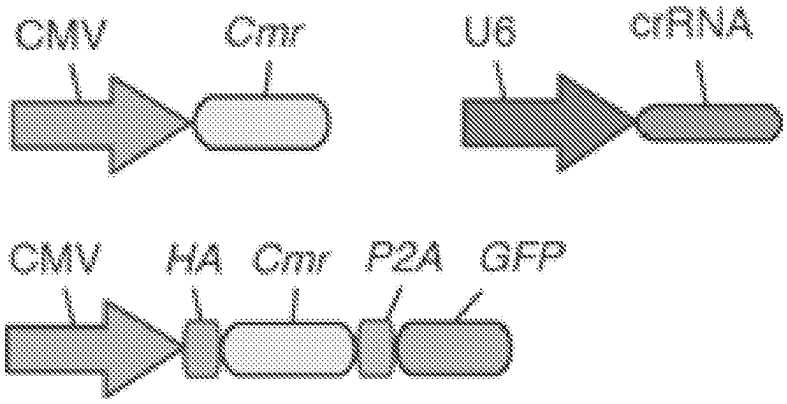


FIG. 36

ENGINEERING AND OPTIMIZATION OF SYSTEMS, METHODS AND COMPOSITIONS FOR SEQUENCE MANIPULATION WITH FUNCTIONAL DOMAINS

RELATED APPLICATIONS AND INCORPORATION BY REFERENCE

[0001] This application is a continuation of U.S. patent application Ser. No. 19/025,722, filed Jan. 16, 2025, which is a continuation of U.S. patent application Ser. No. 14/738,398, filed Jun. 12, 2015, which is a continuation-in-part of international patent application serial no. PCT/US2013/074736 filed Dec. 12, 2013 and published as WO 2014/093655, which claims priority to U.S. provisional patent application 61/835,936 entitled ENGINEERING AND OPTIMIZATION OF SYSTEMS, METHODS AND COMPOSITIONS FOR SEQUENCE MANIPULATION WITH FUNCTIONAL DOMAINS FILED ON Jun. 17, 2013, and which also claims priority to U.S. provisional patent applications 61/758,468; 61/769,046; 61/802,174; 61/806,375; 61/814,263; 61/819,803 and 61/828,130 each entitled ENGINEERING AND OPTIMIZATION OF SYSTEMS, METHODS AND COMPOSITIONS FOR SEQUENCE MANIPULATION, filed on Jan. 30, 2013; Feb. 25, 2013; Mar. 15, 2013; Mar. 28, 2013; Apr. 20, 2013; May 6, 2013 and May 28, 2013 respectively, and which also claims priority to U.S. provisional patent applications 61/736,527 and 61/748,427, both entitled SYSTEMS METHODS AND COMPOSITIONS FOR SEQUENCE MANIPULATION filed on Dec. 12, 2012 and Jan. 2, 2013, respectively, and which also claims priority to U.S. provisional patent applications 61/791,409 and 61/835,931 both entitled BI-2011/008/44790.02.2003 and BI-2011/008/44790.03.2003 filed on Mar. 15, 2013 and Jun. 17, 2013 respectively.

[0002] Reference is also made to international patent applications PCT/US2013/074611 filed Dec. 12, 2013 and published as WO 2014/093595; PCT/US2013/074743 filed Dec. 12, 2013 and published as WO 2014/093661; PCT/US2013/074790 filed Dec. 12, 2013 and published as WO 2014/093694; PCT/US2013/074825 filed Dec. 12, 2013 and published as WO 2014/093718; PCT/US2013/074812 filed Dec. 12, 2013 and published as WO 2014/093709; PCT/US2013/074667 filed Dec. 12, 2013 and published as WO 2014/093622; PCT/US2013/074691 filed Dec. 12, 2013 and published as WO 2014/093635; PCT/US2013/074819 filed Dec. 12, 2013 and published as WO 2014/093712; and PCT/US2013/074800 filed Dec. 12, 2013 and published as WO 2014/093701.

[0003] Reference is also made to U.S. provisional patent applications 61/757,972 filed Jan. 29, 2013, 61/799,800 filed Mar. 15, 2013; 61/835,936, 61/835,973, 61/836,080, 61/836,101, 61/836,123, 61/836,127, and 61/847,537, each filed Jun. 17, 2013; 61/862,468 and 61/862,355, each filed Aug. 5, 2013; 61/871,301 filed Aug. 28, 2013; 61/969,777 filed Sep. 25, 2013; and 61/961,980 filed Oct. 28, 2013.

[0004] The foregoing applications, and all documents cited therein or during their prosecution ("appln cited documents") and all documents cited or referenced in the appln cited documents, and all documents cited or referenced herein ("herein cited documents"), and all documents cited or referenced in herein cited documents, together with any manufacturer's instructions, descriptions, product specifications, and product sheets for any products mentioned herein or in any document incorporated by reference herein, are hereby incorporated herein by reference, and may be employed in the practice of the invention. More specifically, all referenced documents are incorporated by reference to the same extent as if each individual document was specifically and individually indicated to be incorporated by reference.

STATEMENT AS TO FEDERALLY SPONSORED RESEARCH

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

FIELD OF THE INVENTION

[0006] The present invention generally relates to the engineering and optimization of systems, methods and compositions used for the control of gene expression involving sequence targeting, such as genome perturbation or gene-editing, that relate to Clustered Regularly Interspaced Short Palindromic Repeats (CRISPR) and components thereof.

SEQUENCE LISTING

[0007] The instant application contains a Sequence Listing which has been submitted electronically in XML file format and is hereby incorporated by reference in its entirety. Said XML copy, created on Apr. 16, 2025, is named 114203-6219_SL.xml and is 4,728 bytes in size.

BACKGROUND OF THE INVENTION

[0008] Recent advances in genome sequencing techniques and analysis methods have significantly accelerated the ability to catalog and map genetic factors associated with a diverse range of biological functions and diseases. Precise genome targeting technologies are needed to enable systematic reverse engineering of causal genetic variations by allowing selective perturbation of individual genetic elements, as well as to advance synthetic biology, biotechnological, and medical applications. Although genome-editing techniques such as designer zinc fingers, transcription activator-like effectors (TALEs), or homing meganucleases are available for producing targeted genome perturbations, there remains a need for new genome engineering technologies that are affordable, easy to set up, scalable, and amenable to targeting multiple positions within the eukaryotic genome.

SUMMARY OF THE INVENTION

[0009] The CRISPR/Cas or the CRISPR-Cas system (both terms are used interchangeably throughout this application) does not require the generation of customized proteins to target specific sequences but rather a single Cas enzyme can be programmed by a short RNA molecule to recognize a specific DNA target, in other words the Cas enzyme can be recruited to a specific DNA target using said short RNA molecule. Adding the CRISPR-Cas system to the repertoire of genome sequencing techniques and analysis methods may significantly simplify the methodology and accelerate the ability to catalog and map genetic factors associated with a diverse range of biological functions and diseases. To utilize the CRISPR-Cas system effectively for genome editing without deleterious effects, it is critical to understand aspects

of engineering and optimization of these genome engineering tools, which are aspects of the claimed invention.

[0010] There exists a pressing need for alternative and robust systems and techniques for sequence targeting with a wide array of applications. Aspects of this invention address this need and provide related advantages. An exemplary CRISPR complex may comprise a CRISPR enzyme complexed with a guide sequence hybridized to a target sequence within the target polynucleotide. The guide sequence is linked to a tracr mate sequence, which in turn hybridizes to a tracr sequence.

[0011] In one aspect, the invention relates to one or more elements of a CRISPR system having improved or modified functionality. The CRISPR complex of the invention provides an effective means for modifying a target polynucleotide. The CRISPR complex of the invention has a wide variety of utilities including modifying (e.g., deleting, inserting, translocating, inactivating, activating) a target polynucleotide in a multiplicity of cell types. As such the CRISPR complex of the invention has a broad spectrum of applications in, e.g., gene or genome editing, gene therapy, drug discovery, drug screening, disease diagnosis, and prognosis. An exemplary CRISPR complex may comprise a CRISPR enzyme complexed with a guide sequence hybridized to a target sequence within the target polynucleotide. The guide sequence is linked to a tracr mate sequence, which in turn hybridizes to a tracr sequence.

[0012] In some embodiments, a CRISPR/Cas system may comprise: (a) a first regulatory element operably linked to a tracr mate sequence and one or more insertion sites for inserting one or more guide sequences upstream of the tracr mate sequence, wherein when expressed, the guide sequence directs sequence-specific binding of a CRISPR complex to a target sequence in a eukaryotic cell, wherein the CRISPR complex may comprise a CRISPR enzyme complexed with (1) the guide sequence that is hybridized to the target sequence, and (2) the tracr mate sequence that is hybridized to the tracr sequence; and (b) a second regulatory element operably linked to an enzyme-coding sequence encoding said CRISPR enzyme which may comprise a nuclear localization sequence; wherein components (a) and (b) are located on the same or different vectors of the system. In some embodiments, component (a) further may comprise the tracr sequence downstream of the tracr mate sequence under the control of the first regulatory element. In some embodiments, component (a) further may comprise two or more guide sequences operably linked to the first regulatory element, wherein when expressed, each of the two or more guide sequences direct sequence specific binding of a CRISPR complex to a different target sequence in a eukaryotic cell. In some embodiments, the system may comprise the tracr sequence under the control of a third regulatory element, such as a polymerase III promoter. In some embodiments, the tracr sequence exhibits at least 50%, 60%, 70%, 80%, 90%, 95%, or 99% of sequence complementarity along the length of the tracr mate sequence when optimally aligned. In some embodiments, the CRISPR enzyme may comprise one or more nuclear localization sequences of sufficient strength to drive accumulation of said CRISPR enzyme in a detectable amount in the nucleus of a eukaryotic cell. Without wishing to be bound by theory, it is believed that a nuclear localization sequence is not necessary for CRISPR activity in eukaryotes, but that including such sequences enhances activity of the system. In some embodiments, the CRISPR enzyme is a type II CRISPR system enzyme. In some embodiments, the CRISPR enzyme is a Cas9 enzyme. In some embodiments, the Cas9 enzyme is *S. pyogenes* or *S. thermophilus* Cas9, and may include mutated Cas9 derived from either of these organisms. The enzyme may be a Cas9 homolog or ortholog. In some embodiments, the CRISPR enzyme is codon-optimized for expression in a eukaryotic cell. In some embodiments, the CRISPR enzyme directs cleavage of one or two strands at the location of the target sequence. In some embodiments, the CRISPR enzyme lacks DNA strand cleavage activity. In some embodiments, the first regulatory element is a polymerase III promoter. In some embodiments, the second regulatory element is a polymerase II promoter. In some embodiments, the guide sequence is at least 15, 16, 17, 18, 19, 20, 25 nucleotides, or between 10-30, or between 15-25, or between 15-20 nucleotides in length. In general, and throughout this specification, the term "vector" refers to a nucleic acid molecule capable of transporting another nucleic acid to which it has been linked. Vectors include, but are not limited to, nucleic acid molecules that are single-stranded, double-stranded, or partially double-stranded; nucleic acid molecules that comprise one or more free ends, no free ends (e.g. circular); nucleic acid molecules that comprise DNA, RNA, or both; and other varieties of polynucleotides known in the art. One type of vector is a "plasmid," which refers to a circular double stranded DNA loop into which additional DNA segments can be inserted, such as by standard molecular cloning techniques. Another type of vector is a viral vector, wherein virally-derived DNA or RNA sequences are present in the vector for packaging into a virus (e.g. retroviruses, replication defective retroviruses, adenoviruses, replication defective adenoviruses, and adeno-associated viruses). Viral vectors also include polynucleotides carried by a virus for transfection into a host cell. Certain vectors are capable of autonomous replication in a host cell into which they are introduced (e.g. bacterial vectors having a bacterial origin of replication and episomal mammalian vectors). Other vectors (e.g., non-episomal mammalian vectors) are integrated into the genome of a host cell upon introduction into the host cell, and thereby are replicated along with the host genome. Moreover, certain vectors are capable of directing the expression of genes to which they are operatively-linked. Such vectors are referred to herein as "expression vectors." Common expression vectors of utility in recombinant DNA techniques are often in the form of plasmids.

[0013] Recombinant expression vectors can comprise a nucleic acid of the invention in a form suitable for expression of the nucleic acid in a host cell, which means that the recombinant expression vectors include one or more regulatory elements, which may be selected on the basis of the host cells to be used for expression, that is operatively-linked to the nucleic acid sequence to be expressed. Within a recombinant expression vector, "operably linked" is intended to mean that the nucleotide sequence of interest is linked to the regulatory element(s) in a manner that allows for expression of the nucleotide sequence (e.g. in an in vitro transcription/translation system or in a host cell when the vector is introduced into the host cell).

[0014] The term "regulatory element" is intended to include promoters, enhancers, internal ribosomal entry sites (IRES), and other expression control elements (e.g. transcription termination signals, such as polyadenylation signals and poly-U sequences). Such regulatory elements are

described, for example, in Goeddel, GENE EXPRESSION TECHNOLOGY: METHODS IN ENZYMOLOGY 185, Academic Press, San Diego, Calif. (1990). Regulatory elements include those that direct constitutive expression of a nucleotide sequence in many types of host cell and those that direct expression of the nucleotide sequence only in certain host cells (e.g., tissue-specific regulatory sequences). A tissue-specific promoter may direct expression primarily in a desired tissue of interest, such as muscle, neuron, bone, skin, blood, specific organs (e.g. liver, pancreas), or particular cell types (e.g. lymphocytes). Regulatory elements may also direct expression in a temporal-dependent manner, such as in a cell-cycle dependent or developmental stage-dependent manner, which may or may not also be tissue or cell-type specific. In some embodiments, a vector may comprise one or more pol III promoter (e.g. 1, 2, 3, 4, 5, or more pol III promoters), one or more pol II promoters (e.g. 1, 2, 3, 4, 5, or more pol II promoters), one or more pol I promoters (e.g. 1, 2, 3, 4, 5, or more pol I promoters), or combinations thereof. Examples of pol III promoters include, but are not limited to, U6 and H1 promoters. Examples of pol II promoters include, but are not limited to, the retroviral Rous sarcoma virus (RSV) LTR promoter (optionally with the RSV enhancer), the cytomegalovirus (CMV) promoter (optionally with the CMV enhancer) [see, e.g., Boshart et al, Cell, 41:521-530 (1985)], the SV40 promoter, the dihydrofolate reductase promoter, the 3-actin promoter, the phosphoglycerol kinase (PGK) promoter, and the EF1α promoter. Also encompassed by the term "regulatory element" are enhancer elements, such as WPRE; CMV enhancers; the R-U5' segment in LTR of HTLV-I (Mol. Cell. Biol., Vol. 8(1), p. 466-472, 1988); SV40 enhancer; and the intron sequence between exons 2 and 3 of rabbit β-globin (Proc. Natl. Acad. Sci. USA., Vol. 78(3), p. 1527-31, 1981). It will be appreciated by those skilled in the art that the design of the expression vector can depend on such factors as the choice of the host cell to be transformed, the level of expression desired, etc. A vector can be introduced into host cells to thereby produce transcripts, proteins, or peptides, including fusion proteins or peptides, encoded by nucleic acids as described herein (e.g., clustered regularly interspersed short palindromic repeats (CRISPR) transcripts, proteins, enzymes, mutant forms thereof, fusion proteins thereof, etc.).

[0015] Aspects of the invention relate to methods of improving or modifying the target specificity of a CRISPR enzyme (preferably a Cas9 enzyme) which may comprise: a) selecting the CRISPR enzyme having a smaller size for easy packaging into delivery vectors; or b) generating chimeric CRISPR enzymes; or c) utilizing mutated CRISPR (preferably Cas9) enzymes. Further aspects of the invention also relate to methods and compositions for improving target specificity involving two CRISPR enzymes (double nickase), improving the sgRNA scaffold by making improvement to other components aside from the guide RNA.

[0016] The invention comprehends a non-naturally occurring or engineered composition comprising a vector system that may comprise one or more vectors comprising I. a first regulatory element operably linked to a CRISPR/Cas system chimeric RNA (chiRNA) polynucleotide sequence, wherein the polynucleotide sequence may comprise (a) a guide sequence capable of hybridizing to a target sequence in a cell, (b) a tracr mate sequence, and (c) a tracr sequence, and II. a second regulatory element operably linked to an enzyme-coding sequence encoding a CRISPR enzyme comprising at least one or more nuclear localization sequences, wherein (a), (b) and (c) are arranged in a 5' to 3' orientation, wherein components I and II are located on the same or different vectors of the system, wherein when transcribed, the tracr mate sequence hybridizes to the tracr sequence and the guide sequence directs sequence-specific binding of a CRISPR complex to the target sequence, wherein the CRISPR complex comprises the CRISPR enzyme complexed with (1) the guide sequence that is hybridized to the target sequence, and (2) the tracr mate sequence that is hybridized to the tracr sequence, wherein the CRISPR enzyme may comprise two or more mutations, such that the enzyme has altered nuclease activity compared with the wild type enzyme, and wherein the enzyme-coding sequence further encodes one or more heterologous functional domains.

[0017] The invention further comprehends a multiplexed two component CRISPR enzyme system composition comprising a vector system that may comprise one or more vectors comprising I. a first regulatory element operably linked to a CRISPR/Cas system chimeric RNA (chiRNA) polynucleotide sequence, wherein the polynucleotide sequence comprises (a) a guide sequence capable of hybridizing to a target sequence in a cell, (b) a tracr mate sequence, and (c) a tracr sequence, and II. a second regulatory element operably linked to an enzyme-coding sequence encoding a CRISPR enzyme may comprise at least one or more nuclear localization sequences, wherein (a), (b) and (c) are arranged in a 5' to 3' orientation, wherein components I and II are located on the same or different vectors of the system, wherein when transcribed, the tracr mate sequence hybridizes to the tracr sequence and the guide sequence directs sequence-specific binding of a CRISPR complex to the target sequence, wherein the CRISPR complex comprises the CRISPR enzyme complexed with (1) the guide sequence that is hybridized to the target sequence, and (2) the tracr mate sequence that is hybridized to the tracr sequence, wherein the CRISPR enzyme comprises two or more mutations, such that the enzyme has altered nuclease activity compared with the wild type enzyme, wherein the enzyme-coding sequence further encodes one or more heterologous functional domains, and wherein in the multiplexed system composition multiple chiRNA polynucleotide sequences are used.

[0018] The invention further comprehends a non-naturally occurring or engineered composition comprising a vector system that may comprise one or more vectors comprising I. a first regulatory element operably linked to (a) a guide sequence capable of hybridizing to a target sequence in a cell, and (b) at least one or more tracr mate sequences, II. a second regulatory element operably linked to an enzyme-coding sequence encoding a CRISPR enzyme, and III. a third regulatory element operably linked to a tracr sequence, wherein components I, II and III are located on the same or different vectors of the system, wherein when transcribed, the tracr mate sequence hybridizes to the tracr sequence and the guide sequence directs sequence-specific binding of a CRISPR complex to the target sequence, wherein the CRISPR complex comprises the CRISPR enzyme complexed with (1) the guide sequence that is hybridized to the target sequence, and (2) the tracr mate sequence that is hybridized to the tracr sequence, wherein the CRISPR enzyme may comprise two or more mutations, such that the

enzyme has altered nuclease activity compared with the wild type enzyme, and wherein the enzyme-coding sequence further encodes one or more heterologous functional domains.

[0019] The invention also comprehends a multiplexed three component CRISPR enzyme system composition comprising a vector system that may comprise one or more vectors comprising I. a first regulatory element operably linked to (a) a guide sequence capable of hybridizing to a target sequence in a cell, and (b) at least one or more tracr mate sequences, II. a second regulatory element operably linked to an enzyme-coding sequence encoding a CRISPR enzyme, and III. a third regulatory element operably linked to a tracr sequence, wherein components I, II and III are located on the same or different vectors of the system, wherein when transcribed, the tracr mate sequence hybridizes to the tracr sequence and the guide sequence directs sequence-specific binding of a CRISPR complex to the target sequence, wherein the CRISPR complex comprises the CRISPR enzyme complexed with (1) the guide sequence that is hybridized to the target sequence, and (2) the tracr mate sequence that is hybridized to the tracr sequence, wherein the CRISPR enzyme comprises two or more mutations, such that the enzyme has altered nuclease activity compared with the wild type enzyme, wherein the enzyme-coding sequence further encodes one or more heterologous functional domains, and wherein in the multiplexed system composition multiple guide sequences capable of hybridizing to multiple target sequences are used.

[0020] In embodiments of the invention the CRISPR enzyme may comprise one or more mutations in two or more catalytically active domains. In another embodiment the CRISPR enzyme has reduced or abolished nuclease activity compared with the wild type enzyme. In another embodiment the two mutations are D10A SpCas9 in a first catalytically active domain and H840A SpCas9 in a second catalytically active domain or corresponding residues of other CRISPR enzymes. In another embodiment the CRISPR enzyme may comprise two or more mutations in a residue selected from the group consisting of D10, E762, H840, N854, N863, or D986. In another embodiment the CRISPR enzyme may comprise two or more mutations selected from the group comprising D10A, E762A, H840A, N854A, N863A or D986A. In a preferred embodiment the CRISPR enzyme is a DNA binding protein that does not direct cleavage of either strand at the location of the target sequence. In an embodiment, each of the two or more mutations is in a catalytically active domain of the CRISPR enzyme selected from the group comprising RuvCI, RuvCII, RuvCIII or HNH domain. It will be understood throughout this application that many references to specific amino acid residues are to those of the SpCas9 enzyme. The skilled person will understand that the invention is applicable to other Cas enzymes, including Cas9 enzymes from other sources, and that where reference is made to specific SpCas9 enzyme residues corresponding alterations and mutations may be made in those other Cas enzymes. For example, the skilled person will be able to compare sequences of the SpCas9 and other enzymes, and identify corresponding residues and domains, and hence to make appropriate modifications to those enzymes.

[0021] In further embodiments, the compositions of the invention may comprise at least two or more nuclear localization sequences. In an aspect of the invention the functional domain is a transcriptional activation domain, e. g. VP64. In another aspect the functional domain is a transcriptional repressor domain, e.g. a KRAB domain, a SID domain or a SID4X domain. In another embodiment, the enzyme coding sequence encodes one, two, three, four, five or more heterologous functional domains fused to the CRISPR enzyme. The invention also comprehends one or more linker sequences between any two domains. Embodiments of the invention include one or more functional domains having one or more of the following activities: methylase activity, demethylase activity, transcription activation activity, transcription repression activity, transcription release factor activity, histone modification activity, RNA cleavage activity and nucleic acid binding activity. In another embodiment, the functional domain binds DNA and/or affects transcription of the target nucleic acid. In aspects of the invention the cell is a prokaryotic or eukaryotic cell. In a preferred embodiment, the cell is a mammalian cell or a human cell. In some embodiments, the mammalian cell may be a rodent (for example, mouse or rat) cell, an ungulate cell, or a primate cell. In some embodiments, the eukaryotic cell may be an arthropod (eg, insect) or nematode cell. In other embodiments the cell may be a plant cell (including algae) or a fungal cell. In further embodiments of the invention the CRISPR enzyme is codon optimized for expression in a eukaryotic cell; the CRISPR enzyme is a type II CRISPR enzyme; the CRISPR enzyme is a Cas9 enzyme and the Cas9 enzyme is from an organism selected from the group comprising of genus *Streptococcus, Campylobacter, Nitratifractor, Staphylococcus, Parvibaculum, Roseburia, Neisseria, Gluconacetobacter*, Azospirillum, Sphaerochaeta, *Lactobacillus, Eubacterium* or *Corynebacter*. In a further aspect, the vectors of the system are viral vectors selected from the group comprising of a lentiviral vector, an adenoviral vector or an AAV vector.

[0022] The invention also comprehends methods of modulating, i.e. altering, activating, repressing, gene expression at a genomic locus of interest in a cell by contacting the cell with compositions of the invention.

[0023] An aspect of the invention relates to a composition which may comprise a vector system which may comprise one or more vectors which may comprise

[0024] I. a first regulatory element operably linked to a CRISPR/Cas system chimeric RNA (chiRNA) polynucleotide sequence, wherein the polynucleotide sequence may comprise

[0025] (a) a guide sequence capable of hybridizing to a target sequence in a eukaryotic cell,

[0026] (b) a tracr mate sequence, and

[0027] (c) a tracr sequence, and

[0028] II. a second regulatory element operably linked to an enzyme-coding sequence encoding a CRISPR enzyme which may comprise at least one or more nuclear localization sequences,

[0029] wherein (a), (b) and (c) are arranged in a 5' to 3' orientation,

[0030] wherein components I and II are located on the same or different vectors of the system,

[0031] wherein when transcribed, the tracr mate sequence hybridizes to the tracr sequence and the guide sequence directs sequence-specific binding of a CRISPR complex to the target sequence,

[0032] wherein the CRISPR complex may comprise the CRISPR enzyme complexed with (1) the guide

sequence that is hybridized to the target sequence, and (2) the tracr mate sequence that is hybridized to the tracr sequence, and

[0033] wherein the enzyme coding sequence encoding the CRISPR enzyme further encodes a heterologous functional domain.

[0034] The coding sequence may encode one or more heterologous functional domains (e.g. about or more than about 1, 2, 3, 4, 5, 6, 7, 8, 9, 10, or more domains in addition to the CRISPR enzyme). A CRISPR enzyme fusion protein may comprise any additional protein sequence, and optionally a linker sequence between any two domains. Examples of protein domains that may be fused to a CRISPR enzyme include, without limitation, epitope tags, reporter gene sequences, and protein domains having one or more of the following activities: methylase activity, demethylase activity, transcription activation activity, transcription repression activity, transcription release factor activity, histone modification activity, RNA cleavage activity and nucleic acid binding activity. Non-limiting examples of epitope tags include histidine (His) tags, V5 tags, FLAG tags, influenza hemagglutinin (HA) tags, Myc tags, VSV-G tags, and thioredoxin (Trx) tags. Examples of reporter genes include, but are not limited to, glutathione-S-transferase (GST), horseradish peroxidase (HRP), chloramphenicol acetyltransferase (CAT) beta-galactosidase, beta-glucuronidase, luciferase, green fluorescent protein (GFP), HcRed, DsRed, cyan fluorescent protein (CFP), yellow fluorescent protein (YFP), and autofluorescent proteins including blue fluorescent protein (BFP). A CRISPR enzyme may be fused to a gene sequence encoding a protein or a fragment of a protein that bind DNA molecules or bind other cellular molecules, including but not limited to maltose binding protein (MBP), S-tag, Lex A DNA binding domain (DBD) fusions, GAL4 DNA binding domain fusions, and herpes simplex virus (HSV) BP16 protein fusions. Additional domains that may form part of a fusion protein which may comprise a CRISPR enzyme are described in US20110059502, incorporated herein by reference. In Chalasani, US2011/0059502, it is stated, "In certain embodiments, the activity of a second domain in a fusion protein can be about 25% to about 90% more specific than the activity of a second domain not in a fusion protein. In some embodiments, a second domain can comprise an endonuclease activity. In certain embodiments, a second domain can comprise a type II endonuclease activity . . . type IIs endonuclease activity (e.g., Fok I . . .)." Accordingly, a functional domain as herein can be a FokI domain. In some embodiments, a tagged CRISPR enzyme is used to identify the location of a target sequence.

[0035] In an aspect of the invention, the functional domain affects transcription of the target nucleic acid.

[0036] Non-limiting examples of CRISPR enzymes include Cas1, Cas1B, Cas2, Cas3, Cas4, Cas5, Cas6, Cas7, Cas8, Cas9 (also known as Csn1 and Csx12), Cas10, Csy1, Csy2, Csy3, Cse1, Cse2, Csc1, Csc2, Csa5, Csn2, Csm2, Csm3, Csm4, Csm5, Csm6, Cmr1, Cmr3, Cmr4, Cmr5, Cmr6, Csb1, Csb2, Csb3, Csx17, Csx14, Csx10, Csx16, CsaX, Csx3, Csx1, Csx15, Csf1, Csf2, Csf3, Csf4, homologues thereof, or modified versions thereof. These enzymes are known; for example, the amino acid sequence of *S. pyogenes* Cas9 protein may be found in the SwissProt database (available at the website uniprot.org) under accession number Q99ZW2. In some embodiments, the CRISPR enzyme is a Cas9 enzyme. In some embodiments, the Cas9 enzyme is *S. pneumoniae, S. pyogenes* or *S. thermophilus* Cas9, and may include mutated Cas9 derived from these organisms. In some embodiments, the CRISPR enzyme is codon-optimized for expression in a eukaryotic cell. In some embodiments, the CRISPR enzyme directs cleavage of one or two strands at the location of the target sequence. In some embodiments, the CRISPR enzyme lacks DNA strand cleavage activity.

[0037] In some aspects of the invention, a CRISPR enzyme may comprise one or more mutations and may be used as a generic DNA binding protein with or without fusion to a functional domain. The mutations may include but are not limited to catalytic mutations, for instance mutations in one of the catalytic domains or mutation of catalytic residues. Preferred examples of suitable catalytic mutations are the catalytic residue(s) in the N-term RuvC I domain of Cas9 or the catalytic residue(s) in the internal HNH domain. In some embodiments, the Cas9 is (or is derived from) SpCas9. In such embodiments, preferred mutations are at any or all or positions 10, 762, 840, 854, 863 and/or 986 of SpCas9 or corresponding positions in other Cas9s (which may be ascertained for instance by standard sequence comparison tools). In particular, any or all of the following mutations are preferred in SpCas9: D10A, E762A, H840A, N854A, N863A and/or D986A; as well as conservative substitution for any of the replacement amino acids is also envisaged. The same (or conservative substitutions of these mutations) at corresponding positions in other Cas9s are also preferred. Particularly preferred are D10 and H840 in SpCas9. However, in other Cas9s, residues corresponding to SpCas9 D10 and H840 are also preferred.

[0038] In a more advantageous aspect of the invention the mutated CRISPR enzyme may be fused to a transcriptional activation domain. In one aspect of the invention, the transcriptional activation domain may be VP64. Other aspects of the invention relate to the mutated CRISPR enzyme being fused to domains which include but are not limited to a transcriptional repressor, a recombinase, a transposase, a histone remodeler, a DNA methyltransferase, a cryptochrome, a light inducible/controllable domain or a chemically inducible/controllable domain.

[0039] In some aspects of the invention, the CRISPR enzyme is comprised of less than four thousand, and in some aspects less than one thousand, amino acids. Such enzymes may be provided fused to a heterologous functional domain, or not. Where the enzyme is fused to a heterologous functional domain, the size of the enzyme (less than four thousand, less than one thousand amino acids) refers to the CRISPR portion of the fusion protein. In certain embodiments described herein, the invention or method is practiced on an organism or subject. In certain embodiments, the organism or subject is a eukaryote or a non-human eukaryote. In certain embodiments, the organism or subject is a plant. In certain embodiments, the organism or subject is a mammal or a non-human mammal. In certain embodiments, the organism or subject is algae. In some embodiments, the organism or subject may be a rodent (for example, mouse or rat), an ungulate, or a primate. In some embodiments, the organism or subject may be an arthropod (eg, insect) or nematode. In other embodiments the organism or subject may be a plant or a fungus.

[0040] Accordingly, it is an object of the invention to not encompass within the invention any previously known product, process of making the product, or method of using the

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

[0041] It is noted that in this disclosure and particularly in the claims and/or paragraphs, terms such as "comprises", "comprised", "comprising" and the like can have the meaning attributed to it in U.S. Patent law; e.g., they can mean "includes", "included", "including", and the like; and that terms such as "consisting essentially of" and "consists essentially of" have the meaning ascribed to them in U.S. Patent law, e.g., they allow for elements not explicitly recited, but exclude elements that are found in the prior art or that affect a basic or novel characteristic of the invention.

[0042] These and other embodiments are disclosed or are obvious from and encompassed by, the following Detailed Description.

BRIEF DESCRIPTION OF THE DRAWINGS

[0043] The novel features of the invention are set forth with particularity in the appended claims. A better understanding of the features and advantages of the present invention will be obtained by reference to the following detailed description that sets forth illustrative embodiments, in which the principles of the invention are utilized, and the accompanying drawings of which:

[0044] FIG. **1** shows a schematic of RNA-guided Cas9 nuclease. The Cas9 nuclease from *Streptococcus pyogenes* (yellow) is targeted to genomic DNA by a synthetic guide RNA (sgRNA) consisting of a 20-nt guide sequence (blue) and a scaffold (red). The guide sequence base-pairs with the DNA target (blue), directly upstream of a requisite 5'-NGG protospacer adjacent motif (PAM; magenta), and Cas9 mediates a double-stranded break (DSB) ~3 bp upstream of the PAM (red triangle).

[0045] FIGS. **2**A-**2**F show an exemplary CRISPR system, a possible mechanism of action, an example adaptation for expression in eukaryotic cells, and results of tests assessing nuclear localization and CRISPR activity

[0046] FIG. **3**A-D is a phylogenetic tree of Cas genes

[0047] FIGS. **4**A-**4**F show the phylogenetic analysis revealing five families of Cas9s, including three groups of large Cas9s (~1400 amino acids) and two of small Cas9s (~1100 amino acids).

[0048] FIG. **5** shows a schematic construct in which the transcriptional activation domain (VP64) is fused to Cas9 with two mutations in the catalytic domains (D10 and H840).

[0049] FIG. **6** shows a graphical representation of transcriptional activation following co-transfection of the Cas9-VP64 with PCR generated chimeric crispr RNA (chiRNA) in 293 cells. Assessment was carried out 72 hours after transfection using RT-qPCR.

[0050] FIG. **7** shows a number of vectors incorporating mutant Cas9 genes with VP64, NLS, and GFP markers.

[0051] FIG. **8** shows the localization of Cas9-VP64-GFP constructs into 293 cells as assessed by a fluorescent microscope 12 hours post transfection.

[0052] FIG. **9** shows the localization of 16 dCas9-GFP fusions with the same alpha importin NLS sequence on either the N- or C-term looking at zero to three tandem repeats. Each construct was transfected into HEK 293FT cells using Lipofectame 2000 and imaged 24 hours post-transfection

[0053] FIG. **10** shows six versions of a 6×His tag added to dCas9, transfected into 293FT cells, and stained with an anti-6×His antibody. Three were constructed for transcriptional activation (VP64 fusions), and the other three were for transcriptional repression (no functional domain).

[0054] FIG. **11** shows a titrated ratio of chiRNA (Sox2.1 and Sox2.5) to Cas9 (NLS-VP64-NLS-hSpCas9-NLS-VP64-NLS), transfected into 293 cells, and quantified using RT-qPCR.

[0055] FIG. **12** shows the positioning of target sites in the human Sox2 locus with each target being 20 bp long with a neighboring NGG protspacer adjacent motif (PAM).

[0056] FIG. **13** shows co-transfecting of each dCas9 containing construct with pA6 plasmids into HEK 293FT cells using Lipofectame 2000. 72 hours post-transfection total RNA was extracted from the cells. 1 μg of RNA was reverse transcribed into cDNA (qScript Supermix) in a 40 ul reaction. 2 ul of reaction product was added into a single 20 ul TaqMan assay qPCR reaction. Each experiment was performed in biological and technical triplicates. No RT control and no template control reactions showed no amplification.

[0057] FIG. **14** shows testing of constructs (pXRP011, pXRP013, pXRP015) using the same Sox2 targets as FIG. **13**.

[0058] FIGS. **15**A-**15**B shows a list of 31 constructs to explore different linkers, functional domains, and N- and C-term fusions and critical elements.

[0059] FIG. **16** shows co-transfecting of each dCas9 repressor plasmid with two guide RNAs targeted to the coding strand of the beta-catenin gene. RNA was isolated 72 hours after transfection and gene expression was quantified by RT-qPCR. The endogenous control gene was GAPDH. Two validated shRNAs were used as positive controls. Negative controls were certain plasmids transfected without gRNA, these are denoted as "pXRP ##control".

[0060] FIG. **17** shows a graphical representation of transcriptional activation further to the ratio of chiRNA (Sox2.1 and Sox2.5) to Cas9 (NLS-VP64-NLS-hSpCas9-NLS-VP64-NLS) being titrated and transfected in 293 cells. Results were quantified using Rt-qPCR.

[0061] FIG. **18** shows luciferase reporter data for Cas9 activator (top panel) and repressor (bottom panel). Compared to "No Cas9" controls over 3 fold activation was achieved when targeting the promoter of Sox2. When targeting the gene body of beta-catenin (CTNNB1), about 3 fold repression was achieved.

[0062] FIG. **19** shows gene expression of beta-catenin in HEK 293FT cells 72 hours after transfection. Cas9 repressor constructs were targeted to the beta-catenin locus and compared to the gold standard shRNAs. Similar repression could be seen with Cas9 repressors and the shRNA.

[0063] FIG. **20** shows a graphical representation of the fold Neurog2 expression of the 20 bp sgRNA sequences were targeted to the Neurog2 locus in mouse Neuro 2A cells. Neurog2 mRNA levels were measured using RT-qPCR.

[0064] FIG. **21** shows the basal gene expression modulation of dCas9-VP64 that was measured for the indicated gene targets. Italics represent mouse gene targets tested in Neuro 2A cells, all capsrepresent human gene targets tested in 293FT cells. In each case, expression of the gene in GFP transfected cells was compared to expression in cells transfected with Cas9-VP64.

[0065] FIG. **22** shows the changes in expression level of the indicated genes when samples transfected with the dCas9-VP64 construct, but without an sgRNA.

[0066] FIG. **23**A-G shows a, The RNA-guided nuclease Cas9 from the type II *Streptococcus pyogenes* CRISPR/Cas system can be converted into a nucleolytically inactive RNA-guided DNA binding protein (Cas9**) by introducing two alanine substitutions (D10A and H840A). Schematic showing that a synthetic guide RNA (sgRNA) can direct Cas9**-effector fusion to a specific locus in the human genome. The sgRNA contains a 20-bp guide sequence at the 5' end which specifies the target sequence. On the target genomic DNA, the 20-bp target site needs to be followed by a 5'-NGG PAM motif b, c, Schematics showing the sgRNA target sites in the human KLF4 and SOX2 loci, respectively. Each target site is indicated by the blue bar and the corresponding PAM sequence is indicated by the magenta bar. d, e, Schematics of the Cas9**-VP64 transcription activator and SID4X-Cas9** transcription repressor constructs. f, g, Cas9**-VP64- and SID4X-Cas9**-mediated activation of KLF4 and repression of SOX2, respectively. All mRNA levels were measured relative to GFP mock-transfected 293FT cells (mean±s.e.m.; n=3 biological replicates).

[0067] FIG. **24** shows the Cas9 activator sequence.

[0068] FIG. **25** shows the Cas9 repressor sequence.

[0069] FIG. **26** shows the graphical representation of fold activation of different genes targeted by the Cas9 activator (pXRP57) and guide RNA.

[0070] FIG. **27** shows the graphical representation of fold repression of the hSox2 gene targeted by the Cas9 activator (pXRP57) and guide RNA.

[0071] FIG. **28** shows a pAAV-EF1a-dCas9-GS-CIB1 (mNLS d318-334)_WPRE_hGHpolyA plasmid map.

[0072] FIG. **29** shows a pAAV-EF1a-dCas9-GS-NLS-cib1-WPRE-hGHpA plasmid map.

[0073] FIG. **30** shows a pAAV-EF1a-dCas9-GS-NLS-NLS-cib1-WPRE-hGHpA plasmid map.

[0074] FIG. **31** shows a graphical representation of CasLITE constructs exhibiting varying levels of light-inducible transcriptional activation.

[0075] FIG. **32** shows the validation of Rosa26 Cre-dependent Cas9 knockin mouse embryonic stem cells.

[0076] FIG. **33** shows a gel image indicating the genotyping results for Cas9 mice.

[0077] FIG. **34** shows CRISPR/Cmr mediated silencing of RNA. Cmr proteins form a complex with mature crRNA to site-specifically target and cleave RNA. Mature crRNAs from *P. furiosus* exist in two forms of different lengths, long (45 nt) and short (39 nt), consisting of a 5' handle and a guide sequence. The 5' handle is required for the crRNA to be included in the Cmr complex. The guide sequence programs the target site and can be either long (37 nt) or short (31 nt). RNA cleavage occurs 14 nucleotides from the 3' end of the crRNA. This platform could be repurposed to target mammalian genes expressed from a genomic locus by only changing the guide sequence.

[0078] FIG. **35**A-B shows Cmr proteins are expressed within mammalian cells. Six Cmr genes (Cmr1-6) and one Cas gene (Cas6) from *P. furiosus* were cloned into mammalian expression vectors and transfected into HEK 293FT cells. 72 hours post-transfection fluorescence images were taken and protein lysates were prepared. A) EGFP expression is strongly observed suggesting that the exogenous proteins are robustly expressed. B) Western blot images show protein bands located at the expected size. The second band within each lane is 30 kDa larger in each case and is likely the result of uncleaved P2A sequence.

[0079] FIG. **36** shows CRISPR/Cmr expression vectors.

[0080] The figures herein are for illustrative purposes only and are not necessarily drawn to scale.

DETAILED DESCRIPTION OF THE INVENTION

[0081] The invention relates to the engineering and optimization of systems, methods and compositions used for the control of gene expression involving sequence targeting, such as genome perturbation or gene-editing, that relate to the CRISPR/Cas system and components thereof. In advantageous embodiments, the Cas enzyme is Cas9; for example, Cas9 from *S. pyogenes* or *S. thermophilus.*

[0082] The terms "polynucleotide", "nucleotide", "nucleotide sequence", "nucleic acid" and "oligonucleotide" are used interchangeably. They refer to a polymeric form of nucleotides of any length, either deoxyribonucleotides or ribonucleotides, or analogs thereof. Polynucleotides may have any three dimensional structure, and may perform any function, known or unknown. The following are non-limiting examples of polynucleotides: coding or non-coding regions of a gene or gene fragment, loci (locus) defined from linkage analysis, exons, introns, messenger RNA (mRNA), transfer RNA, ribosomal RNA, short interfering RNA (siRNA), short-hairpin RNA (shRNA), micro-RNA (miRNA), ribozymes, cDNA, recombinant polynucleotides, branched polynucleotides, plasmids, vectors, isolated DNA of any sequence, isolated RNA of any sequence, nucleic acid probes, and primers. The term also encompasses nucleic-acid-like structures with synthetic backbones, see, e.g., Eckstein, 1991; Baserga et al., 1992; Milligan, 1993; WO 97/03211; WO 96/39154; Mata, 1997; Strauss-Soukup, 1997; and Samstag, 1996. A polynucleotide may comprise one or more modified nucleotides, such as methylated nucleotides and nucleotide analogs. If present, modifications to the nucleotide structure may be imparted before or after assembly of the polymer. The sequence of nucleotides may be interrupted by non-nucleotide components. A polynucleotide may be further modified after polymerization, such as by conjugation with a labeling component.

[0083] In aspects of the invention the terms "chimeric RNA", "chimeric guide RNA", "guide RNA", "single guide RNA" and "synthetic guide RNA" are used interchangeably and refer to the polynucleotide sequence comprising the guide sequence, the tracr sequence and the tracr mate sequence. The term "guide sequence" refers to the about 20 bp sequence within the guide RNA that specifies the target site and may be used interchangeably with the terms "guide" or "spacer". The term "tracr mate sequence" may also be used interchangeably with the term "direct repeat(s)".

[0084] As used herein the term "wild type" is a term of the art understood by skilled persons and means the typical form of an organism, strain, gene or characteristic as it occurs in

nature as distinguished from mutant or variant forms. For example, "wild type StCas9" refers to wild type Cas9 from *S thermophilus*, the protein sequence of which is given in the SwissProt database under accession number G3ECR1. Similarly, *S pyogenes* Cas9 is included in SwissProt under accession number Q99ZW2.

[0085] As used herein the term "variant" should be taken to mean the exhibition of qualities that have a pattern that deviates from what occurs in nature.

[0086] The terms "non-naturally occurring" or "engineered" are used interchangeably and indicate the involvement of the hand of man. The terms, when referring to nucleic acid molecules or polypeptides mean that the nucleic acid molecule or the polypeptide is at least substantially free from at least one other component with which they are naturally associated in nature and as found in nature.

[0087] "Complementarity" refers to the ability of a nucleic acid to form hydrogen bond(s) with another nucleic acid sequence by either traditional Watson-Crick base pairing or other non-traditional types. A percent complementarity indicates the percentage of residues in a nucleic acid molecule which can form hydrogen bonds (e.g., Watson-Crick base pairing) with a second nucleic acid sequence (e.g., 5, 6, 7, 8, 9, 10 out of 10 being 50%, 60%, 70%, 80%, 90%, and 100% complementary). "Perfectly complementary" means that all the contiguous residues of a nucleic acid sequence will hydrogen bond with the same number of contiguous residues in a second nucleic acid sequence. "Substantially complementary" as used herein refers to a degree of complementarity that is at least 60%, 65%, 70%, 75%, 80%, 85%, 90%, 95%, 97%, 98%, 99%, or 100% over a region of 8, 9, 10, 11, 12, 13, 14, 15, 16, 17, 18, 19, 20, 21, 22, 23, 24, 25, 30, 35, 40, 45, 50, or more nucleotides, or refers to two nucleic acids that hybridize under stringent conditions.

[0088] As used herein, "stringent conditions" for hybridization refer to conditions under which a nucleic acid having complementarity to a target sequence predominantly hybridizes with the target sequence, and substantially does not hybridize to non-target sequences. Stringent conditions are generally sequence-dependent, and vary depending on a number of factors. In general, the longer the sequence, the higher the temperature at which the sequence specifically hybridizes to its target sequence. Non-limiting examples of stringent conditions are described in detail in Tijssen (1993), Laboratory Techniques In Biochemistry And Molecular Biology-Hybridization With Nucleic Acid Probes Part I, Second Chapter "Overview of principles of hybridization and the strategy of nucleic acid probe assay", Elsevier, N.Y.

[0089] "Hybridization" refers to a reaction in which one or more polynucleotides react to form a complex that is stabilized via hydrogen bonding between the bases of the nucleotide residues. The hydrogen bonding may occur by Watson Crick base pairing, Hoogstein binding, or in any other sequence specific manner. The complex may comprise two strands forming a duplex structure, three or more strands forming a multi stranded complex, a single self-hybridizing strand, or any combination of these. A hybridization reaction may constitute a step in a more extensive process, such as the initiation of PCR, or the cleavage of a polynucleotide by an enzyme. A sequence capable of hybridizing with a given sequence is referred to as the "complement" of the given sequence.

[0090] As used herein, the term "genomic locus" or "locus" (plural loci) is the specific location of a gene or DNA sequence on a chromosome. A "gene" refers to stretches of DNA or RNA that encode a polypeptide or an RNA chain that has functional role to play in an organism and hence is the molecular unit of heredity in living organisms. For the purpose of this invention it may be considered that genes include regions which regulate the production of the gene product, whether or not such regulatory sequences are adjacent to coding and/or transcribed sequences. Accordingly, a gene includes, but is not necessarily limited to, promoter sequences, terminators, translational regulatory sequences such as ribosome binding sites and internal ribosome entry sites, enhancers, silencers, insulators, boundary elements, replication origins, matrix attachment sites and locus control regions.

[0091] As used herein, "expression of a genomic locus" or "gene expression" is the process by which information from a gene is used in the synthesis of a functional gene product. The products of gene expression are often proteins, but in non-protein coding genes such as rRNA genes or tRNA genes, the product is functional RNA. The process of gene expression is used by all known life—eukaryotes (including multicellular organisms), prokaryotes (bacteria and archaea) and viruses to generate functional products to survive. As used herein "expression" of a gene or nucleic acid encompasses not only cellular gene expression, but also the transcription and translation of nucleic acid(s) in cloning systems and in any other context. As used herein, "expression" also refers to the process by which a polynucleotide is transcribed from a DNA template (such as into and mRNA or other RNA transcript) and/or the process by which a transcribed mRNA is subsequently translated into peptides, polypeptides, or proteins. Transcripts and encoded polypeptides may be collectively referred to as "gene product." If the polynucleotide is derived from genomic DNA, expression may include splicing of the mRNA in a eukaryotic cell.

[0092] The terms "polypeptide", "peptide" and "protein" are used interchangeably herein to refer to polymers of amino acids of any length. The polymer may be linear or branched, it may comprise modified amino acids, and it may be interrupted by non amino acids. The terms also encompass an amino acid polymer that has been modified; for example, disulfide bond formation, glycosylation, lipidation, acetylation, phosphorylation, or any other manipulation, such as conjugation with a labeling component. As used herein the term "amino acid" includes natural and/or unnatural or synthetic amino acids, including glycine and both the D or L optical isomers, and amino acid analogs and peptidomimetics.

[0093] As used herein, the term "domain" or "protein domain" refers to a part of a protein sequence that may exist and function independently of the rest of the protein chain.

[0094] As described in aspects of the invention, sequence identity is related to sequence homology. Homology comparisons may be conducted by eye, or more usually, with the aid of readily available sequence comparison programs. These commercially available computer programs may calculate percent (%) homology between two or more sequences and may also calculate the sequence identity shared by two or more amino acid or nucleic acid sequences. Sequence homologies may be generated by any of a number of computer programs known in the art, for example BLAST or FASTA, etc. A suitable computer program for carrying out such an alignment is the GCG Wisconsin Bestfit package (University of Wisconsin, U.S.A; Devereux et al., 1984,

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

[0095] % homology may be calculated over contiguous sequences, i.e., one sequence is aligned with the other sequence and each amino acid or nucleotide in one sequence is directly compared with the corresponding amino acid or nucleotide in the other sequence, one residue at a time. This is called an "ungapped" alignment. Typically, such ungapped alignments are performed only over a relatively short number of residues.

[0096] Although this is a very simple and consistent method, it fails to take into consideration that, for example, in an otherwise identical pair of sequences, one insertion or deletion may cause the following amino acid residues to be put out of alignment, thus potentially resulting in a large reduction in % homology when a global alignment is performed. Consequently, most sequence comparison methods are designed to produce optimal alignments that take into consideration possible insertions and deletions without unduly penalizing the overall homology or identity score. This is achieved by inserting "gaps" in the sequence alignment to try to maximize local homology or identity.

[0097] However, these more complex methods assign "gap penalties" to each gap that occurs in the alignment so that, for the same number of identical amino acids, a sequence alignment with as few gaps as possible—reflecting higher relatedness between the two compared sequences—may achieve a higher score than one with many gaps. "Affinity gap costs" are typically used that charge a relatively high cost for the existence of a gap and a smaller penalty for each subsequent residue in the gap. This is the most commonly used gap scoring system. High gap penalties may, of course, produce optimized alignments with fewer gaps. Most alignment programs allow the gap penalties to be modified. However, it is preferred to use the default values when using such software for sequence comparisons. For example, when using the GCG Wisconsin Bestfit package the default gap penalty for amino acid sequences is −12 for a gap and −4 for each extension.

[0098] Calculation of maximum % homology therefore first requires the production of an optimal alignment, taking into consideration gap penalties. A suitable computer program for carrying out such an alignment is the GCG Wisconsin Bestfit package (Devereux et al., 1984 *Nuc. Acids Research* 12 p387). Examples of other software than may perform sequence comparisons include, but are not limited to, the BLAST package (see Ausubel et al., 1999 *Short Protocols in Molecular Biology,* 4th Ed.—Chapter 18), FASTA (Altschul et al., 1990 *J. Mol. Biol.* 403-410) and the GENEWORKS suite of comparison tools. Both BLAST and FASTA are available for offline and online searching (see Ausubel et al., 1999*, Short Protocols in Molecular Biology*, pages 7-58 to 7-60). However, for some applications, it is preferred to use the GCG Bestfit program. A new tool, called BLAST 2 Sequences is also available for comparing protein and nucleotide sequences (see *FEMS Microbiol Lett.* 1999 174(2): 247-50*; FEMS Microbiol Lett.* 1999 177(1): 187-8 and the website of the National Center for Biotechnology information at the website of the National Institutes for Health).

[0099] Although the final % homology may be measured in terms of identity, the alignment process itself is typically not based on an all-or-nothing pair comparison. Instead, a scaled similarity score matrix is generally used that assigns scores to each pair-wise comparison based on chemical similarity or evolutionary distance. An example of such a matrix commonly used is the BLOSUM62 matrix—the default matrix for the BLAST suite of programs. GCG Wisconsin programs generally use either the public default values or a custom symbol comparison table, if supplied (see user manual for further details). For some applications, it is preferred to use the public default values for the GCG package, or in the case of other software, the default matrix, such as BLOSUM62.

[0100] Alternatively, percentage homologies may be calculated using the multiple alignment feature in DNASIS™ (Hitachi Software), based on an algorithm, analogous to CLUSTAL (Higgins D G & Sharp P M (1988), Gene 73(1), 237-244). Once the software has produced an optimal alignment, it is possible to calculate % homology, preferably % sequence identity. The software typically does this as part of the sequence comparison and generates a numerical result.

[0101] The sequences may also have deletions, insertions or substitutions of amino acid residues which produce a silent change and result in a functionally equivalent substance. Deliberate amino acid substitutions may be made on the basis of similarity in amino acid properties (such as polarity, charge, solubility, hydrophobicity, hydrophilicity, and/or the amphipathic nature of the residues) and it is therefore useful to group amino acids together in functional groups. Amino acids may be grouped together based on the properties of their side chains alone. However, it is more useful to include mutation data as well. The sets of amino acids thus derived are likely to be conserved for structural reasons. These sets may be described in the form of a Venn diagram (Livingstone C. D. and Barton G. J. (1993) "Protein sequence alignments: a strategy for the hierarchical analysis of residue conservation" *Comput. Appl. Biosci.* 9: 745-756) (Taylor W. R. (1986) "The classification of amino acid conservation" *J. Theor. Biol.* 119; 205-218). Conservative substitutions may be made, for example according to the table below which describes a generally accepted Venn diagram grouping of amino acids.

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

[0102] Embodiments of the invention include sequences (both polynucleotide or polypeptide) which may comprise homologous substitution (substitution and replacement are both used herein to mean the interchange of an existing amino acid residue or nucleotide, with an alternative residue

or nucleotide) that may occur i.e., like-for-like substitution in the case of amino acids such as basic for basic, acidic for acidic, polar for polar, etc. Non-homologous substitution may also occur i.e., from one class of residue to another or alternatively involving the inclusion of unnatural amino acids such as ornithine (hereinafter referred to as Z), diaminobutyric acid ornithine (hereinafter referred to as B), norleucine ornithine (hereinafter referred to as O), pyriylalanine, thienylalanine, naphthylalanine and phenylglycine.

[0103] Variant amino acid sequences may include suitable spacer groups that may be inserted between any two amino acid residues of the sequence including alkyl groups such as methyl, ethyl or propyl groups in addition to amino acid spacers such as glycine or β-alanine residues. A further form of variation, which involves the presence of one or more amino acid residues in peptoid form, may be well understood by those skilled in the art. For the avoidance of doubt, "the peptoid form" is used to refer to variant amino acid residues wherein the α-carbon substituent group is on the residue's nitrogen atom rather than the α-carbon. Processes for preparing peptides in the peptoid form are known in the art, for example Simon R J et al., *PNAS* (1992) 89(20), 9367-9371 and Horwell D C, *Trends Biotechnol.* (1995) 13(4), 132-134.

[0104] The practice of the present invention employs, unless otherwise indicated, conventional techniques of immunology, biochemistry, chemistry, molecular biology, microbiology, cell biology, genomics and recombinant DNA, which are within the skill of the art. See Sambrook, Fritsch and Maniatis, MOLECULAR CLONING: A LABORATORY MANUAL, 2nd edition (1989); CURRENT PROTOCOLS IN MOLECULAR BIOLOGY (F. M. Ausubel, et al. eds., (1987)); the series METHODS IN ENZYMOLOGY (Academic Press, Inc.): PCR 2: A PRACTICAL APPROACH (M. J. MacPherson, B. D. Hames and G. R. Taylor eds. (1995)), Harlow and Lane, eds. (1988) ANTIBODIES, A LABORATORY MANUAL, and ANIMAL CELL CULTURE (R. I. Freshney, ed. (1987)).

[0105] In one aspect, the invention provides for vectors that are used in the engineering and optimization of CRISPR/Cas systems.

[0106] A used herein, a "vector" is a tool that allows or facilitates the transfer of an entity from one environment to another. It is a replicon, such as a plasmid, phage, or cosmid, into which another DNA segment may be inserted so as to bring about the replication of the inserted segment. Generally, a vector is capable of replication when associated with the proper control elements. In general, the term "vector" refers to a nucleic acid molecule capable of transporting another nucleic acid to which it has been linked. Vectors include, but are not limited to, nucleic acid molecules that are single-stranded, double-stranded, or partially double-stranded; nucleic acid molecules that comprise one or more free ends, no free ends (e.g. circular); nucleic acid molecules that comprise DNA, RNA, or both; and other varieties of polynucleotides known in the art. One type of vector is a "plasmid," which refers to a circular double stranded DNA loop into which additional DNA segments can be inserted, such as by standard molecular cloning techniques. Another type of vector is a viral vector, wherein virally-derived DNA or RNA sequences are present in the vector for packaging into a virus (e.g. retroviruses, replication defective retroviruses, adenoviruses, replication defective adenoviruses, and adeno-associated viruses). Viral vectors also include polynucleotides carried by a virus for transfection into a host cell. Certain vectors are capable of autonomous replication in a host cell into which they are introduced (e.g. bacterial vectors having a bacterial origin of replication and episomal mammalian vectors). Other vectors (e.g., non-episomal mammalian vectors) are integrated into the genome of a host cell upon introduction into the host cell, and thereby are replicated along with the host genome. Moreover, certain vectors are capable of directing the expression of genes to which they are operatively-linked. Such vectors are referred to herein as "expression vectors." Common expression vectors of utility in recombinant DNA techniques are often in the form of plasmids.

[0107] Recombinant expression vectors can comprise a nucleic acid of the invention in a form suitable for expression of the nucleic acid in a host cell, which means that the recombinant expression vectors include one or more regulatory elements, which may be selected on the basis of the host cells to be used for expression, that is operatively-linked to the nucleic acid sequence to be expressed. Within a recombinant expression vector, "operably linked" is intended to mean that the nucleotide sequence of interest is linked to the regulatory element(s) in a manner that allows for expression of the nucleotide sequence (e.g. in an in vitro transcription/translation system or in a host cell when the vector is introduced into the host cell). With regards to recombination and cloning methods, mention is made of U.S. patent application Ser. No. 10/815,730, the contents of which are herein incorporated by reference in their entirety.

[0108] Aspects of the invention relate to bicistronic vectors for chimeric RNA and Cas9. Bicistronic expression vectors for chimeric RNA and Cas9 are preferred. In general and particularly in this embodiment Cas9 is preferably driven by the CBh promoter. The chimeric RNA may preferably be driven by a U6 promoter. Ideally the two are combined. The chimeric guide RNA typically consists of a 20 bp guide sequence (Ns) and this may be joined to the tracr sequence (running from the first "U" of the lower strand to the end of the transcript). The tracr sequence may be truncated at various positions as indicated. The guide and tracr sequences are separated by the tracr-mate sequence, which may be GUUUUAGAGCUA. This may be followed by the loop sequence GAAA as shown. Both of these are preferred examples. Applicants have demonstrated Cas9-mediated indels at the human EMX1 and PVALB loci by SURVEYOR assays. ChiRNAs are indicated by their "+n" designation, and crRNA refers to a hybrid RNA where guide and tracr sequences are expressed as separate transcripts. Throughout this application, chimeric RNA may also be called single guide, or synthetic guide RNA (sgRNA). The loop is preferably GAAA, but it is not limited to this sequence or indeed to being only 4 bp in length. Indeed, preferred loop forming sequences for use in hairpin structures are four nucleotides in length, and most preferably have the sequence GAAA. However, longer or shorter loop sequences may be used, as may alternative sequences. The sequences preferably include a nucleotide triplet (for example, AAA), and an additional nucleotide (for example C or G). Examples of loop forming sequences include CAAA and AAAG.

[0109] The term "regulatory element" is intended to include promoters, enhancers, internal ribosomal entry sites (IRES), and other expression control elements (e.g. transcription termination signals, such as polyadenylation sig-

nals and poly-U sequences). Such regulatory elements are described, for example, in Goeddel, GENE EXPRESSION TECHNOLOGY: METHODS IN ENZYMOLOGY 185, Academic Press, San Diego, Calif. (1990). Regulatory elements include those that direct constitutive expression of a nucleotide sequence in many types of host cell and those that direct expression of the nucleotide sequence only in certain host cells (e.g., tissue-specific regulatory sequences). A tissue-specific promoter may direct expression primarily in a desired tissue of interest, such as muscle, neuron, bone, skin, blood, specific organs (e.g. liver, pancreas), or particular cell types (e.g. lymphocytes). Regulatory elements may also direct expression in a temporal-dependent manner, such as in a cell-cycle dependent or developmental stage-dependent manner, which may or may not also be tissue or cell-type specific. In some embodiments, a vector may comprise one or more pol III promoter (e.g. 1, 2, 3, 4, 5, or more pol III promoters), one or more pol II promoters (e.g. 1, 2, 3, 4, 5, or more pol II promoters), one or more pol I promoters (e.g. 1, 2, 3, 4, 5, or more pol I promoters), or combinations thereof. Examples of pol III promoters include, but are not limited to, U6 and H1 promoters. Examples of pol II promoters include, but are not limited to, the retroviral Rous sarcoma virus (RSV) LTR promoter (optionally with the RSV enhancer), the cytomegalovirus (CMV) promoter (optionally with the CMV enhancer) [see, e.g., Boshart et al, *Cell,* 41:521-530 (1985)], the SV40 promoter, the dihydrofolate reductase promoter, the 3-actin promoter, the phosphoglycerol kinase (PGK) promoter, and the EF1α promoter. Also encompassed by the term "regulatory element" are enhancer elements, such as WPRE; CMV enhancers; the R-U5' segment in LTR of HTLV-I (Mol. Cell. Biol., Vol. 8(1), p. 466-472, 1988); SV40 enhancer; and the intron sequence between exons 2 and 3 of rabbit β-globin (Proc. Natl. Acad. Sci. USA., Vol. 78(3), p. 1527-31, 1981). It will be appreciated by those skilled in the art that the design of the expression vector can depend on such factors as the choice of the host cell to be transformed, the level of expression desired, etc. A vector can be introduced into host cells to thereby produce transcripts, proteins, or peptides, including fusion proteins or peptides, encoded by nucleic acids as described herein (e.g., clustered regularly interspersed short palindromic repeats (CRISPR) transcripts, proteins, enzymes, mutant forms thereof, fusion proteins thereof, etc.). With regards to regulatory sequences, mention is made of U.S. patent application Ser. No. 10/491,026, the contents of which are incorporated by reference herein in their entirety. With regards to promoters, mention is made of PCT publication WO 2011/028929 and U.S. application Ser. No. 12/511,940, the contents of which are incorporated by reference herein in their entirety.

[0110] Vectors can be designed for expression of CRISPR transcripts (e.g. nucleic acid transcripts, proteins, or enzymes) in prokaryotic or eukaryotic cells. For example, CRISPR transcripts can be expressed in bacterial cells such as *Escherichia coli*, insect cells (using baculovirus expression vectors), yeast cells, or mammalian cells. Suitable host cells are discussed further in Goeddel, GENE EXPRESSION TECHNOLOGY: METHODS IN ENZYMOLOGY 185, Academic Press, San Diego, Calif. (1990). Alternatively, the recombinant expression vector can be transcribed and translated in vitro, for example using T7 promoter regulatory sequences and T7 polymerase.

[0111] Vectors may be introduced and propagated in a prokaryote or prokaryotic cell. In some embodiments, a prokaryote is used to amplify copies of a vector to be introduced into a eukaryotic cell or as an intermediate vector in the production of a vector to be introduced into a eukaryotic cell (e.g. amplifying a plasmid as part of a viral vector packaging system). In some embodiments, a prokaryote is used to amplify copies of a vector and express one or more nucleic acids, such as to provide a source of one or more proteins for delivery to a host cell or host organism. Expression of proteins in prokaryotes is most often carried out in *Escherichia coli* with vectors containing constitutive or inducible promoters directing the expression of either fusion or non-fusion proteins. Fusion vectors add a number of amino acids to a protein encoded therein, such as to the amino terminus of the recombinant protein. Such fusion vectors may serve one or more purposes, such as: (i) to increase expression of recombinant protein; (ii) to increase the solubility of the recombinant protein; and (iii) to aid in the purification of the recombinant protein by acting as a ligand in affinity purification. Often, in fusion expression vectors, a proteolytic cleavage site is introduced at the junction of the fusion moiety and the recombinant protein to enable separation of the recombinant protein from the fusion moiety subsequent to purification of the fusion protein. Such enzymes, and their cognate recognition sequences, include Factor Xa, thrombin and enterokinase. Example fusion expression vectors include pGEX (Pharmacia Biotech Inc; Smith and Johnson, 1988. *Gene* 67: 31-40), pMAL (New England Biolabs, Beverly, Mass.) and pRIT5 (Pharmacia, Piscataway, N.J.) that fuse glutathione S-transferase (GST), maltose E binding protein, or protein A, respectively, to the target recombinant protein.

[0112] Examples of suitable inducible non-fusion *E. coli* expression vectors include pTrc (Amrann et al., (1988) Gene 69:301-315) and pET lid (Studier et al., GENE EXPRESSION TECHNOLOGY: METHODS IN ENZYMOLOGY 185, Academic Press, San Diego, Calif. (1990) 60-89).

[0113] In some embodiments, a vector is a yeast expression vector. Examples of vectors for expression in yeast *Saccharomyces cerivisae* include pYepSec1 (Baldari, et al., 1987. EMBO J. 6: 229-234), pMFa (Kuijan and Herskowitz, 1982. Cell 30: 933-943), pJRY88 (Schultz et al., 1987. Gene 54: 113-123), pYES2 (Invitrogen Corporation, San Diego, Calif.), and picZ (InVitrogen Corp, San Diego, Calif.).

[0114] In some embodiments, a vector drives protein expression in insect cells using baculovirus expression vectors. Baculovirus vectors available for expression of proteins in cultured insect cells (e.g., SF9 cells) include the pAc series (Smith, et al., 1983. Mol. Cell. Biol. 3: 2156-2165) and the pVL series (Lucklow and Summers, 1989. Virology 170: 31-39).

[0115] In some embodiments, a vector is capable of driving expression of one or more sequences in mammalian cells using a mammalian expression vector. Examples of mammalian expression vectors include pCDM8 (Seed, 1987. Nature 329: 840) and pMT2PC (Kaufman, et al., 1987. EMBO J. 6: 187-195). When used in mammalian cells, the expression vector's control functions are typically provided by one or more regulatory elements. For example, commonly used promoters are derived from polyoma, adenovirus 2, cytomegalovirus, simian virus 40, and others disclosed herein and known in the art. For other suitable expression systems for both prokaryotic and eukaryotic cells

see, e.g., Chapters 16 and 17 of Sambrook, et al., MOLECULAR CLONING: A LABORATORY MANUAL. 2nd ed., Cold Spring Harbor Laboratory, Cold Spring Harbor Laboratory Press, Cold Spring Harbor, N.Y., 1989.

[0116] In some embodiments, the recombinant mammalian expression vector is capable of directing expression of the nucleic acid preferentially in a particular cell type (e.g., tissue-specific regulatory elements are used to express the nucleic acid). Tissue-specific regulatory elements are known in the art. Non-limiting examples of suitable tissue-specific promoters include the albumin promoter (liver-specific; Pinkert, et al., 1987. *Genes Dev.* 1: 268-277), lymphoid-specific promoters (Calame and Eaton, 1988. *Adv. Immunol.* 43: 235-275), in particular promoters of T cell receptors (Winoto and Baltimore, 1989. *EMBO J.* 8: 729-733) and immunoglobulins (Baneiji, et al., 1983. *Cell* 33: 729-740; Queen and Baltimore, 1983. *Cell* 33: 741-748), neuron-specific promoters (e.g., the neurofilament promoter; Byrne and Ruddle, 1989. *Proc. Natl. Acad. Sci. USA* 86: 5473-5477), pancreas-specific promoters (Edlund, et al., 1985. *Science* 230: 912-916), and mammary gland-specific promoters (e.g., milk whey promoter; U.S. Pat. No. 4,873,316 and European Application Publication No. 264,166). Developmentally-regulated promoters are also encompassed, e.g., the murine hox promoters (Kessel and Gruss, 1990. *Science* 249: 374-379) and the α-fetoprotein promoter (Campes and Tilghman, 1989. *Genes Dev.* 3: 537-546). With regards to these prokaryotic and eukaryotic vectors, mention is made of U.S. Pat. No. 6,750,059, the contents of which are incorporated by reference herein in their entirety. Other embodiments of the invention may relate to the use of viral vectors, with regards to which mention is made of U.S. patent application Ser. No. 13/092,085, the contents of which are incorporated by reference herein in their entirety. Tissue-specific regulatory elements are known in the art and in this regard, mention is made of U.S. Pat. No. 7,776,321, the contents of which are incorporated by reference herein in their entirety.

[0117] In some embodiments, a regulatory element is operably linked to one or more elements of a CRISPR system so as to drive expression of the one or more elements of the CRISPR system. In general, CRISPRs (Clustered Regularly Interspaced Short Palindromic Repeats), also known as SPIDRs (SPacer Interspersed Direct Repeats), constitute a family of DNA loci that are usually specific to a particular bacterial species. The CRISPR locus may comprise a distinct class of interspersed short sequence repeats (SSRs) that were recognized in *E. coli* (Ishino et al., J. Bacteriol., 169:5429-5433 [1987]; and Nakata et al., J. Bacteriol., 171:3553-3556 [1989]), and associated genes. Similar interspersed SSRs have been identified in *Haloferax mediterranei, Streptococcus pyogenes, Anabaena*, and *Mycobacterium tuberculosis* (See, Groenen et al., Mol. Microbiol., 10:1057-1065 [1993]; Hoe et al., *Emerg. Infect. Dis.,* 5:254-263 [1999]; Masepohl et al., Biochim. Biophys. Acta 1307:26-30 [1996]; and Möjica et al., Mol. Microbiol., 17:85-93 [1995]). The CRISPR loci typically differ from other SSRs by the structure of the repeats, which have been termed short regularly spaced repeats (SRSRs) (Janssen et al., OMICS J. Integ. Biol., 6:23-33 [2002]; and Mojica et al., Mol. Microbiol., 36:244-246 [2000]). In general, the repeats are short elements that occur in clusters that are regularly spaced by unique intervening sequences with a substantially constant length (Mojica et al., [2000], supra). Although the repeat sequences are highly conserved between strains, the number of interspersed repeats and the sequences of the spacer regions typically differ from strain to strain (van Embden et al., J. Bacteriol., 182:2393-2401 [2000]). CRISPR loci have been identified in more than 40 prokaryotes (See e.g., Jansen et al., Mol. Microbiol., 43:1565-1575 [2002]; and Mojica et al., [2005]) including, but not limited to Aeropyrum, *Pyrobaculum, Sulfolobus, Archaeoglobus, Halocarcula, Methanobacterium, Methanococcus, Methanosarcina, Methanopyrus, Pyrococcus, Picrophilus, Thermoplasma, Corynebacterium, Mycobacterium, Streptomyces, Aquifex, Porphyromonas, Chlorobium, Thermus, Bacillus, Listeria, Staphylococcus, Clostridium, Thermoanaerobacter, Mycoplasma, Fusobacterium, Azarcus, Chromobacterium, Neisseria, Nitrosomonas, Desulfovibrio, Geobacter, Myxococcus, Campylobacter, Wolinella, Acinetobacter, Erwinia, Escherichia, Legionella, Methylococcus, Pasteurella, Photobacterium, Salmonella, Xanthomonas, Yersinia, Treponema*, and *Thermotoga.*

[0118] In general, "CRISPR system" refers collectively to transcripts and other elements involved in the expression of or directing the activity of CRISPR-associated ("Cas") genes, including sequences encoding a Cas gene, a tracr (trans-activating CRISPR) sequence (e.g. tracrRNA or an active partial tracrRNA), a tracr-mate sequence (encompassing a "direct repeat" and a tracrRNA-processed partial direct repeat in the context of an endogenous CRISPR system), a guide sequence (also referred to as a "spacer" in the context of an endogenous CRISPR system), or other sequences and transcripts from a CRISPR locus. In embodiments of the invention the terms guide sequence and guide RNA are used interchangeably. In some embodiments, one or more elements of a CRISPR system is derived from a type I, type II, or type III CRISPR system. In some embodiments, one or more elements of a CRISPR system is derived from a particular organism which may comprise an endogenous CRISPR system, such as *Streptococcus pyogenes*. In general, a CRISPR system is characterized by elements that promote the formation of a CRISPR complex at the site of a target sequence (also referred to as a protospacer in the context of an endogenous CRISPR system). In the context of formation of a CRISPR complex, "target sequence" refers to a sequence to which a guide sequence is designed to have complementarity, where hybridization between a target sequence and a guide sequence promotes the formation of a CRISPR complex. A target sequence may comprise any polynucleotide, such as DNA or RNA polynucleotides. In some embodiments, a target sequence is located in the nucleus or cytoplasm of a cell.

[0119] In preferred embodiments of the invention, the CRISPR system is a type II CRISPR system and the Cas enzyme is Cas9, which catalyzes DNA cleavage. Enzymatic action by Cas9 derived from *Streptococcus pyogenes* or any closely related Cas9 generates double stranded breaks at target site sequences which hybridize to 20 nucleotides of the guide sequence and that have a protospacer-adjacent motif (PAM) sequence NGG following the 20 nucleotides of the target sequence. CRISPR activity through Cas9 for site-specific DNA recognition and cleavage is defined by the guide sequence, the tracr sequence that hybridizes in part to the guide sequence and the PAM sequence. More aspects of the CRISPR system are described in Karginov and Hannon, The CRISPR system: small RNA-guided defense in bacteria and archaea, Mole Cell 2010, January 15; 37(1): 7.

[0120] The type II CRISPR locus from *Streptococcus pyogenes* SF370 contains a cluster of four genes Cas9, Cas1, Cas2, and Csn1, as well as two non-coding RNA elements, tracrRNA and a characteristic array of repetitive sequences (direct repeats) interspaced by short stretches of non-repetitive sequences (spacers, about 30 bp each). In this system, targeted DNA double-strand break (DSB) is generated in four sequential steps (FIG. **2**A). First, two non-coding RNAs, the pre-crRNA array and tracrRNA, are transcribed from the CRISPR locus. Second, tracrRNA hybridizes to the direct repeats of pre-crRNA, which is then processed into mature crRNAs containing individual spacer sequences. Third, the mature crRNA:tracrRNA complex directs Cas9 to the DNA target consisting of the protospacer and the corresponding PAM via heteroduplex formation between the spacer region of the crRNA and the protospacer DNA. Finally, Cas9 mediates cleavage of target DNA upstream of PAM to create a DSB within the protospacer (FIG. **2**A). FIG. **2**B demonstrates the nuclear localization of the codon optimized Cas9. To promote precise transcriptional initiation, the RNA polymerase III-based U6 promoter was selected to drive the expression of tracrRNA (FIG. **2**C). Similarly, a U6 promoter-based construct was developed to express a pre-crRNA array consisting of a single spacer flanked by two direct repeats (DRs, also encompassed by the term "tracr-mate sequences"; FIG. **2**C). The initial spacer was designed to target a 33-base-pair (bp) target site (30-bp protospacer plus a 3-bp CRISPR motif (PAM) sequence satisfying the NGG recognition motif of Cas9) in the human EMX1 locus (FIG. **2**C), a key gene in the development of the cerebral cortex.

[0121] Typically, in the context of an endogenous CRISPR system, formation of a CRISPR complex (which may comprise a guide sequence hybridized to a target sequence and complexed with one or more Cas proteins) results in cleavage of one or both strands in or near (e.g. within 1, 2, 3, 4, 5, 6, 7, 8, 9, 10, 20, 50, or more base pairs from) the target sequence. Without wishing to be bound by theory, the tracr sequence, which may comprise or consist of all or a portion of a wild-type tracr sequence (e.g. about or more than about 20, 26, 32, 45, 48, 54, 63, 67, 85, or more nucleotides of a wild-type tracr sequence), may also form part of a CRISPR complex, such as by hybridization along at least a portion of the tracr sequence to all or a portion of a tracr mate sequence that is operably linked to the guide sequence. In some embodiments, one or more vectors driving expression of one or more elements of a CRISPR system are introduced into a host cell such that expression of the elements of the CRISPR system direct formation of a CRISPR complex at one or more target sites. For example, a Cas enzyme, a guide sequence linked to a tracr-mate sequence, and a tracr sequence could each be operably linked to separate regulatory elements on separate vectors. Alternatively, two or more of the elements expressed from the same or different regulatory elements, may be combined in a single vector, with one or more additional vectors providing any components of the CRISPR system not included in the first vector. CRISPR system elements that are combined in a single vector may be arranged in any suitable orientation, such as one element located 5' with respect to ("upstream" of) or 3' with respect to ("downstream" of) a second element. The coding sequence of one element may be located on the same or opposite strand of the coding sequence of a second element, and oriented in the same or opposite direction. In some embodiments, a single promoter drives expression of a transcript encoding a CRISPR enzyme and one or more of the guide sequence, tracr mate sequence (optionally operably linked to the guide sequence), and a tracr sequence embedded within one or more intron sequences (e.g. each in a different intron, two or more in at least one intron, or all in a single intron). In some embodiments, the CRISPR enzyme, guide sequence, tracr mate sequence, and tracr sequence are operably linked to and expressed from the same promoter.

[0122] In some embodiments, a vector may comprise one or more insertion sites, such as a restriction endonuclease recognition sequence (also referred to as a "cloning site"). In some embodiments, one or more insertion sites (e.g. about or more than about 1, 2, 3, 4, 5, 6, 7, 8, 9, 10, or more insertion sites) are located upstream and/or downstream of one or more sequence elements of one or more vectors. In some embodiments, a vector may comprise an insertion site upstream of a tracr mate sequence, and optionally downstream of a regulatory element operably linked to the tracr mate sequence, such that following insertion of a guide sequence into the insertion site and upon expression the guide sequence directs sequence-specific binding of a CRISPR complex to a target sequence in a eukaryotic cell. In some embodiments, a vector may comprise two or more insertion sites, each insertion site being located between two tracr mate sequences so as to allow insertion of a guide sequence at each site. In such an arrangement, the two or more guide sequences may comprise two or more copies of a single guide sequence, two or more different guide sequences, or combinations of these. When multiple different guide sequences are used, a single expression construct may be used to target CRISPR activity to multiple different, corresponding target sequences within a cell. For example, a single vector may comprise about or more than about 1, 2, 3, 4, 5, 6, 7, 8, 9, 10, 15, 20, or more guide sequences. In some embodiments, about or more than about 1, 2, 3, 4, 5, 6, 7, 8, 9, 10, or more such guide-sequence-containing vectors may be provided, and optionally delivered to a cell.

[0123] In some embodiments, a vector may comprise a regulatory element operably linked to an enzyme-coding sequence encoding a CRISPR enzyme, such as a Cas protein. Non-limiting examples of Cas proteins include Cas1, Cas1B, Cas2, Cas3, Cas4, Cas5, Cas6, Cas7, Cas8, Cas9 (also known as Csn1 and Csx12), Cas10, Csy1, Csy2, Csy3, Cse1, Cse2, Csc1, Csc2, Csa5, Csn2, Csm2, Csm3, Csm4, Csm5, Csm6, Cmr1, Cmr3, Cmr4, Cmr5, Cmr6, Csb1, Csb2, Csb3, Csx17, Csx14, Csx10, Csx16, CsaX, Csx3, Csx1, Csx15, Csf1, Csf2, Csf3, Csf4, homologues thereof, or modified versions thereof. In some embodiments, the unmodified CRISPR enzyme has DNA cleavage activity, such as Cas9. In some embodiments, the CRISPR enzyme directs cleavage of one or both strands at the location of a target sequence, such as within the target sequence and/or within the complement of the target sequence. In some embodiments, the CRISPR enzyme directs cleavage of one or both strands within about 1, 2, 3, 4, 5, 6, 7, 8, 9, 10, 15, 20, 25, 50, 100, 200, 500, or more base pairs from the first or last nucleotide of a target sequence. In some embodiments, a vector encodes a CRISPR enzyme that is mutated to with respect to a corresponding wild-type enzyme such that the mutated CRISPR enzyme lacks the ability to cleave one or both strands of a target polynucleotide containing a target sequence. For example, an aspartate-to-alanine sub-

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

[0124] An aspartate-to-alanine substitution (D10A) in the RuvC I catalytic domain of SpCas9 was engineered to convert the nuclease into a nickase (SpCas9n) (see e.g. Sapranauskas et al., 2011, Nucleic Acis Research, 39: 9275; Gasiunas et al., 2012, Proc. Natl. Acad. Sci. USA, 109: E2579), such that nicked genomic DNA undergoes the high-fidelity homology-directed repair (HDR). Surveyor assay confirmed that SpCas9n does not generate indels at the EMX1 protospacer target. Co-expression of EMX1-targeting chimeric crRNA (having the tracrRNA component as well) with SpCas9 produced indels in the target site, whereas co-expression with SpCas9n did not (n=3). Moreover, sequencing of 327 amplicons did not detect any indels induced by SpCas9n. The same locus was selected to test CRISPR-mediated HR by co-transfecting HEK 293FT cells with the chimeric RNA targeting EMX1, hSpCas9 or hSpCas9n, as well as a HR template to introduce a pair of restriction sites (HindIII and NheI) near the protospacer.

[0125] Preferred orthologs are described herein. A Cas enzyme may be identified Cas9 as this can refer to the general class of enzymes that share homology to the biggest nuclease with multiple nuclease domains from the type II CRISPR system. Most preferably, the Cas9 enzyme is from, or is derived from, spCas9 or saCas9. By derived, Applicants mean that the derived enzyme is largely based, in the sense of having a high degree of sequence homology with, a wildtype enzyme, but that it has been mutated (modified) in some way as described herein.

[0126] In some embodiments, an enzyme coding sequence encoding a CRISPR enzyme is codon optimized for expression in particular cells, such as eukaryotic cells. The eukaryotic cells may be those of or derived from a particular organism, such as a mammal, including but not limited to human, mouse, rat, rabbit, dog, or non-human primate. In general, codon optimization refers to a process of modifying a nucleic acid sequence for enhanced expression in the host cells of interest by replacing at least one codon (e.g. about or more than about 1, 2, 3, 4, 5, 10, 15, 20, 25, 50, or more codons) of the native sequence with codons that are more frequently or most frequently used in the genes of that host cell while maintaining the native amino acid sequence. Various species exhibit particular bias for certain codons of a particular amino acid. Codon bias (differences in codon usage between organisms) often correlates with the efficiency of translation of messenger RNA (mRNA), which is in turn believed to be dependent on, among other things, the properties of the codons being translated and the availability of particular transfer RNA (tRNA) molecules. The predominance of selected tRNAs in a cell is generally a reflection of the codons used most frequently in peptide synthesis. Accordingly, genes can be tailored for optimal gene expression in a given organism based on codon optimization. Codon usage tables are readily available, for example, at the "Codon Usage Database" available at www.kazusa.orjp/codon/(visited Jul. 9, 2002), and these tables can be adapted in a number of ways. See Nakamura, Y., et al. "Codon usage tabulated from the international DNA sequence databases: status for the year 2000" Nucl. Acids Res. 28:292 (2000). Computer algorithms for codon optimizing a particular sequence for expression in a particular host cell are also available, such as Gene Forge (Aptagen; Jacobus, PA), are also available. In some embodiments, one or more codons (e.g. 1, 2, 3, 4, 5, 10, 15, 20, 25, 50, or more, or all codons) in a sequence encoding a CRISPR enzyme correspond to the most frequently used codon for a particular amino acid.

[0127] In some embodiments, a vector encodes a CRISPR enzyme which may comprise one or more nuclear localization sequences (NLSs), such as about or more than about 1, 2, 3, 4, 5, 6, 7, 8, 9, 10, or more NLSs. In some embodiments, the CRISPR enzyme may comprise about or more than about 1, 2, 3, 4, 5, 6, 7, 8, 9, 10, or more NLSs at or near the amino-terminus, about or more than about 1, 2, 3, 4, 5, 6, 7, 8, 9, 10, or more NLSs at or near the carboxy-terminus, or a combination of these (e.g. one or more NLS at the amino-terminus and one or more NLS at the carboxy terminus). When more than one NLS is present, each may be selected independently of the others, such that a single NLS may be present in more than one copy and/or in combination with one or more other NLSs present in one or more copies. In a preferred embodiment of the invention, the CRISPR enzyme may comprise at most 6 NLSs. In some embodiments, an NLS is considered near the N- or C-terminus when the nearest amino acid of the NLS is within about 1, 2, 3, 4, 5, 10, 15, 20, 25, 30, 40, 50, or more amino acids along the polypeptide chain from the N-or C-terminus. Non-limiting examples of NLSs include an NLS sequence derived from: the NLS of the SV40 virus large T-antigen, having the amino acid sequence PKKKRKV; the NLS from nucleoplasmin (e.g. the nucleoplasmin bipartite NLS with the sequence KRPAATKKAGQAKKKK); the c-myc NLS having the amino acid sequence PAAKRVKLD or RQRRNELKRSP; the hRNPA1 M9 NLS having the sequence NQSSNFGPMKGGNFGGRSSGPYGGGGQYFAK-PRNQGGY; the sequence RMRIZFKNKGKDTAEL-RRRRVEVSVELRKAKKDEQILKRRNV of the IBB domain from importin-alpha; the sequences VSRKRPRP and PPKKARED of the myoma T protein; the sequence POPKKKPL of human p53; the sequence SALIKKKKK-MAP of mouse c-abl IV; the sequences DRLRR and

PKQKKRK of the influenza virus NS1; the sequence RKLKKKIKKL of the Hepatitis virus delta antigen; the sequence REKKKFLKRR of the mouse Mxl protein; the sequence KRKGDEVDGVDEVAKKKSKK of the human poly(ADP-ribose) polymerase; and the sequence RKCLQAGMNLEARKTKK of the steroid hormone receptors (human) glucocorticoid.

[0128] In general, the one or more NLSs are of sufficient strength to drive accumulation of the CRISPR enzyme in a detectable amount in the nucleus of a eukaryotic cell. In general, strength of nuclear localization activity may derive from the number of NLSs in the CRISPR enzyme, the particular NLS(s) used, or a combination of these factors. Detection of accumulation in the nucleus may be performed by any suitable technique. For example, a detectable marker may be fused to the CRISPR enzyme, such that location within a cell may be visualized, such as in combination with a means for detecting the location of the nucleus (e.g. a stain specific for the nucleus such as DAPI). Cell nuclei may also be isolated from cells, the contents of which may then be analyzed by any suitable process for detecting protein, such as immunohistochemistry, Western blot, or enzyme activity assay. Accumulation in the nucleus may also be determined indirectly, such as by an assay for the effect of CRISPR complex formation (e.g. assay for DNA cleavage or mutation at the target sequence, or assay for altered gene expression activity affected by CRISPR complex formation and/or CRISPR enzyme activity), as compared to a control no exposed to the CRISPR enzyme or complex, or exposed to a CRISPR enzyme lacking the one or more NLSs.

[0129] In general, a guide sequence is any polynucleotide sequence having sufficient complementarity with a target polynucleotide sequence to hybridize with the target sequence and direct sequence-specific binding of a CRISPR complex to the target sequence. In some embodiments, the degree of complementarity between a guide sequence and its corresponding target sequence, when optimally aligned using a suitable alignment algorithm, is about or more than about 50%, 60%, 75%, 80%, 85%, 90%, 95%, 97.5%, 99%, or more. Optimal alignment may be determined with the use of any suitable algorithm for aligning sequences, non-limiting example of which include the Smith-Waterman algorithm, the Needleman-Wunsch algorithm, algorithms based on the Burrows-Wheeler Transform (e.g. the Burrows Wheeler Aligner), ClustalW, Clustal X, BLAT, Novoalign (Novocraft Technologies; available at www.novocraft.com), ELAND (Illumina, San Diego, CA), SOAP (available at soap.genomics.org.cn), and Maq (available at maq.sourceforge.net). In some embodiments, a guide sequence is about or more than about 5, 10, 11, 12, 13, 14, 15, 16, 17, 18, 19, 20, 21, 22, 23, 24, 25, 26, 27, 28, 29, 30, 35, 40, 45, 50, 75, or more nucleotides in length. In some embodiments, a guide sequence is less than about 75, 50, 45, 40, 35, 30, 25, 20, 15, 12, or fewer nucleotides in length. The ability of a guide sequence to direct sequence-specific binding of a CRISPR complex to a target sequence may be assessed by any suitable assay. For example, the components of a CRISPR system sufficient to form a CRISPR complex, including the guide sequence to be tested, may be provided to a host cell having the corresponding target sequence, such as by transfection with vectors encoding the components of the CRISPR sequence, followed by an assessment of preferential cleavage within the target sequence, such as by Surveyor assay as described herein. Similarly, cleavage of a target polynucleotide sequence may be evaluated in a test tube by providing the target sequence, components of a CRISPR complex, including the guide sequence to be tested and a control guide sequence different from the test guide sequence, and comparing binding or rate of cleavage at the target sequence between the test and control guide sequence reactions. Other assays are possible, and will occur to those skilled in the art.

[0130] A guide sequence may be selected to target any target sequence. In some embodiments, the target sequence is a sequence within a genome of a cell. Exemplary target sequences include those that are unique in the target genome. For example, for the *S. pyogenes* Cas9, a unique target sequence in a genome may include a Cas9 target site of the form MMMMMMMMNNNNNNNNNNNNXGG where NNNNNNNNNNNNXGG (N is A, G, T, or C; and X can be anything) has a single occurrence in the genome. A unique target sequence in a genome may include an *S. pyogenes* Cas9 target site of the form MMMMMMMMNNNNNNNNNNNNXGG where NNNNNNNNNNNNXGG (N is A, G, T, or C; and X can be anything) has a single occurrence in the genome. For the *S. thermophilus* CRISPR1 Cas9, a unique target sequence in a genome may include a Cas9 target site of the form MMMMMMMMNNNNNNNNNNNNXXAGAAW where NNNNNNNNNNNNXXAGAAW (N is A, G, T, or C; X can be anything; and W is A or T) has a single occurrence in the genome. A unique target sequence in a genome may include an *S. thermophilus* CRISPR1 Cas9 target site of the form MMMMMMMMNNNNNNNNNNNNXXAGAAW where NNNNNNNNNNNNXXAGAAW (N is A, G, T, or C; X can be anything; and W is A or T) has a single occurrence in the genome. For the *S. pyogenes* Cas9, a unique target sequence in a genome may include a Cas9 target site of the form MMMMMMMMNNNNNNNNNNNNXGGXG where NNNNNNNNNNNNXGGXG (N is A, G, T, or C; and X can be anything) has a single occurrence in the genome. A unique target sequence in a genome may include an *S. pyogenes* Cas9 target site of the form MMMMMMMMNNNNNNNNNNNNXGGXG where NNNNNNNNNNNNXGGXG (N is A, G, T, or C; and X can be anything) has a single occurrence in the genome. In each of these sequences "M" may be A, G, T, or C, and need not be considered in identifying a sequence as unique.

[0131] In some embodiments, a guide sequence is selected to reduce the degree of secondary structure within the guide sequence. In some embodiments, about or less than about 75%, 50%, 40%, 30%, 25%, 20%, 15%, 10%, 5%, 1%, or fewer of the nucleotides of the guide sequence participate in self-complementary base pairing when optimally folded. Optimal folding may be determined by any suitable polynucleotide folding algorithm. Some programs are based on calculating the minimal Gibbs free energy. An example of one such algorithm is mFold, as described by Zuker and Stiegler (Nucleic Acids Res. 9 (1981), 133-148). Another example folding algorithm is the online webserver RNAfold, developed at Institute for Theoretical Chemistry at the University of Vienna, using the centroid structure prediction algorithm (see e.g. A. R. Gruber et al., 2008, *Cell* 106(1): 23-24; and PA Carr and GM Church, 2009, *Nature Biotechnology* 27(12): 1151-62).

[0132] In general, a tracr mate sequence includes any sequence that has sufficient complementarity with a tracr sequence to promote one or more of: (1) excision of a guide

sequence flanked by tracr mate sequences in a cell containing the corresponding tracr sequence; and (2) formation of a CRISPR complex at a target sequence, wherein the CRISPR complex may comprise the tracr mate sequence hybridized to the tracr sequence. In general, degree of complementarity is with reference to the optimal alignment of the tracr mate sequence and tracr sequence, along the length of the shorter of the two sequences. Optimal alignment may be determined by any suitable alignment algorithm, and may further account for secondary structures, such as self-complementarity within either the tracr sequence or tracr mate sequence. In some embodiments, the degree of complementarity between the tracr sequence and tracr mate sequence along the length of the shorter of the two when optimally aligned is about or more than about 25%, 30%, 40%, 50%, 60%, 70%, 80%, 90%, 95%, 97.5%, 99%, or higher. In some embodiments, the tracr sequence is about or more than about 5, 6, 7, 8, 9, 10, 11, 12, 13, 14, 15, 16, 17, 18, 19, 20, 25, 30, 40, 50, or more nucleotides in length. In some embodiments, the tracr sequence and tracr mate sequence are contained within a single transcript, such that hybridization between the two produces a transcript having a secondary structure, such as a hairpin. In an embodiment of the invention, the transcript or transcribed polynucleotide sequence has at least two or more hairpins. In preferred embodiments, the transcript has two, three, four or five hairpins. In a further embodiment of the invention, the transcript has at most five hairpins. In a hairpin structure the portion of the sequence 5' of the final "N" and upstream of the loop corresponds to the tracr mate sequence, and the portion of the sequence 3' of the loop corresponds to the tracr sequence. Further non-limiting examples of single polynucleotides which may comprise a guide sequence, a tracr mate sequence, and a tracr sequence are as follows (listed 5' to 3'), where "N" represents a base of a guide sequence, the first block of lower case letters represent the tracr mate sequence, and the second block of lower case letters represent the tracr sequence, and the final poly-T sequence represents the transcription terminator: (1) NNNNNNNNNNNNNNNNNNNNgtttttgtactctcaagattaGAAAtaaatcttgcagaagctacaaagataaggctt catgccgaaatcaacaccctgtcattttatggcagggtgttttcgttatttaaTTTTTT; (2) NNNNNNNNNNNNNNNNNNNNgtttttgtactctcaGAAAtgcagaagctacaaagataaggcttcatgccgaaatca acaccctgtcatttatggcagggtgttttcgttatttaaTTTTTT; (3) NNNNNNNNNNNNNNNNNNNNgtttttgtactctcaGAAAtgcagaagctacaaagataaggcttcatgccgaaatca acaccctgtcatttatggcagggtgtTTTTTT; (4) NNNNNNNNNNNNNNNNNNNNgtttagagctaGAAAtagcaagttaaaataaggctagtccgttatcaacttgaaaaagtggcaccgagtcggtgcTTTTTT; (5) NNNNNNNNNNNNNNNNNNNNgtttagagctaGAAATAGcaagttaaaataaggctagtccgttatcaacttgaaaaagtgTTTTTTT; and (6) NNNNNNNNNNNNNNNNNNNNgtttagagctagAAATAGcaagttaaaataaggctagtccgttatcaTTTTTTTT. In some embodiments, sequences (1) to (3) are used in combination with Cas9 from *S. thermophilus* CRISPR1. In some embodiments, sequences (4) to (6) are used in combination with Cas9 from *S. pyogenes*. In some embodiments, the tracr sequence is a separate transcript from a transcript which may comprise the tracr mate sequence.

[0133] In some embodiments, a recombination template is also provided. A recombination template may be a component of another vector as described herein, contained in a separate vector, or provided as a separate polynucleotide. In some embodiments, a recombination template is designed to serve as a template in homologous recombination, such as within or near a target sequence nicked or cleaved by a CRISPR enzyme as a part of a CRISPR complex. A template polynucleotide may be of any suitable length, such as about or more than about 10, 15, 20, 25, 50, 75, 100, 150, 200, 500, 1000, or more nucleotides in length. In some embodiments, the template polynucleotide is complementary to a portion of a polynucleotide which may comprise the target sequence. When optimally aligned, a template polynucleotide might overlap with one or more nucleotides of a target sequences (e.g. about or more than about 1, 5, 10, 15, 20, or more nucleotides). In some embodiments, when a template sequence and a polynucleotide which may comprise a target sequence are optimally aligned, the nearest nucleotide of the template polynucleotide is within about 1, 5, 10, 15, 20, 25, 50, 75, 100, 200, 300, 400, 500, 1000, 5000, 10000, or more nucleotides from the target sequence.

[0134] In some embodiments, the CRISPR enzyme is part of a fusion protein which may comprise one or more heterologous protein domains (e.g. about or more than about 1, 2, 3, 4, 5, 6, 7, 8, 9, 10, or more domains in addition to the CRISPR enzyme). A CRISPR enzyme fusion protein may comprise any additional protein sequence, and optionally a linker sequence between any two domains. Examples of protein domains that may be fused to a CRISPR enzyme include, without limitation, epitope tags, reporter gene sequences, and protein domains having one or more of the following activities: methylase activity, demethylase activity, transcription activation activity, transcription repression activity, transcription release factor activity, histone modification activity, RNA cleavage activity and nucleic acid binding activity. Non-limiting examples of epitope tags include histidine (His) tags, V5 tags, FLAG tags, influenza hemagglutinin (HA) tags, Myc tags, VSV-G tags, and thioredoxin (Trx) tags. Examples of reporter genes include, but are not limited to, glutathione-S-transferase (GST), horseradish peroxidase (HRP), chloramphenicol acetyltransferase (CAT) beta-galactosidase, beta-glucuronidase, luciferase, green fluorescent protein (GFP), HcRed, DsRed, cyan fluorescent protein (CFP), yellow fluorescent protein (YFP), and autofluorescent proteins including blue fluorescent protein (BFP). A CRISPR enzyme may be fused to a gene sequence encoding a protein or a fragment of a protein that bind DNA molecules or bind other cellular molecules, including but not limited to maltose binding protein (MBP), S-tag, Lex A DNA binding domain (DBD) fusions, GAL4 DNA binding domain fusions, and herpes simplex virus (HSV) BP16 protein fusions. Additional domains that may form part of a fusion protein which may comprise a CRISPR enzyme are described in US20110059502, incorporated herein by reference. In some embodiments, a tagged CRISPR enzyme is used to identify the location of a target sequence.

[0135] In some aspects, the invention provides methods which may comprise delivering one or more polynucleotides, such as or one or more vectors as described herein, one or more transcripts thereof, and/or one or proteins transcribed therefrom, to a host cell. In some aspects, the invention further provides cells produced by such methods, and animals which may comprise or produced from such cells. In some embodiments, a CRISPR enzyme in combination with (and optionally complexed with) a guide

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

[0136] Methods of non-viral delivery of nucleic acids include lipofection, microinjection, biolistics, virosomes, liposomes, immunoliposomes, polycation or lipid:nucleic acid conjugates, naked DNA, artificial virions, and agent-enhanced uptake of DNA. Lipofection is described in e.g., U.S. Pat. Nos. 5,049,386, 4,946,787; and 4,897,355) and lipofection reagents are sold commercially (e.g., Transfectam™ and Lipofectin™). Cationic and neutral lipids that are suitable for efficient receptor-recognition lipofection of polynucleotides include those of Felgner, WO 91/17424; WO 91/16024. Delivery can be to cells (e.g. in vitro or ex vivo administration) or target tissues (e.g. in vivo administration).

[0137] The preparation of lipid:nucleic acid complexes, including targeted liposomes such as immunolipid complexes, is well known to one of skill in the art (see, e.g., Crystal, Science 270:404-410 (1995); Blaese et al., Cancer Gene Ther. 2:291-297 (1995); Behr et al., Bioconjugate Chem. 5:382-389 (1994); Remy et al., Bioconjugate Chem. 5:647-654 (1994); Gao et al., Gene Therapy 2:710-722 (1995); Ahmad et al., *Cancer Res.* 52:4817-4820 (1992); U.S. Pat. Nos. 4,186,183, 4,217,344, 4,235,871, 4,261,975, 4,485,054, 4,501,728, 4,774,085, 4,837,028, and 4,946,787).

[0138] The use of RNA or DNA viral based systems for the delivery of nucleic acids take advantage of highly evolved processes for targeting a virus to specific cells in the body and trafficking the viral payload to the nucleus. Viral vectors can be administered directly to patients (in vivo) or they can be used to treat cells in vitro, and the modified cells may optionally be administered to patients (ex vivo). Conventional viral based systems could include retroviral, lentivirus, adenoviral, adeno-associated and herpes simplex virus vectors for gene transfer. Integration in the host genome is possible with the retrovirus, lentivirus, and adeno-associated virus gene transfer methods, often resulting in long term expression of the inserted transgene. Additionally, high transduction efficiencies have been observed in many different cell types and target tissues.

[0139] The tropism of a retrovirus can be altered by incorporating foreign envelope proteins, expanding the potential target population of target cells. Lentiviral vectors are retroviral vectors that are able to transduce or infect non-dividing cells and typically produce high viral titers. Selection of a retroviral gene transfer system would therefore depend on the target tissue. Retroviral vectors are comprised of cis-acting long terminal repeats with packaging capacity for up to 6-10 kb of foreign sequence. The minimum cis-acting LTRs are sufficient for replication and packaging of the vectors, which are then used to integrate the therapeutic gene into the target cell to provide permanent transgene expression. Widely used retroviral vectors include those based upon murine leukemia virus (MuLV), gibbon ape leukemia virus (GaLV), Simian Immuno deficiency virus (SIV), human immuno deficiency virus (HIV), and combinations thereof (see, e.g., Buchscher et al., J. Virol. 66:2731-2739 (1992); Johann et al., J. Virol. 66:1635-1640 (1992); Sommnerfelt et al., Virol. 176:58-59 (1990); Wilson et al., J. Virol. 63:2374-2378 (1989); Miller et al., J. Virol. 65:2220-2224 (1991); PCT/US94/05700).

[0140] In applications where transient expression is preferred, adenoviral based systems may be used. Adenoviral based vectors are capable of very high transduction efficiency in many cell types and do not require cell division. With such vectors, high titer and levels of expression have been obtained. This vector can be produced in large quantities in a relatively simple system. Adeno-associated virus ("AAV") vectors may also be used to transduce cells with target nucleic acids, e.g., in the in vitro production of nucleic acids and peptides, and for in vivo and ex vivo gene therapy procedures (see, e.g., West et al., Virology 160:38-47 (1987); U.S. Pat. No. 4,797,368; WO 93/24641; Kotin, Human Gene Therapy 5:793-801 (1994); Muzyczka, J. Clin. Invest. 94:1351 (1994). Construction of recombinant AAV vectors are described in a number of publications, including U.S. Pat. No. 5,173,414; Tratschin et al., Mol. Cell. Biol. 5:3251-3260 (1985); Tratschin, et al., Mol. Cell. Biol. 4:2072-2081 (1984); Hermonat & Muzyczka, PNAS 81:6466-6470 (1984); and Samulski et al., J. Virol. 63:03822-3828 (1989).

[0141] Packaging cells are typically used to form virus particles that are capable of infecting a host cell. Such cells include 293 cells, which package adenovirus, and ψ2 cells or PA317 cells, which package retrovirus. Viral vectors used in gene therapy are usually generated by producer a cell line that packages a nucleic acid vector into a viral particle. The vectors typically contain the minimal viral sequences required for packaging and subsequent integration into a host, other viral sequences being replaced by an expression cassette for the polynucleotide(s) to be expressed. The missing viral functions are typically supplied in trans by the packaging cell line. For example, AAV vectors used in gene therapy typically only possess ITR sequences from the AAV genome which are required for packaging and integration into the host genome. Viral DNA is packaged in a cell line, which contains a helper plasmid encoding the other AAV genes, namely rep and cap, but lacking ITR sequences. The cell line may also infected with adenovirus as a helper. The helper virus promotes replication of the AAV vector and expression of AAV genes from the helper plasmid. The helper plasmid is not packaged in significant amounts due to a lack of ITR sequences. Contamination with adenovirus can be reduced by, e.g., heat treatment to which adenovirus is more sensitive than AAV. Additional methods for the deliv-

ery of nucleic acids to cells are known to those skilled in the art. See, for example, US20030087817, incorporated herein by reference.

[0142] In some embodiments, a host cell is transiently or non-transiently transfected with one or more vectors described herein. In some embodiments, a cell is transfected as it naturally occurs in a subject. In some embodiments, a cell that is transfected is taken from a subject. In some embodiments, the cell is derived from cells taken from a subject, such as a cell line. A wide variety of cell lines for tissue culture are known in the art. Examples of cell lines include, but are not limited to, C8161, CCRF-CEM, MOLT, mIMCD-3, NHDF, HeLa-S3, Huh1, Huh4, Huh7, HUVEC, HASMC, HEKn, HEKa, MiaPaCell, Panc1, PC-3, TF1, CTLL-2, C1R, Rat6, CV1, RPTE, A10, T24, J82, A375, ARH-77, Calu1, SW480, SW620, SKOV3, SK-UT, CaCo2, P388D1, SEM-K2, WEHI-231, HB56, TIB55, Jurkat, J45.01, LRMB, Bcl-1, BC-3, IC21, DLD2, Raw264.7, NRK, NRK-52E, MRC5, MEF, Hep G2, HeLa B, HeLa T4, COS, COS-1, COS-6, COS-M6A, BS-C-1 monkey kidney epithelial, BALB/3T3 mouse embryo fibroblast, 3T3 Swiss, 3T3-L1, 132-d5 human fetal fibroblasts; 10.1 mouse fibroblasts, 293-T, 3T3, 721, 9L, A2780, A2780ADR, A2780cis, A172, A20, A253, A431, A-549, ALC, B16, B35, BCP-1 cells, BEAS-2B, bEnd.3, BHK-21, BR 293, BxPC3, C3H-10T1/2, C6/36, Cal-27, CHO, CHO-7, CHO-IR, CHO-K1, CHO-K2, CHO-T, CHO Dhfr −/−, COR-L23, COR-L23/CPR, COR-L23/5010, COR-L23/R23, COS-7, COV-434, CML T1, CMT, CT26, D17, DH82, DU145, DuCaP, EL4, EM2, EM3, EMT6/AR1, EMT6/AR10.0, FM3, H1299, H69, HB54, HB55, HCA2, HEK-293, HeLa, Hepa1c1c7, HL-60, HMEC, HT-29, Jurkat, JY cells, K562 cells, Ku812, KCL22, KG1, KYO1, LNCap, Ma-Mel 1-48, MC-38, MCF-7, MCF-10A, MDA-MB-231, MDA-MB-468, MDA-MB-435, MDCK II, MDCK II, MOR/0.2R, MONO-MAC 6, MTD-1A, MyEnd, NCI-H69/CPR, NCI-H69/LX10, NCI-H69/LX20, NCI-H69/LX4, NIH-3T3, NALM-1, NW-145, OPCN/OPCT cell lines, Peer, PNT-1A/PNT 2, RenCa, RIN-5F, RMA/RMAS, Saos-2 cells, Sf-9, SkBr3, T2, T-47D, T84, THP1 cell line, U373, U87, U937, VCaP, Vero cells, WM39, WT-49, X63, YAC-1, YAR, and transgenic varieties thereof. Cell lines are available from a variety of sources known to those with skill in the art (see, e.g., the American Type Culture Collection (ATCC) (Manassas, Va.)). In some embodiments, a cell transfected with one or more vectors described herein is used to establish a new cell line which may comprise one or more vector-derived sequences. In some embodiments, a cell transiently transfected with the components of a CRISPR system as described herein (such as by transient transfection of one or more vectors, or transfection with RNA), and modified through the activity of a CRISPR complex, is used to establish a new cell line which may comprise cells containing the modification but lacking any other exogenous sequence. In some embodiments, cells transiently or non-transiently transfected with one or more vectors described herein, or cell lines derived from such cells are used in assessing one or more test compounds.

[0143] In some embodiments, one or more vectors described herein are used to produce a non-human transgenic animal or transgenic plant. In some embodiments, the transgenic animal is a mammal, such as a mouse, rat, or rabbit. Methods for producing transgenic plants and animals are known in the art, and generally begin with a method of cell transfection, such as described herein.

[0144] With recent advances in crop genomics, the ability to use CRISPR-Cas systems to perform efficient and cost effective gene editing and manipulation will allow the rapid selection and comparison of single and and multiplexed genetic manipulations to transform such genomes for improved production and enhanced traits. In this regard reference is made to US patents and publications: U.S. Pat. No. 6,603,061 —*Agrobacterium*-Mediated Plant Transformation Method; U.S. Pat. No. 7,868,149—Plant Genome Sequences and Uses Thereof and US 2009/0100536—Transgenic Plants with Enhanced Agronomic Traits, all the contents and disclosure of each of which are herein incorporated by reference in their entirety. In the practice of the invention, the contents and disclosure of Morrell et al "Crop genomics:advances and applications" Nat Rev Genet. 2011 Dec. 29; 13(2):85-96 are also herein incorporated by reference in their entirety. In an advantageous embodiment of the invention, the CRISPR/Cas9 system is used to engineer microalgae. Accordingly, reference herein to animal cells may also apply, mutatis mutandis, to plant cells unless otherwise apparent.

[0145] In plants, pathogens are often host-specific. For example, *Fusarium oxysporum* f. sp. *lycopersici* causes tomato wilt but attacks only tomato, and *F. oxysporum* f *dianthii Puccinia graminis* f. sp. *tritici* attacks only wheat. Plants have existing and induced defenses to resist most pathogens. Mutations and recombination events across plant generations lead to genetic variability that gives rise to susceptibility, especially as pathogens reproduce with more frequency than plants. In plants there can be non-host resistance, e.g., the host and pathogen are incompatible. There can also be Horizontal Resistance, e.g., partial resistance against all races of a pathogen, typically controlled by many genes and Vertical Resistance, e.g., complete resistance to some races of a pathogen but not to other races, typically controlled by a few genes. In a Gene-for-Gene level, plants and pathogens evolve together, and the genetic changes in one balance changes in other. Accordingly, using Natural Variability, breeders combine most useful genes for Yield, Quality, Uniformity, Hardiness, Resistance. The sources of resistance genes include native or foreign Varieties, Heirloom Varieties, Wild Plant Relatives, and Induced Mutations, e.g., treating plant material with mutagenic agents. Using the present invention, plant breeders are provided with a new tool to induce mutations. Accordingly, one skilled in the art can analyze the genome of sources of resistance genes, and in Varieties having desired characteristics or traits employ the present invention to induce the rise of resistance genes, with more precision than previous mutagenic agents and hence accelerate and improve plant breeding programs.

[0146] In one aspect, the invention provides for methods of modifying a target polynucleotide in a eukaryotic cell, which may be in vivo, ex vivo or in vitro. In some embodiments, the method comprises sampling a cell or population of cells from a human or non-human animal or plant (including micro-algae), and modifying the cell or cells. Culturing may occur at any stage ex vivo. The cell or cells may even be re-introduced into the non-human animal or plant (including micro-algae). For re-introduced cells it is particularly preferred that the cells are stem cells.

[0147] In one aspect, the invention provides for methods of modifying a target polynucleotide in a eukaryotic cell. In some embodiments, the method may comprise allowing a CRISPR complex to bind to the target polynucleotide to effect cleavage of said target polynucleotide thereby modifying the target polynucleotide, wherein the CRISPR complex may comprise a CRISPR enzyme complexed with a guide sequence hybridized to a target sequence within said target polynucleotide, wherein said guide sequence is linked to a tracr mate sequence which in turn hybridizes to a tracr sequence.

[0148] In one aspect, the invention provides a method of modifying expression of a polynucleotide in a eukaryotic cell. In some embodiments, the method may comprise allowing a CRISPR complex to bind to the polynucleotide such that said binding results in increased or decreased expression of said polynucleotide; wherein the CRISPR complex may comprise a CRISPR enzyme complexed with a guide sequence hybridized to a target sequence within said polynucleotide, wherein said guide sequence is linked to a tracr mate sequence which in turn hybridizes to a tracr sequence.

[0149] In one aspect, the invention provides kits containing any one or more of the elements disclosed in the above methods and compositions. Elements may be provided individually or in combinations, and may be provided in any suitable container, such as a vial, a bottle, or a tube. In some embodiments, the kit includes instructions in one or more languages, for example in more than one language.

[0150] In some embodiments, a kit may comprise one or more reagents for use in a process utilizing one or more of the elements described herein. Reagents may be provided in any suitable container. For example, a kit may provide one or more reaction or storage buffers. Reagents may be provided in a form that is usable in a particular assay, or in a form that requires addition of one or more other components before use (e.g. in concentrate or lyophilized form). A buffer can be any buffer, including but not limited to a sodium carbonate buffer, a sodium bicarbonate buffer, a borate buffer, a Tris buffer, a MOPS buffer, a HEPES buffer, and combinations thereof. In some embodiments, the buffer is alkaline. In some embodiments, the buffer has a pH from about 7 to about 10. In some embodiments, the kit may comprise one or more oligonucleotides corresponding to a guide sequence for insertion into a vector so as to operably link the guide sequence and a regulatory element. In some embodiments, the kit may comprise a homologous recombination template polynucleotide.

[0151] In one aspect, the invention provides methods for using one or more elements of a CRISPR system. The CRISPR complex of the invention provides an effective means for modifying a target polynucleotide. The CRISPR complex of the invention has a wide variety of utility including modifying (e.g., deleting, inserting, translocating, inactivating, activating) a target polynucleotide in a multiplicity of cell types. As such the CRISPR complex of the invention has a broad spectrum of applications in, e.g., gene therapy, drug screening, disease diagnosis, and prognosis. An exemplary CRISPR complex may comprise a CRISPR enzyme complexed with a guide sequence hybridized to a target sequence within the target polynucleotide. The guide sequence is linked to a tracr mate sequence, which in turn hybridizes to a tracr sequence.

[0152] In one embodiment, this invention provides a method of cleaving a target polynucleotide. The method may comprise modifying a target polynucleotide using a CRISPR complex that binds to the target polynucleotide and effect cleavage of said target polynucleotide. Typically, the CRISPR complex of the invention, when introduced into a cell, creates a break (e.g., a single or a double strand break) in the genome sequence. For example, the method can be used to cleave a disease gene in a cell.

[0153] The break created by the CRISPR complex can be repaired by a repair processes such as the error prone non-homologous end joining (NHEJ) pathway or the high fidelity homology-directed repair (HDR). During these repair process, an exogenous polynucleotide template can be introduced into the genome sequence. In some methods, the HDR process is used modify genome sequence. For example, an exogenous polynucleotide template which may comprise a sequence to be integrated flanked by an upstream sequence and a downstream sequence is introduced into a cell. The upstream and downstream sequences share sequence similarity with either side of the site of integration in the chromosome.

[0154] Where desired, a donor polynucleotide can be DNA, e.g., a DNA plasmid, a bacterial artificial chromosome (BAC), a yeast artificial chromosome (YAC), a viral vector, a linear piece of DNA, a PCR fragment, a naked nucleic acid, or a nucleic acid complexed with a delivery vehicle such as a liposome or poloxamer.

[0155] The exogenous polynucleotide template may comprise a sequence to be integrated (e.g, a mutated gene). The sequence for integration may be a sequence endogenous or exogenous to the cell. Examples of a sequence to be integrated include polynucleotides encoding a protein or a non-coding RNA (e.g., a microRNA). Thus, the sequence for integration may be operably linked to an appropriate control sequence or sequences. Alternatively, the sequence to be integrated may provide a regulatory function.

[0156] The upstream and downstream sequences in the exogenous polynucleotide template are selected to promote recombination between the chromosomal sequence of interest and the donor polynucleotide. The upstream sequence is a nucleic acid sequence that shares sequence similarity with the genome sequence upstream of the targeted site for integration. Similarly, the downstream sequence is a nucleic acid sequence that shares sequence similarity with the chromosomal sequence downstream of the targeted site of integration. The upstream and downstream sequences in the exogenous polynucleotide template can have 75%, 80%, 85%, 90%, 95%, or 100% sequence identity with the targeted genome sequence. Preferably, the upstream and downstream sequences in the exogenous polynucleotide template have about 95%, 96%, 97%, 98%, 99%, or 100% sequence identity with the targeted genome sequence. In some methods, the upstream and downstream sequences in the exogenous polynucleotide template have about 99% or 100% sequence identity with the targeted genome sequence.

[0157] An upstream or downstream sequence may comprise from about 20 bp to about 2500 bp, for example, about 50, 100, 200, 300, 400, 500, 600, 700, 800, 900, 1000, 1100, 1200, 1300, 1400, 1500, 1600, 1700, 1800, 1900, 2000, 2100, 2200, 2300, 2400, or 2500 bp. In some methods, the exemplary upstream or downstream sequence have about 200 bp to about 2000 bp, about 600 bp to about 1000 bp, or more particularly about 700 bp to about 1000 bp.

[0158] In some methods, the exogenous polynucleotide template may further comprise a marker. Such a marker may make it easy to screen for targeted integrations. Examples of suitable markers include restriction sites, fluorescent proteins, or selectable markers. The exogenous polynucleotide template of the invention can be constructed using recombinant techniques (see, for example, Sambrook et al., 2001 and Ausubel et al., 1996).

[0159] In an exemplary method for modifying a target polynucleotide by integrating an exogenous polynucleotide template, a double stranded break is introduced into the genome sequence by the CRISPR complex, the break is repaired via homologous recombination an exogenous polynucleotide template such that the template is integrated into the genome. The presence of a double-stranded break facilitates integration of the template.

[0160] In other embodiments, this invention provides a method of modifying expression of a polynucleotide in a eukaryotic cell. The method may comprise increasing or decreasing expression of a target polynucleotide by using a CRISPR complex that binds to the polynucleotide.

[0161] In some methods, a target polynucleotide can be inactivated to effect the modification of the expression in a cell. For example, upon the binding of a CRISPR complex to a target sequence in a cell, the target polynucleotide is inactivated such that the sequence is not transcribed, the coded protein is not produced, or the sequence does not function as the wild-type sequence does. For example, a protein or microRNA coding sequence may be inactivated such that the protein is not produced.

[0162] In some methods, a control sequence can be inactivated such that it no longer functions as a control sequence. As used herein, "control sequence" refers to any nucleic acid sequence that effects the transcription, translation, or accessibility of a nucleic acid sequence. Examples of a control sequence include, a promoter, a transcription terminator, and an enhancer are control sequences.

[0163] The inactivated target sequence may include a deletion mutation (i.e., deletion of one or more nucleotides), an insertion mutation (i.e., insertion of one or more nucleotides), or a nonsense mutation (i.e., substitution of a single nucleotide for another nucleotide such that a stop codon is introduced). In some methods, the inactivation of a target sequence results in "knock-out" of the target sequence.

[0164] A method of the invention may be used to create a plant, an animal or cell that may be used as a disease model. As used herein, "disease" refers to a disease, disorder, or indication in a subject. For example, a method of the invention may be used to create an animal or cell that may comprise a modification in one or more nucleic acid sequences associated with a disease, or an animal or cell in which the expression of one or more nucleic acid sequences associated with a disease are altered. Such a nucleic acid sequence may encode a disease associated protein sequence or may be a disease associated control sequence.

[0165] In some methods, the disease model can be used to study the effects of mutations on the animal or cell and development and/or progression of the disease using measures commonly used in the study of the disease. Alternatively, such a disease model is useful for studying the effect of a pharmaceutically active compound on the disease.

[0166] In some methods, the disease model can be used to assess the efficacy of a potential gene therapy strategy. That is, a disease-associated gene or polynucleotide can be modified such that the disease development and/or progression is inhibited or reduced. In particular, the method may comprise modifying a disease-associated gene or polynucleotide such that an altered protein is produced and, as a result, the animal or cell has an altered response. Accordingly, in some methods, a genetically modified animal may be compared with an animal predisposed to development of the disease such that the effect of the gene therapy event may be assessed.

[0167] In another embodiment, this invention provides a method of developing a biologically active agent that modulates a cell signaling event associated with a disease gene. The method may comprise contacting a test compound with a cell which may comprise one or more vectors that drive expression of one or more of a CRISPR enzyme, a guide sequence linked to a tracr mate sequence, and a tracr sequence; and detecting a change in a readout that is indicative of a reduction or an augmentation of a cell signaling event associated with, e.g., a mutation in a disease gene contained in the cell.

[0168] A cell model or animal model can be constructed in combination with the method of the invention for screening a cellular function change. Such a model may be used to study the effects of a genome sequence modified by the CRISPR complex of the invention on a cellular function of interest. For example, a cellular function model may be used to study the effect of a modified genome sequence on intracellular signaling or extracellular signaling. Alternatively, a cellular function model may be used to study the effects of a modified genome sequence on sensory perception. In some such models, one or more genome sequences associated with a signaling biochemical pathway in the model are modified.

[0169] An altered expression of one or more genome sequences associated with a signaling biochemical pathway can be determined by assaying for a difference in the mRNA levels of the corresponding genes between the test model cell and a control cell, when they are contacted with a candidate agent. Alternatively, the differential expression of the sequences associated with a signaling biochemical pathway is determined by detecting a difference in the level of the encoded polypeptide or gene product.

[0170] To assay for an agent-induced alteration in the level of mRNA transcripts or corresponding polynucleotides, nucleic acid contained in a sample is first extracted according to standard methods in the art. For instance, mRNA can be isolated using various lytic enzymes or chemical solutions according to the procedures set forth in Sambrook et al. (1989), or extracted by nucleic-acid-binding resins following the accompanying instructions provided by the manufacturers. The mRNA contained in the extracted nucleic acid sample is then detected by amplification procedures or conventional hybridization assays (e.g. Northern blot analysis) according to methods widely known in the art or based on the methods exemplified herein.

[0171] For purpose of this invention, amplification means any method employing a primer and a polymerase capable of replicating a target sequence with reasonable fidelity. Amplification may be carried out by natural or recombinant DNA polymerases such as TaqGold™, T7 DNA polymerase, Klenow fragment of *E. coli* DNA polymerase, and reverse transcriptase. A preferred amplification method is PCR. In particular, the isolated RNA can be subjected to a reverse transcription assay that is coupled with a quantitative poly-

merase chain reaction (RT-PCR) in order to quantify the expression level of a sequence associated with a signaling biochemical pathway.

[0172] Detection of the gene expression level can be conducted in real time in an amplification assay. In one aspect, the amplified products can be directly visualized with fluorescent DNA-binding agents including but not limited to DNA intercalators and DNA groove binders. Because the amount of the intercalators incorporated into the double-stranded DNA molecules is typically proportional to the amount of the amplified DNA products, one can conveniently determine the amount of the amplified products by quantifying the fluorescence of the intercalated dye using conventional optical systems in the art. DNA-binding dye suitable for this application include SYBR green, SYBR blue, DAPI, propidium iodine, Hoeste, SYBR gold, ethidium bromide, acridines, proflavine, acridine orange, acriflavine, fluorcoumanin, ellipticine, daunomycin, chloroquine, distamycin D, chromomycin, homidium, mithramycin, ruthenium polypyridyls, anthramycin, and the like.

[0173] In another aspect, other fluorescent labels such as sequence specific probes can be employed in the amplification reaction to facilitate the detection and quantification of the amplified products. Probe-based quantitative amplification relies on the sequence-specific detection of a desired amplified product. It utilizes fluorescent, target-specific probes (e.g., TaqMan® probes) resulting in increased specificity and sensitivity. Methods for performing probe-based quantitative amplification are well established in the art and are taught in U.S. Pat. No. 5,210,015.

[0174] In yet another aspect, conventional hybridization assays using hybridization probes that share sequence homology with sequences associated with a signaling biochemical pathway can be performed. Typically, probes are allowed to form stable complexes with the sequences associated with a signaling biochemical pathway contained within the biological sample derived from the test subject in a hybridization reaction. It will be appreciated by one of skill in the art that where antisense is used as the probe nucleic acid, the target polynucleotides provided in the sample are chosen to be complementary to sequences of the antisense nucleic acids. Conversely, where the nucleotide probe is a sense nucleic acid, the target polynucleotide is selected to be complementary to sequences of the sense nucleic acid.

[0175] Hybridization can be performed under conditions of various stringency. Suitable hybridization conditions for the practice of the present invention are such that the recognition interaction between the probe and sequences associated with a signaling biochemical pathway is both sufficiently specific and sufficiently stable. Conditions that increase the stringency of a hybridization reaction are widely known and published in the art. See, for example, Sambrook, et al., (1989); Nonradioactive In Situ Hybridization Application Manual, Boehringer Mannheim, second edition. The hybridization assay can be formed using probes immobilized on any solid support, including but are not limited to nitrocellulose, glass, silicon, and a variety of gene arrays. A preferred hybridization assay is conducted on high-density gene chips as described in U.S. Pat. No. 5,445,934.

[0176] For a convenient detection of the probe-target complexes formed during the hybridization assay, the nucleotide probes are conjugated to a detectable label. Detectable labels suitable for use in the present invention include any composition detectable by photochemical, biochemical, spectroscopic, immunochemical, electrical, optical or chemical means. A wide variety of appropriate detectable labels are known in the art, which include fluorescent or chemiluminescent labels, radioactive isotope labels, enzymatic or other ligands. In preferred embodiments, one will likely desire to employ a fluorescent label or an enzyme tag, such as digoxigenin, 3-galactosidase, urease, alkaline phosphatase or peroxidase, avidin/biotin complex.

[0177] The detection methods used to detect or quantify the hybridization intensity will typically depend upon the label selected above. For example, radiolabels may be detected using photographic film or a phosphoimager. Fluorescent markers may be detected and quantified using a photodetector to detect emitted light. Enzymatic labels are typically detected by providing the enzyme with a substrate and measuring the reaction product produced by the action of the enzyme on the substrate; and finally colorimetric labels are detected by simply visualizing the colored label.

[0178] An agent-induced change in expression of sequences associated with a signaling biochemical pathway can also be determined by examining the corresponding gene products. Determining the protein level typically involves a) contacting the protein contained in a biological sample with an agent that specifically bind to a protein associated with a signaling biochemical pathway; and (b) identifying any agent:protein complex so formed. In one aspect of this embodiment, the agent that specifically binds a protein associated with a signaling biochemical pathway is an antibody, preferably a monoclonal antibody.

[0179] The reaction is performed by contacting the agent with a sample of the proteins associated with a signaling biochemical pathway derived from the test samples under conditions that will allow a complex to form between the agent and the proteins associated with a signaling biochemical pathway. The formation of the complex can be detected directly or indirectly according to standard procedures in the art. In the direct detection method, the agents are supplied with a detectable label and unreacted agents may be removed from the complex; the amount of remaining label thereby indicating the amount of complex formed. For such method, it is preferable to select labels that remain attached to the agents even during stringent washing conditions. It is preferable that the label does not interfere with the binding reaction. In the alternative, an indirect detection procedure may use an agent that contains a label introduced either chemically or enzymatically. A desirable label generally does not interfere with binding or the stability of the resulting agent:polypeptide complex. However, the label is typically designed to be accessible to an antibody for an effective binding and hence generating a detectable signal.

[0180] A wide variety of labels suitable for detecting protein levels are known in the art. Non-limiting examples include radioisotopes, enzymes, colloidal metals, fluorescent compounds, bioluminescent compounds, and chemiluminescent compounds.

[0181] The amount of agent:polypeptide complexes formed during the binding reaction can be quantified by standard quantitative assays. As illustrated above, the formation of agent:polypeptide complex can be measured directly by the amount of label remained at the site of binding. In an alternative, the protein associated with a signaling biochemical pathway is tested for its ability to compete with a labeled analog for binding sites on the

specific agent. In this competitive assay, the amount of label captured is inversely proportional to the amount of protein sequences associated with a signaling biochemical pathway present in a test sample.

[0182] A number of techniques for protein analysis based on the general principles outlined above are available in the art. They include but are not limited to radioimmunoassays, ELISA (enzyme linked immunoradiometric assays), "sandwich" immunoassays, immunoradiometric assays, in situ immunoassays (using e.g., colloidal gold, enzyme or radioisotope labels), western blot analysis, immunoprecipitation assays, immunofluorescent assays, and SDS-PAGE.

[0183] Antibodies that specifically recognize or bind to proteins associated with a signaling biochemical pathway are preferable for conducting the aforementioned protein analyses. Where desired, antibodies that recognize a specific type of post-translational modifications (e.g., signaling biochemical pathway inducible modifications) can be used. Post-translational modifications include but are not limited to glycosylation, lipidation, acetylation, and phosphorylation. These antibodies may be purchased from commercial vendors. For example, anti-phosphotyrosine antibodies that specifically recognize tyrosine-phosphorylated proteins are available from a number of vendors including Invitrogen and Perkin Elmer. Anti-phosphotyrosine antibodies are particularly useful in detecting proteins that are differentially phosphorylated on their tyrosine residues in response to an ER stress. Such proteins include but are not limited to eukaryotic translation initiation factor 2 alpha (eIF-2a). Alternatively, these antibodies can be generated using conventional polyclonal or monoclonal antibody technologies by immunizing a host animal or an antibody-producing cell with a target protein that exhibits the desired post-translational modification.

[0184] In practicing the subject method, it may be desirable to discern the expression pattern of an protein associated with a signaling biochemical pathway in different bodily tissue, in different cell types, and/or in different subcellular structures. These studies can be performed with the use of tissue-specific, cell-specific or subcellular structure specific antibodies capable of binding to protein markers that are preferentially expressed in certain tissues, cell types, or subcellular structures.

[0185] An altered expression of a gene associated with a signaling biochemical pathway can also be determined by examining a change in activity of the gene product relative to a control cell. The assay for an agent-induced change in the activity of a protein associated with a signaling biochemical pathway will dependent on the biological activity and/or the signal transduction pathway that is under investigation. For example, where the protein is a kinase, a change in its ability to phosphorylate the downstream substrate(s) can be determined by a variety of assays known in the art. Representative assays include but are not limited to immunoblotting and immunoprecipitation with antibodies such as anti-phosphotyrosine antibodies that recognize phosphorylated proteins. In addition, kinase activity can be detected by high throughput chemiluminescent assays such as AlphaScreen™ (available from Perkin Elmer) and eTag™ assay (Chan-Hui, et al. (2003) Clinical Immunology 111: 162-174).

[0186] Where the protein associated with a signaling biochemical pathway is part of a signaling cascade leading to a fluctuation of intracellular pH condition, pH sensitive molecules such as fluorescent pH dyes can be used as the reporter molecules. In another example where the protein associated with a signaling biochemical pathway is an ion channel, fluctuations in membrane potential and/or intracellular ion concentration can be monitored. A number of commercial kits and high-throughput devices are particularly suited for a rapid and robust screening for modulators of ion channels. Representative instruments include FLIPR™ (Molecular Devices, Inc.) and VIPR (Aurora Biosciences). These instruments are capable of detecting reactions in over 1000 sample wells of a microplate simultaneously, and providing real-time measurement and functional data within a second or even a minisecond.

[0187] In practicing any of the methods disclosed herein, a suitable vector can be introduced to a cell or an embryo via one or more methods known in the art, including without limitation, microinjection, electroporation, sonoporation, biolistics, calcium phosphate-mediated transfection, cationic transfection, liposome transfection, dendrimer transfection, heat shock transfection, nucleofection transfection, magnetofection, lipofection, impalefection, optical transfection, proprietary agent-enhanced uptake of nucleic acids, and delivery via liposomes, immunoliposomes, virosomes, or artificial virions. In some methods, the vector is introduced into an embryo by microinjection. The vector or vectors may be microinjected into the nucleus or the cytoplasm of the embryo. In some methods, the vector or vectors may be introduced into a cell by nucleofection.

[0188] The target polynucleotide of a CRISPR complex can be any polynucleotide endogenous or exogenous to the eukaryotic cell. For example, the target polynucleotide can be a polynucleotide residing in the nucleus of the eukaryotic cell. The target polynucleotide can be a sequence coding a gene product (e.g., a protein) or a non-coding sequence (e.g., a regulatory polynucleotide or a junk DNA).

[0189] Examples of target polynucleotides include a sequence associated with a signaling biochemical pathway, e.g., a signaling biochemical pathway-associated gene or polynucleotide. Examples of target polynucleotides include a disease associated gene or polynucleotide. A "disease-associated" gene or polynucleotide refers to any gene or polynucleotide which is yielding transcription or translation products at an abnormal level or in an abnormal form in cells derived from a disease-affected tissues compared with tissues or cells of a non disease control. It may be a gene that becomes expressed at an abnormally high level; it may be a gene that becomes expressed at an abnormally low level, where the altered expression correlates with the occurrence and/or progression of the disease. A disease-associated gene also refers to a gene possessing mutation(s) or genetic variation that is directly responsible or is in linkage disequilibrium with a gene(s) that is responsible for the etiology of a disease. The transcribed or translated products may be known or unknown, and may be at a normal or abnormal level.

[0190] The target polynucleotide of a CRISPR complex can be any polynucleotide endogenous or exogenous to the eukaryotic cell. For example, the target polynucleotide can be a polynucleotide residing in the nucleus of the eukaryotic cell. The target polynucleotide can be a sequence coding a gene product (e.g., a protein) or a non-coding sequence (e.g., a regulatory polynucleotide or a junk DNA). Without wishing to be bound by theory, it is believed that the target sequence should be associated with a PAM (protospacer

adjacent motif); that is, a short sequence recognized by the CRISPR complex. The precise sequence and length requirements for the PAM differ depending on the CRISPR enzyme used, but PAMs are typically 2-5 base pair sequences adjacent the protospacer (that is, the target sequence) Examples of PAM sequences are given in the examples section below, and the skilled person will be able to identify further PAM sequences for use with a given CRISPR enzyme.

[0191] Examples of target polynucleotides include a sequence associated with a signaling biochemical pathway, e.g., a signaling biochemical pathway-associated gene or polynucleotide. Examples of target polynucleotides include a disease associated gene or polynucleotide. A "disease-associated" gene or polynucleotide refers to any gene or polynucleotide which is yielding transcription or translation products at an abnormal level or in an abnormal form in cells derived from a disease-affected tissues compared with tissues or cells of a non disease control. It may be a gene that becomes expressed at an abnormally high level; it may be a gene that becomes expressed at an abnormally low level, where the altered expression correlates with the occurrence and/or progression of the disease. A disease-associated gene also refers to a gene possessing mutation(s) or genetic variation that is directly responsible or is in linkage disequilibrium with a gene(s) that is responsible for the etiology of a disease. The transcribed or translated products may be known or unknown, and may be at a normal or abnormal level.

[0192] The target polynucleotide of a CRISPR complex may include a number of disease-associated genes and polynucleotides as well as signaling biochemical pathway-associated genes and polynucleotides as listed in U.S. provisional patent applications 61/736,527 and 61/748,427 having Broad reference BI-2011/008/WSGR Docket No. 44063-701.101 and BI-2011/008/WSGR Docket No. 44063-701.102 respectively, both entitled SYSTEMS METHODS AND COMPOSITIONS FOR SEQUENCE MANIPULATION filed on Dec. 12, 2012 and Jan. 2, 2013, respectively, the contents of all of which are herein incorporated by reference in their entirety.

[0193] Examples of target polynucleotides include a sequence associated with a signaling biochemical pathway, e.g., a signaling biochemical pathway-associated gene or polynucleotide. Examples of target polynucleotides include a disease associated gene or polynucleotide. A "disease-associated" gene or polynucleotide refers to any gene or polynucleotide which is yielding transcription or translation products at an abnormal level or in an abnormal form in cells derived from a disease-affected tissues compared with tissues or cells of a non disease control. It may be a gene that becomes expressed at an abnormally high level; it may be a gene that becomes expressed at an abnormally low level, where the altered expression correlates with the occurrence and/or progression of the disease. A disease-associated gene also refers to a gene possessing mutation(s) or genetic variation that is directly responsible or is in linkage disequilibrium with a gene(s) that is responsible for the etiology of a disease. The transcribed or translated products may be known or unknown, and may be at a normal or abnormal level.

[0194] Examples of disease-associated genes and polynucleotides are available from McKusick-Nathans Institute of Genetic Medicine, Johns Hopkins University (Baltimore, Md.) and National Center for Biotechnology Information, National Library of Medicine (Bethesda, Md.), available on the World Wide Web.

[0195] Examples of disease-associated genes and polynucleotides are listed in Tables A and B. Disease specific information is available from McKusick-Nathans Institute of Genetic Medicine, Johns Hopkins University (Baltimore, Md.) and National Center for Biotechnology Information, National Library of Medicine (Bethesda, Md.), available on the World Wide Web. Examples of signaling biochemical pathway-associated genes and polynucleotides are listed in Table C.

[0196] Mutations in these genes and pathways can result in production of improper proteins or proteins in improper amounts which affect function. Further examples of genes, diseases and proteins are hereby incorporated by reference from U.S. Provisional applications 61/736,527 filed Dec. 12, 2012 and 61/748,427 filed Jan. 2, 2013. Such genes, proteins and pathways may be the target polynucleotide of a CRISPR complex.

TABLE A

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

TABLE A-continued

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

TABLE B

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

TABLE B-continued

Cell dysregulation and oncology diseases and disorders	B-cell non-Hodgkin lymphoma (BCL7A, BCL7); Leukemia (TAL1 TCL5, SCL, TAL2, FLT3, NBS1, NBS, ZNFN1A1, IK1, LYF1, HOXD4, HOX4B, BCR, CML, PHL, ALL, ARNT, KRAS2, RASK2, GMPS, AF10, ARHGEF12, LARG, KIAA0382, CALM, CLTH, CEBPA, CEBP, CHIC2, BTL, FLT3, KIT, PBT, LPP, NPM1, NUP214, D9S46E, CAN, CAIN, RUNX1, CBFA2, AML1, WHSC1L1, NSD3, FLT3, AF1Q, NPM1, NUMA1, ZNF145, PLZF, PML, MYL, STAT5B, AF10, CALM, CLTH, ARL11, ARLTS1, P2RX7, P2X7, BCR, CML, PHL, ALL, GRAF, NF1, VRNF, WSS, NFNS, PTPN11, PTP2C, SHP2, NS1, BCL2, CCND1, PRAD1, BCL1, TCRA, GATA1, GF1, ERYF1, NFE1, ABL1, NQO1, DIA4, NMOR1, NUP214, D9S46E, CAN, CAIN).
Inflammation and immune related diseases and disorders	AIDS (KIR3DL1, NKAT3, NKB1, AMB11, KIR3DS1, IFNG, CXCL12, SDF1); Autoimmune lymphoproliferative syndrome (TNFRSF6, APT1, FAS, CD95, ALPS1A); Combined immunodeficiency, (IL2RG, SCIDX1, SCIDX, IMD4); HIV-1 (CCL5, SCYA5, D17S136E, TCP228), HIV susceptibility or infection (IL10, CSIF, CMKBR2, CCR2, CMKBR5, CCCKR5 (CCR5)); Immunodeficiencies (CD3E, CD3G, AICDA, AID, HIGM2, TNFRSF5, CD40, UNG, DGU, HIGM4, TNFSF5, CD40LG, HIGM1, IGM, FOXP3, IPEX, AIID, XPID, PIDX, TNFRSF14B, TACI); Inflammation (IL-10, IL-1 (IL-1a, IL-1b), IL-13, IL-17 (IL-17a (CTLA8), IL-17b, IL-17c, IL-17d, IL-17f), II-23, Cx3cr1, ptpn22, TNFa, NOD2/CARD15 for IBD, IL-6, IL-12 (IL-12a, IL-12b), CTLA4, Cx3cl1); Severe combined immunodeficiencies (SCIDs)(JAK3, JAKL, DCLRE1C, ARTEMIS, SCIDA, RAG1, RAG2, ADA, PTPRC, CD45, LCA, IL7R, CD3D, T3D, IL2RG, SCIDX1, SCIDX, IMD4).
Metabolic, liver, kidney and protein diseases and disorders	Amyloid neuropathy (TTR, PALB); Amyloidosis (APOA1, APP, AAA, CVAP, AD1, GSN, FGA, LYZ, TTR, PALB); Cirrhosis (KRT18, KRT8, CIRH1A, NAIC, TEX292, KIAA1988); Cystic fibrosis (CFTR, ABCC7, CF, MRP7); Glycogen storage diseases (SLC2A2, GLUT2, G6PC, G6PT, G6PT1, GAA, LAMP2, LAMPB, AGL, GDE, GBE1, GYS2, PYGL, PFKM); Hepatic adenoma, 142330 (TCF1, HNF1A, MODY3), Hepatic failure, early onset, and neurologic disorder (SCOD1, SCO1), Hepatic lipase deficiency (LIPC), Hepatoblastoma, cancer and carcinomas (CTNNB1, PDGFRL, PDGRL, PRLTS, AXIN1, AXIN, CTNNB1, TP53, P53, LFS1, IGF2R, MPRI, MET, CASP8, MCH5; Medullary cystic kidney disease (UMOD, HNFJ, FJHN, MCKD2, ADMCKD2); Phenylketonuria (PAH, PKU1, QDPR, DHPR, PTS); Polycystic kidney and hepatic disease (FCYT, PKHD1, ARPKD, PKD1,

TABLE B-continued

	PKD2, PKD4, PKDTS, PRKCSH, G19P1, PCLD, SEC63).
Muscular/Skeletal diseases and disorders	Becker muscular dystrophy (DMD, BMD, MYF6), Duchenne Muscular Dystrophy (DMD, BMD); Emery-Dreifuss muscular dystrophy (LMNA, LMN1, EMD2, FPLD, CMD1A, HGPS, LGMD1B, LMNA, LMN1, EMD2, FPLD, CMD1A); Facioscapulohumeral muscular dystrophy (FSHMD1A, FSHD1A); Muscular dystrophy (FKRP, MDC1C, LGMD2I, LAMA2, LAMM, LARGE, KIAA0609, MDC1D, FCMD, TTID, MYOT, CAPN3, CANP3, DYSF, LGMD2B, SGCG, LGMD2C, DMDA1, SCG3, SGCA, ADL, DAG2, LGMD2D, DMDA2, SGCB, LGMD2E, SGCD, SGD, LGMD2F, CMD1L, TCAP, LGMD2G, CMD1N, TRIM32, HT2A, LGMD2H, FKRP, MDC1C, LGMD2I, TTN, CMD1G, TMD, LGMD2J, POMT1, CAV3, LGMD1C, SEPN1, SELN, RSMD1, PLEC1, PLTN, EBS1); Osteopetrosis (LRP5, BMND1, LRP7, LR3, OPPG, VBCH2, CLCN7, CLC7, OPTA2, OSTM1, GL, TCIRG1, TIRC7, OC116, OPTB1); Muscular atrophy (VAPB, VAPC, ALS8, SMN1, SMA1, SMA2, SMA3, SMA4, BSCL2, SPG17, GARS, SMAD1, CMT2D, HEXB, IGHMBP2, SMUBP2, CATF1, SMARD1).
Neurological and neuronal diseases and disorders	ALS (SOD1, ALS2, STEX, FUS, TARDBP, VEGF (VEGF-a, VEGF-b, VEGF-c); Alzheimer disease (APP, AAA, CVAP, AD1, APOE, AD2, PSEN2, AD4, STM2, APBB2, FE65L1, NOS3, PLAU, URK, ACE, DCP1, ACE1, MPO, PACIP1, PAXIP1L, PTIP, A2M, BLMH, BMH, PSEN1, AD3); Autism (Mecp2, BZRAP1, MDGA2, Sema5A, Neurexin 1, GLO1, MECP2, RTT, PPMX, MRX16, MRX79, NLGN3, NLGN4, KIAA1260, AUTSX2); Fragile X Syndrome (FMR2, FXR1, FXR2, mGLUR5); Huntington's disease and disease like disorders (HD, IT15, PRNP, PRIP, JPH3, JP3, HDL2, TBP, SCA17); Parkinson disease (NR4A2, NURR1, NOT, TINUR, SNCAIP, TBP, SCA17, SNCA, NACP, PARK1, PARK4, DJ1, PARK7, LRRK2, PARK8, PINK1, PARK6, UCHL1, PARK5, SNCA, NACP, PARK1, PARK4, PRKN, PARK2, PDJ, DBH, NDUFV2); Rett syndrome (MECP2, RTT, PPMX, MRX16, MRX79, CDKL5, STK9, MECP2, RTT, PPMX, MRX16, MRX79, x-Synuclein, DJ-1); Schizophrenia (Neuregulin1 (Nrg1), Erb4 (receptor for Neuregulin), Complexin1 (Cplx1), Tph1 Tryptophan hydroxylase, Tph2, Tryptophan hydroxylase 2, Neurexin 1, GSK3, GSK3a, GSK3b, 5-HTT (Slc6a4), COMT, DRD (Drd1a), SLC6A3, DAOA, DTNBP1, Dao (Dao1)); Secretase Related Disorders (APH-1 (alpha and beta), Presenilin (Psen1), nicastrin, (Ncstn), PEN-2, Nos1, Parp1, Nat1, Nat2); Trinucleotide Repeat Disorders (HTT (Huntington's Dx), SBMA/SMAX1/AR (Kennedy's Dx),

TABLE B-continued

	FXN/X25 (Friedrich's Ataxia), ATX3 (Machado-Joseph's Dx), ATXN1 and ATXN2 (spinocerebellar ataxias), DMPK (myotonic dystrophy), Atrophin-1 and Atn1 (DRPLA Dx), CBP (Creb-BP-global instability), VLDLR (Alzheimer's), Atxn7, Atxn10).
Occular diseases and disorders	Age-related macular degeneration (Abcr, Ccl2, Cc2, cp (ceruloplasmin), Timp3, cathepsinD, Vldlr, Ccr2); Cataract (CRYAA, CRYA1, CRYBB2, CRYB2, PITX3, BFSP2, CP49, CP47, CRYAA, CRYA1, PAX6, AN2, MGDA, CRYBA1, CRYB1, CRYGC, CRYG3, CCL, LIM2, MP19, CRYGD, CRYG4, BFSP2, CP49, CP47, HSF4, CTM, HSF4, CTM, MIP, AQP0, CRYAB, CRYA2, CTPP2, CRYBB1, CRYGD, CRYG4, CRYBB2, CRYB2, CRYGC, CRYG3, CCL, CRYAA, CRYA1, GJA8, CX50, CAE1, GJA3, CX46, CZP3, CAE3, CCM1, CAM, KRIT1); Corneal clouding and dystrophy (APOA1, TGFBI, CSD2, CDGG1, CSD, BIGH3, CDG2, TACSTD2, TROP2, M1S1, VSX1, RINX, PPCD, PPD, KTCN, COL8A2, FECD, PPCD2, PIP5K3, CFD); Cornea plana congenital (KERA, CNA2); Glaucoma (MYOC, TIGR, GLC1A, JOAG, GPOA, OPTN, GLC1E, FIP2, HYPL, NRP, CYP1B1, GLC3A, OPA1, NTG, NPG, CYP1B1, GLC3A); Leber congenital amaurosis (CRB1, RP12, CRX, CORD2, CRD, RPGRIP1, LCA6, CORD9, RPE65, RP20, AIPL1, LCA4, GUCY2D, GUC2D, LCA1, CORD6, RDH12, LCA3); Macular dystrophy (ELOVL4, ADMD, STGD2, STGD3, RDS, RP7, PRPH2, PRPH, AVMD, AOFMD, VMD2).

TABLE C

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

TABLE C-continued

CELLULAR FUNCTION	GENES
	EIF2AK2; RAC1; RAP1A; TLN1; EIF4E; ELK1; GRK6; MAPK1; RAC2; PLK1; AKT2; PIK3CA; CDK8; CREB1; PRKCI; PTK2; FOS; RPS6KA4; PIK3CB; PPP2R1A; PIK3C3; MAPK8; MAPK3; ITGA1; ETS1; KRAS; MYCN; EIF4EBP1; PPARG; PRKCD; PRKAA1; MAPK9; SRC; CDK2; PPP2CA; PIM1; PIK3C2A; ITGB7; YWHAZ; PPP1CC; KSR1; PXN; RAF1; FYN; DYRK1A; ITGB1; MAP2K2; PAK4; PIK3R1; STAT3; PPP2R5C; MAP2K1; PAK3; ITGB3; ESR1; ITGA2; MYC; TTK; CSNK1A1; CRKL; BRAF; ATF4; PRKCA; SRF; STAT1; SGK
Glucocorticoid Receptor Signaling	RAC1; TAF4B; EP300; SMAD2; TRAF6; PCAF; ELK1; MAPK1; SMAD3; AKT2; IKBKB; NCOR2; UBE2I; PIK3CA; CREB1; FOS; HSPA5; NFKB2; BCL2; MAP3K14; STAT5B; PIK3CB; PIK3C3; MAPK8; BCL2L1; MAPK3; TSC22D3; MAPK10; NRIP1; KRAS; MAPK13; RELA; STAT5A; MAPK9; NOS2A; PBX1; NR3C1; PIK3C2A; CDKN1C; TRAF2; SERPINE1; NCOA3; MAPK14; TNF; RAF1; IKBKG; MAP3K7; CREBBP; CDKN1A; MAP2K2; JAK1; IL8; NCOA2; AKT1; JAK2; PIK3R1; CHUK; STAT3; MAP2K1; NFKB1; TGFBR1; ESR1; SMAD4; CEBPB; JUN; AR; AKT3; CCL2; MMP1; STAT1; IL6; HSP90AA1
Axonal Guidance Signaling	PRKCE; ITGAM; ROCK1; ITGA5; CXCR4; ADAM12; IGF1; RAC1; RAP1A; EIF4E; PRKCZ; NRP1; NTRK2; ARHGEF7; SMO; ROCK2; MAPK1; PGF; RAC2; PTPN11; GNAS; AKT2; PIK3CA; ERBB2; PRKCI; PTK2; CFL1; GNAQ; PIK3CB; CXCL12; PIK3C3; WNT11; PRKD1; GNB2L1; ABL1; MAPK3; ITGA1; KRAS; RHOA; PRKCD; PIK3C2A; ITGB7; GLI2; PXN; VASP; RAF1; FYN; ITGB1; MAP2K2; PAK4; ADAM17; AKT1; PIK3R1; GLI1; WNT5A; ADAM10; MAP2K1; PAK3; ITGB3; CDC42; VEGFA; ITGA2; EPHA8; CRKL; RND1; GSK3B; AKT3; PRKCA
Ephrin Receptor Signaling	PRKCE; ITGAM; ROCK1; ITGA5; CXCR4; IRAK1; PRKAA2; EIF2AK2; RAC1; RAP1A; GRK6; ROCK2; MAPK1; PGF; RAC2; PTPN11; GNAS; PLK1; AKT2; DOK1; CDK8; CREB1; PTK2; CFL1; GNAQ; MAP3K14; CXCL12; MAPK8; GNB2L1; ABL1; MAPK3; ITGA1;

TABLE C-continued

CELLULAR FUNCTION	GENES
	KRAS; RHOA; PRKCD; PRKAA1; MAPK9; SRC; CDK2; PIM1; ITGB7; PXN; RAF1; FYN; DYRK1A; ITGB1; MAP2K2; PAK4, AKT1; JAK2; STAT3; ADAM10; MAP2K1; PAK3; ITGB3; CDC42; VEGFA; ITGA2; EPHA8; TTK; CSNK1A1; CRKL; BRAF; PTPN13; ATF4; AKT3; SGK
Actin Cytoskeleton Signaling	ACTN4; PRKCE; ITGAM; ROCK1; ITGA5; IRAK1; PRKAA2; EIF2AK2; RAC1; INS; ARHGEF7; GRK6; ROCK2; MAPK1; RAC2; PLK1; AKT2; PIK3CA; CDK8; PTK2; CFL1; PIK3CB; MYH9; DIAPH1; PIK3C3; MAPK8; F2R; MAPK3; SLC9A1; ITGA1; KRAS; RHOA; PRKCD; PRKAA1; MAPK9; CDK2; PIM1; PIK3C2A; ITGB7; PPP1CC; PXN; VIL2; RAF1; GSN; DYRK1A; ITGB1; MAP2K2; PAK4; PIP5K1A; PIK3R1; MAP2K1; PAK3; ITGB3; CDC42; APC; ITGA2; TTK; CSNK1A1; CRKL; BRAF; VAV3; SGK
Huntington's Disease Signaling	PRKCE; IGF1; EP300; RCOR1; PRKCZ; HDAC4; TGM2; MAPK1; CAPNS1; AKT2; EGFR; NCOR2; SP1; CAPN2; PIK3CA; HDAC5; CREB1; PRKC1; HSPA5; REST; GNAQ; PIK3CB; PIK3C3; MAPK8; IGF1R; PRKD1; GNB2L1; BCL2L1; CAPN1; MAPK3; CASP8; HDAC2; HDAC7A; PRKCD; HDAC11; MAPK9; HDAC9; PIK3C2A; HDAC3; TP53; CASP9; CREBBP; AKT1; PIK3R1; PDPK1; CASP1; APAF1; FRAP1; CASP2; JUN; BAX; ATF4; AKT3; PRKCA; CLTC; SGK; HDAC6; CASP3
Apoptosis Signaling	PRKCE; ROCK1; BID; IRAK1; PRKAA2; EIF2AK2; BAK1; BIRC4; GRK6; MAPK1; CAPNS1; PLK1; AKT2; IKBKB; CAPN2; CDK8; FAS; NFKB2; BCL2; MAP3K14; MAPK8; BCL2L1; CAPN1; MAPK3; CASP8; KRAS; RELA; PRKCD; PRKAA1; MAPK9; CDK2; PIM1; TP53; TNF; RAF1; IKBKG; RELB; CASP9; DYRK1A; MAP2K2; CHUK; APAF1; MAP2K1; NFKB1; PAK3; LMNA; CASP2; BIRC2; TTK; CSNK1A1; BRAF; BAX; PRKCA; SGK; CASP3; BIRC3; PARP1
B Cell Receptor Signaling	RAC1; PTEN; LYN; ELK1; MAPK1; RAC2; PTPN11; AKT2; IKBKB; PIK3CA; CREB1; SYK; NFKB2; CAMK2A; MAP3K14; PIK3CB; PIK3C3; MAPK8; BCL2L1; ABL1; MAPK3; ETS1; KRAS; MAPK13; RELA; PTPN6; MAPK9; EGR1; PIK3C2A; BTK; MAPK14;

TABLE C-continued

CELLULAR FUNCTION	GENES
	RAF1; IKBKG; RELB; MAP3K7; MAP2K2; AKT1; PIK3R1; CHUK; MAP2K1; NFKB1; CDC42; GSK3A; FRAP1; BCL6; BCL10; JUN; GSK3B; ATF4; AKT3; VAV3; RPS6KB1
Leukocyte Extravasation Signaling	ACTN4; CD44; PRKCE; ITGAM; ROCK1; CXCR4; CYBA; RAC1; RAP1A; PRKCZ; ROCK2; RAC2; PTPN11; MMP14; PIK3CA; PRKCI; PTK2; PIK3CB; CXCL12; PIK3C3; MAPK8; PRKD1; ABL1; MAPK10; CYBB; MAPK13; RHOA; PRKCD; MAPK9; SRC; PIK3C2A; BTK; MAPK14; NOX1; PXN; VIL2; VASP; ITGB1; MAP2K2; CTNND1; PIK3R1; CTNNB1; CLDN1; CDC42; F11R; ITK; CRKL; VAV3; CTTN; PRKCA; MMP1; MMP9
Integrin Signaling	ACTN4; ITGAM; ROCK1; ITGA5; RAC1; PTEN; RAP1A; TLN1; ARHGEF7; MAPK1; RAC2; CAPNS1; AKT2; CAPN2; PIK3CA; PTK2; PIK3CB; PIK3C3; MAPK8; CAV1; CAPN1; ABL1; MAPK3; ITGA1; KRAS; RHOA; SRC; PIK3C2A; ITGB7; PPP1CC; ILK; PXN; VASP; RAF1; FYN; ITGB1; MAP2K2; PAK4; AKT1; PIK3R1; TNK2; MAP2K1; PAK3; ITGB3; CDC42; RND3; ITGA2; CRKL; BRAF; GSK3B; AKT3
Acute Phase Response Signaling	IRAK1; SOD2; MYD88; TRAF6; ELK1; MAPK1; PTPN11; AKT2; IKBKB; PIK3CA; FOS; NFKB2; MAP3K14; PIK3CB; MAPK8; RIPK1; MAPK3; IL6ST; KRAS; MAPK13; IL6R; RELA; SOCS1; MAPK9; FTL; NR3C1; TRAF2; SERPINE1; MAPK14; TNF; RAF1; PDK1; IKBKG; RELB; MAP3K7; MAP2K2; AKT1; JAK2; PIK3R1; CHUK; STAT3; MAP2K1; NFKB1; FRAP1; CEBPB; JUN; AKT3; IL1R1; IL6
PTEN Signaling	ITGAM; ITGA5; RAC1; PTEN; PRKCZ; BCL2L11; MAPK1; RAC2; AKT2; EGFR; IKBKB; CBL; PIK3CA; CDKN1B; PTK2; NFKB2; BCL2; PIK3CB; BCL2L1; MAPK3; ITGA1; KRAS; ITGB7; ILK; PDGFRB; INSR; RAF1; IKBKG; CASP9; CDKN1A; ITGB1; MAP2K2; AKT1; PIK3R1; CHUK; PDGFRA; PDPK1; MAP2K1; NFKB1; ITGB3; CDC42; CCND1; GSK3A; ITGA2; GSK3B; AKT3; FOXO1; CASP3; RPS6KB1
p53 Signaling	PTEN; EP300; BBC3; PCAF; FASN; BRCA1; GADD45A; BIRC5; AKT2; PIK3CA; CHEK1; TP53INP1; BCL2; PIK3CB; PIK3C3; MAPK8; THBS1; ATR; BCL2L1; E2F1;

TABLE C-continued

CELLULAR FUNCTION	GENES
	PMAIP1; CHEK2; TNFRSF10B; TP73; RB1; HDAC9; CDK2; PIK3C2A; MAPK14; TP53; LRDD; CDKN1A; HIPK2; AKT1; PIK3R1; RRM2B; APAF1; CTNNB1; SIRT1; CCND1; PRKDC; ATM; SFN; CDKN2A; JUN; SNAI2; GSK3B; BAX; AKT3
Aryl Hydrocarbon Receptor Signaling	HSPB1; EP300; FASN; TGM2; RXRA; MAPK1; NQO1; NCOR2; SP1; ARNT; CDKN1B; FOS; CHEK1; SMARCA4; NFKB2; MAPK8; ALDH1A1; ATR; E2F1; MAPK3; NRIP1; CHEK2; RELA; TP73; GSTP1; RB1; SRC; CDK2; AHR; NFE2L2; NCOA3; TP53; TNF; CDKN1A; NCOA2; APAF1; NFKB1; CCND1; ATM; ESR1; CDKN2A; MYC; JUN; ESR2; BAX; IL6; CYP1B1; HSP90AA1
Xenobiotic Metabolism Signaling	PRKCE; EP300; PRKCZ; RXRA; MAPK1; NQO1; NCOR2; PIK3CA; ARNT; PRKCI; NFKB2; CAMK2A; PIK3CB; PPP2R1A; PIK3C3; MAPK8; PRKD1; ALDH1A1; MAPK3; NRIP1; KRAS; MAPK13; PRKCD; GSTP1; MAPK9; NOS2A; ABCB1; AHR; PPP2CA; FTL; NFE2L2; PIK3C2A; PPARGC1A; MAPK14; TNF; RAF1; CREBBP; MAP2K2; PIK3R1; PPP2R5C; MAP2K1; NFKB1; KEAP1; PRKCA; EIF2AK3; IL6; CYP1B1; HSP90AA1
SAPK/JNK Signaling	PRKCE; IRAK1; PRKAA2; EIF2AK2; RAC1; ELK1; GRK6; MAPK1; GADD45A; RAC2; PLK1; AKT2; PIK3CA; FADD; CDK8; PIK3CB; PIK3C3; MAPK8; RIPK1; GNB2L1; IRS1; MAPK3; MAPK10; DAXX; KRAS; PRKCD; PRKAA1; MAPK9; CDK2; PIM1; PIK3C2A; TRAF2; TP53; LCK; MAP3K7; DYRK1A; MAP2K2; PIK3R1; MAP2K1; PAK3; CDC42; JUN; TTK; CSNK1A1; CRKL; BRAF; SGK
PPAr/RXR Signaling	PRKAA2; EP300; INS; SMAD2; TRAF6; PPARA; FASN; RXRA; MAPK1; SMAD3; GNAS; IKBKB; NCOR2; ABCA1; GNAQ; NFKB2; MAP3K14; STAT5B; MAPK8; IRS1; MAPK3; KRAS; RELA; PRKAA1; PPARGC1A; NCOA3; MAPK14; INSR; RAF1; IKBKG; RELB; MAP3K7; CREBBP; MAP2K2; JAK2; CHUK; MAP2K1; NFKB1; TGFBR1; SMAD4; JUN; IL1R1; PRKCA; IL6; HSP90AA1; ADIPOQ
NF-KB Signaling	IRAK1; EIF2AK2; EP300; INS; MYD88; PRKCZ: TRAF6; TBK1; AKT2; EGFR; IKBKB;

TABLE C-continued

CELLULAR FUNCTION	GENES
	PIK3CA; BTRC; NFKB2; MAP3K14; PIK3CB; PIK3C3; MAPK8; RIPK1; HDAC2; KRAS; RELA; PIK3C2A; TRAF2; TLR4: PDGFRB; TNF; INSR; LCK; IKBKG; RELB; MAP3K7; CREBBP; AKT1; PIK3R1; CHUK; PDGFRA; NFKB1; TLR2; BCL10; GSK3B; AKT3; TNFAIP3; IL1R1
Neuregulin Signaling	ERBB4; PRKCE; ITGAM; ITGA5: PTEN; PRKCZ; ELK1; MAPK1; PTPN11; AKT2; EGFR; ERBB2; PRKCI; CDKN1B; STAT5B; PRKD1; MAPK3; ITGA1; KRAS; PRKCD; STAT5A; SRC; ITGB7; RAF1; ITGB1; MAP2K2; ADAM17; AKT1; PIK3R1; PDPK1; MAP2K1; ITGB3; EREG; FRAP1; PSEN1; ITGA2; MYC; NRG1; CRKL; AKT3; PRKCA; HSP90AA1; RPS6KB1
Wnt & Beta catenin Signaling	CD44; EP300; LRP6; DVL3; CSNK1E; GJA1; SMO; AKT2; PIN1; CDH1; BTRC; GNAQ; MARK2; PPP2R1A; WNT11; SRC; DKK1; PPP2CA; SOX6; SFRP2: ILK; LEF1; SOX9; TP53; MAP3K7; CREBBP; TCF7L2; AKT1; PPP2R5C; WNT5A; LRP5; CTNNB1; TGFBR1; CCND1; GSK3A; DVL1; APC; CDKN2A; MYC; CSNK1A1; GSK3B; AKT3; SOX2
Insulin Receptor Signaling	PTEN; INS; EIF4E; PTPN1; PRKCZ; MAPK1; TSC1; PTPN11; AKT2; CBL; PIK3CA; PRKCI; PIK3CB; PIK3C3; MAPK8; IRS1; MAPK3; TSC2; KRAS; EIF4EBP1; SLC2A4; PIK3C2A; PPP1CC; INSR; RAF1; FYN; MAP2K2; JAK1; AKT1; JAK2; PIK3R1; PDPK1; MAP2K1; GSK3A; FRAP1; CRKL; GSK3B; AKT3; FOXO1; SGK; RPS6KB1
IL-6 Signaling	HSPB1; TRAF6; MAPKAPK2; ELK1; MAPK1; PTPN11; IKBKB; FOS; NFKB2: MAP3K14; MAPK8; MAPK3; MAPK10; IL6ST; KRAS; MAPK13; IL6R; RELA; SOCS1; MAPK9; ABCB1; TRAF2; MAPK14; TNF; RAF1; IKBKG; RELB; MAP3K7; MAP2K2; IL8; JAK2; CHUK; STAT3; MAP2K1; NFKB1; CEBPB; JUN; IL1R1; SRF; IL6
Hepatic Cholestasis	PRKCE; IRAK1; INS; MYD88; PRKCZ; TRAF6; PPARA; RXRA; IKBKB; PRKCI; NFKB2; MAP3K14; MAPK8; PRKD1; MAPK10; RELA; PRKCD; MAPK9; ABCB1; TRAF2; TLR4; TNF; INSR; IKBKG; RELB; MAP3K7; IL8; CHUK; NR1H2; TJP2; NFKB1; ESR1; SREBF1; FGFR4; JUN; IL1R1; PRKCA; IL6
IGF-1 Signaling	IGF1; PRKCZ; ELK1; MAPK1; PTPN11; NEDD4; AKT2;

TABLE C-continued

CELLULAR FUNCTION	GENES
	PIK3CA; PRKCI; PTK2; FOS; PIK3CB; PIK3C3; MAPK8; IGF1R; IRS1; MAPK3; IGFBP7; KRAS; PIK3C2A; YWHAZ; PXN; RAF1; CASP9; MAP2K2; AKT1; PIK3R1; PDPK1; MAP2K1; IGFBP2; SFN; JUN; CYR61; AKT3; FOXO1; SRF; CTGF; RPS6KB1
NRF2-mediated Oxidative Stress Response	PRKCE; EP300; SOD2; PRKCZ; MAPK1; SQSTM1; NQO1; PIK3CA; PRKCI; FOS; PIK3CB; PIK3C3; MAPK8; PRKD1; MAPK3; KRAS; PRKCD; GSTP1; MAPK9; FTL; NFE2L2; PIK3C2A; MAPK14; RAF1; MAP3K7; CREBBP; MAP2K2; AKT1; PIK3R1; MAP2K1; PPIB; JUN; KEAP1; GSK3B; ATF4; PRKCA; EIF2AK3; HSP90AA1
Hepatic Fibrosis/ Hepatic Stellate Cell Activation	EDN1; IGF1; KDR; FLT1; SMAD2; FGFR1; MET; PGF; SMAD3; EGFR; FAS; CSF1; NFKB2; BCL2; MYH9; IGF1R; IL6R; RELA; TLR4; PDGFRB; TNF; RELB; IL8; PDGFRA; NFKB1; TGFBR1; SMAD4; VEGFA; BAX; IL1R1; CCL2; HGF; MMP1; STAT1; IL6; CTGF; MMP9
PPAR Signaling	EP300; INS; TRAF6; PPARA; RXRA; MAPK1; IKBKB; NCOR2; FOS; NFKB2; MAP3K14; STAT5B; MAPK3; NRIP1; KRAS; PPARG; RELA; STAT5A; TRAF2; PPARGC1A; PDGFRB; TNF; INSR; RAF1; IKBKG; RELB; MAP3K7; CREBBP; MAP2K2; CHUK; PDGFRA; MAP2K1; NFKB1; JUN; IL1R1; HSP90AA1
Fc Epsilon RI Signaling	PRKCE; RAC1; PRKCZ; LYN; MAPK1; RAC2; PTPN11; AKT2; PIK3CA; SYK; PRKCI; PIK3CB; PIK3C3; MAPK8; PRKD1; MAPK3; MAPK10; KRAS; MAPK13; PRKCD; MAPK9; PIK3C2A; BTK; MAPK14; TNF; RAF1; FYN; MAP2K2; AKT1; PIK3R1; PDPK1; MAP2K1; AKT3; VAV3; PRKCA
G-Protein Coupled Receptor Signaling	PRKCE; RAP1A; RGS16; MAPK1; GNAS; AKT2; IKBKB; PIK3CA; CREB1; GNAQ; NFKB2; CAMK2A; PIK3CB; PIK3C3; MAPK3; KRAS; RELA; SRC; PIK3C2A; RAF1; IKBKG; RELB; FYN; MAP2K2; AKT1; PIK3R1; CHUK; PDPK1; STAT3; MAP2K1; NFKB1; BRAF; ATF4; AKT3; PRKCA
Inositol Phosphate Metabolism	PRKCE; IRAK1; PRKAA2; EIF2AK2; PTEN; GRK6; MAPK1; PLK1; AKT2; PIK3CA; CDK8; PIK3CB; PIK3C3; MAPK8; MAPK3; PRKCD; PRKAA1; MAPK9; CDK2; PIM1; PIK3C2A; DYRK1A; MAP2K2; PIP5K1A; PIK3R1; MAP2K1; PAK3; ATM; TTK;

TABLE C-continued

CELLULAR FUNCTION	GENES
	CSNK1A1; BRAF; SGK
PDGF Signaling	EIF2AK2; ELK1; ABL2; MAPK1; PIK3CA; FOS; PIK3CB; PIK3C3; MAPK8; CAV1; ABL1; MAPK3; KRAS; SRC; PIK3C2A; PDGFRB; RAF1; MAP2K2; JAK1; JAK2; PIK3R1; PDGFRA; STAT3; SPHK1; MAP2K1; MYC; JUN; CRKL; PRKCA; SRF; STAT1; SPHK2
VEGF Signaling	ACTN4; ROCK1; KDR; FLT1; ROCK2; MAPK1; PGF; AKT2; PIK3CA; ARNT; PTK2; BCL2; PIK3CB; PIK3C3; BCL2L1; MAPK3; KRAS; HIF1A; NOS3; PIK3C2A; PXN; RAF1; MAP2K2; ELAVL1; AKT1; PIK3R1; MAP2K1; SFN; VEGFA; AKT3; FOXO1; PRKCA
Natural Killer Cell Signaling	PRKCE; RAC1; PRKCZ; MAPK1; RAC2; PTPN11; KIR2DL3; AKT2; PIK3CA; SYK; PRKCI; PIK3CB; PIK3C3; PRKD1; MAPK3; KRAS; PRKCD; PTPN6; PIK3C2A; LCK; RAF1; FYN; MAP2K2; PAK4; AKT1; PIK3R1; MAP2K1; PAK3; AKT3; VAV3; PRKCA
Cell Cycle: G1/S Checkpoint Regulation	HDAC4; SMAD3; SUV39H1; HDAC5; CDKN1B; BTRC; ATR; ABL1; E2F1; HDAC2; HDAC7A; RB1; HDAC11; HDAC9; CDK2; E2F2; HDAC3; TP53; CDKN1A; CCND1; E2F4; ATM; RBL2; SMAD4; CDKN2A; MYC; NRG1; GSK3B; RBL1; HDAC6
T Cell Receptor Signaling	RAC1; ELK1; MAPK1; IKBKB; CBL; PIK3CA; FOS; NFKB2; PIK3CB; PIK3C3; MAPK8; MAPK3; KRAS; RELA, PIK3C2A; BTK; LCK; RAF1; IKBKG; RELB, FYN; MAP2K2; PIK3R1; CHUK; MAP2K1; NFKB1; ITK; BCL10; JUN; VAV3
Death Receptor Signaling	CRADD; HSPB1; BID; BIRC4; TBK1; IKBKB; FADD; FAS; NFKB2; BCL2; MAP3K14; MAPK8; RIPK1; CASP8; DAXX; TNFRSF10B; RELA; TRAF2; TNF; IKBKG; RELB; CASP9; CHUK; APAF1; NFKB1; CASP2; BIRC2; CASP3; BIRC3
FGF Signaling	RAC1; FGFR1; MET; MAPKAPK2; MAPK1; PTPN11; AKT2; PIK3CA; CREB1; PIK3CB; PIK3C3; MAPK8; MAPK3; MAPK13; PTPN6; PIK3C2A; MAPK14; RAF1; AKT1; PIK3R1; STAT3; MAP2K1; FGFR4; CRKL; ATF4; AKT3; PRKCA; HGF
GM-CSF Signaling	LYN; ELK1; MAPK1; PTPN11; AKT2; PIK3CA; CAMK2A; STAT5B; PIK3CB; PIK3C3; GNB2L1; BCL2L1; MAPK3; ETS1; KRAS; RUNX1; PIM1; PIK3C2A; RAF1; MAP2K2; AKT1; JAK2; PIK3R1; STAT3; MAP2K1; CCND1; AKT3; STAT1
Amyotrophic Lateral Sclerosis Signaling	BID; IGF1; RAC1; BIRC4; PGF; CAPNS1; CAPN2; PIK3CA; BCL2; PIK3CB; PIK3C3; BCL2L1; CAPN1; PIK3C2A; TP53; CASP9; PIK3R1; RAB5A; CASP1; APAF1; VEGFA; BIRC2; BAX; AKT3; CASP3; BIRC3
JAK/Stat Signaling	PTPN1; MAPK1; PTPN11; AKT2; PIK3CA; STAT5B; PIK3CB; PIK3C3; MAPK3; KRAS; SOCS1; STAT5A; PTPN6; PIK3C2A; RAF1; CDKN1A; MAP2K2; JAK1; AKT1; JAK2; PIK3R1; STAT3; MAP2K1; FRAP1; AKT3; STAT1
Nicotinate and Nicotinamide Metabolism	PRKCE; IRAK1; PRKAA2; EIF2AK2; GRK6; MAPK1; PLK1; AKT2; CDK8; MAPK8; MAPK3; PRKCD; PRKAA1; PBEF1; MAPK9; CDK2; PIM1; DYRK1A; MAP2K2; MAP2K1; PAK3; NT5E; TTK; CSNK1A1; BRAF; SGK
Chemokine Signaling	CXCR4; ROCK2; MAPK1; PTK2; FOS; CFL1; GNAQ; CAMK2A; CXCL12; MAPK8; MAPK3; KRAS; MAPK13; RHOA; CCR3; SRC; PPP1CC; MAPK14; NOX1; RAF1; MAP2K2; MAP2K1; JUN; CCL2; PRKCA
IL-2 Signaling	ELK1; MAPK1; PTPN11; AKT2; PIK3CA; SYK; FOS; STAT5B; PIK3CB; PIK3C3; MAPK8; MAPK3; KRAS; SOCS1; STAT5A; PIK3C2A; LCK; RAF1; MAP2K2; JAK1; AKT1; PIK3R1; MAP2K1; JUN; AKT3
Synaptic Long Term Depression	PRKCE; IGF1; PRKCZ; PRDX6; LYN; MAPK1; GNAS; PRKCI; GNAQ; PPP2R1A; IGF1R; PRKD1; MAPK3; KRAS; GRN; PRKCD; NOS3; NOS2A; PPP2CA; YWHAZ; RAF1; MAP2K2; PPP2R5C; MAP2K1; PRKCA
Estrogen Receptor Signaling	TAF4B; EP300; CARM1; PCAF; MAPK1; NCOR2; SMARCA4; MAPK3; NRIP1; KRAS; SRC; NR3C1; HDAC3; PPARGC1A; RBM9; NCOA3; RAF1; CREBBP; MAP2K2; NCOA2; MAP2K1; PRKDC; ESR1; ESR2
Protein Ubiquitination Pathway	TRAF6; SMURF1; BIRC4; BRCA1; UCHL1; NEDD4; CBL; UBE2I; BTRC; HSPA5; USP7; USP10; FBXW7; USP9X; STUB1; USP22; B2M; BIRC2; PARK2; USP8; USP1; VHL; HSP90AA1; BIRC3
IL-10 Signaling	TRAF6; CCR1; ELK1; IKBKB; SP1; FOS; NFKB2; MAP3K14; MAPK8; MAPK13; RELA; MAPK14; TNF; IKBKG; RELB; MAP3K7; JAK1;

TABLE C-continued

CELLULAR FUNCTION	GENES
	CHUK; STAT3; NFKB1; JUN; IL1R1; IL6
VDR/RXR Activation	PRKCE; EP300; PRKCZ; RXRA; GADD45A; HES1; NCOR2; SP1; PRKC1; CDKN1B; PRKD1; PRKCD; RUNX2; KLF4; YY1; NCOA3; CDKN1A; NCOA2; SPP1; LRP5; CEBPB; FOXO1; PRKCA
TGF-beta Signaling	EP300; SMAD2; SMURF1; MAPK1; SMAD3; SMAD1; FOS; MAPK8; MAPK3; KRAS; MAPK9; RUNX2; SERPINE1; RAF1; MAP3K7; CREBBP; MAP2K2; MAP2K1; TGFBR1; SMAD4; JUN; SMAD5
Toll-like Receptor Signaling	IRAK1; EIF2AK2; MYD88; TRAF6; PPARA; ELK1; IKBKB; FOS; NFKB2; MAP3K14; MAPK8; MAPK13; RELA; TLR4; MAPK14; IKBKG; RELB; MAP3K7; CHUK; NFKB1; TLR2; JUN
p38 MAPK Signaling	HSPB1; IRAK1; TRAF6; MAPKAPK2; ELK1; FADD; FAS; CREB1; DDIT3; RPS6KA4; DAXX; MAPK13; TRAF2; MAPK14; TNF; MAP3K7; TGFBR1; MYC; ATF4; IL1R1; SRF; STAT1
Neurotrophin/TRK Signaling	NTRK2; MAPK1; PTPN11; PIK3CA; CREB1; FOS; PIK3CB; PIK3C3; MAPK8; MAPK3; KRAS; PIK3C2A; RAF1; MAP2K2; AKT1; PIK3R1; PDPK1; MAP2K1; CDC42; JUN; ATF4
FXR/RXR Activation	INS; PPARA; FASN; RXRA; AKT2; SDC1; MAPK8; APOB; MAPK10; PPARG; MTTP; MAPK9; PPARGC1A; TNF; CREBBP; AKT1; SREBF1; FGFR4; AKT3; FOXO1
Synaptic Long Term Potentiation	PRKCE; RAP1A; EP300; PRKCZ; MAPK1; CREB1; PRKCI; GNAQ; CAMK2A; PRKD1; MAPK3; KRAS; PRKCD; PPP1CC; RAF1; CREBBP; MAP2K2; MAP2K1; ATF4; PRKCA
Calcium Signaling	RAP1A; EP300; HDAC4; MAPK1; HDAC5; CREB1; CAMK2A; MYH9; MAPK3; HDAC2; HDAC7A; HDAC11; HDAC9; HDAC3; CREBBP; CALR; CAMKK2; ATF4; HDAC6
EGF Signaling	ELK1; MAPK1; EGFR; PIK3CA; FOS; PIK3CB; PIK3C3; MAPK8; MAPK3; PIK3C2A; RAF1; JAK1; PIK3R1; STAT3; MAP2K1; JUN; PRKCA; SRF; STAT1
Hypoxia Signaling in the Cardiovascular System	EDN1; PTEN; EP300; NQO1; UBE2I; CREB1; ARNT; HIF1A; SLC2A4; NOS3; TP53; LDHA; AKT1; ATM; VEGFA; JUN; ATF4; VHL; HSP90AA1
LPS/IL-1 Mediated Inhibition of RXR Function	IRAK1; MYD88; TRAF6; PPARA; RXRA; ABCA1, MAPK8; ALDH1A1; GSTP1; MAPK9; ABCB1; TRAF2;

TABLE C-continued

CELLULAR FUNCTION	GENES
	TLR4; TNF; MAP3K7; NR1H2; SREBF1; JUN; IL1R1
LXR/RXR Activation	FASN; RXRA; NCOR2; ABCA1; NFKB2; IRF3; RELA; NOS2A; TLR4; TNF; RELB; LDLR; NR1H2; NFKB1; SREBF1; IL1R1; CCL2; IL6; MMP9
Amyloid Processing	PRKCE; CSNK1E; MAPK1; CAPNS1; AKT2; CAPN2; CAPN1; MAPK3; MAPK13; MAPT; MAPK14; AKT1; PSEN1; CSNK1A1; GSK3B; AKT3; APP
IL-4 Signaling	AKT2; PIK3CA; PIK3CB; PIK3C3; IRS1; KRAS; SOCS1; PTPN6; NR3C1; PIK3C2A; JAK1; AKT1; JAK2; PIK3R1; FRAP1; AKT3; RPS6KB1
Cell Cycle: G2/M DNA Damage Checkpoint Regulation	EP300; PCAF; BRCA1; GADD45A; PLK1; BTRC; CHEK1; ATR; CHEK2; YWHAZ; TP53; CDKN1A; PRKDC; ATM; SFN; CDKN2A
Nitric Oxide Signaling in the Cardiovascular System	KDR; FLT1; PGF; AKT2; PIK3CA; PIK3CB; PIK3C3; CAV1; PRKCD; NOS3; PIK3C2A; AKT1; PIK3R1; VEGFA; AKT3; HSP90AA1
Purine Metabolism	NME2; SMARCA4; MYH9; RRM2; ADAR; EIF2AK4; PKM2; ENTPD1; RAD51; RRM2B; TJP2; RAD51C; NT5E; POLD1; NME1
cAMP-mediated Signaling	RAP1A; MAPK1; GNAS; CREB1; CAMK2A; MAPK3; SRC; RAF1; MAP2K2; STAT3; MAP2K1; BRAF; ATF4
Mitochondrial Dysfunction	SOD2; MAPK8; CASP8; MAPK10; MAPK9; CASP9; PARK7; PSEN1; PARK2; APP; CASP3
Notch Signaling	HES1; JAG1; NUMB; NOTCH4; ADAM17; NOTCH2; PSEN1; NOTCH3; NOTCH1; DLL4
Endoplasmic Reticulum Stress Pathway	HSPA5; MAPK8; XBP1; TRAF2; ATF6; CASP9; ATF4; EIF2AK3; CASP3
Pyrimidine Metabolism	NME2; AICDA; RRM2; EIF2AK4; ENTPD1; RRM2B; NT5E; POLD1; NME1
Parkinson's Signaling	UCHL1; MAPK8; MAPK13; MAPK14; CASP9; PARK7; PARK2; CASP3
Cardiac & Beta Adrenergic Signaling	GNAS; GNAQ; PPP2R1A; GNB2L1; PPP2CA; PPP1CC; PPP2R5C
Glycolysis/ Gluconeogenesis	HK2; GCK; GPI; ALDH1A1; PKM2; LDHA; HK1
Interferon Signaling	IRF1; SOCS1; JAK1; JAK2; IFITM1; STAT1; IFIT3
Sonic Hedgehog Signaling	ARRB2; SMO; GLI2; DYRK1A; GLI1; GSK3B; DYRK1B
Glycerophospholipid Metabolism	PLD1; GRN; GPAM; YWHAZ; SPHK1; SPHK2
Phospholipid Degradation	PRDX6; PLD1; GRN; YWHAZ; SPHK1; SPHK2
Tryptophan Metabolism	SIAH2; PRMT5; NEDD4; ALDH1A1; CYP1B1; SIAH1
Lysine Degradation	SUV39H1; EHMT2; NSD1; SETD7; PPP2R5C
Nucleotide Excision Repair Pathway	ERCC5; ERCC4; XPA; XPC; ERCC1
Starch and Sucrose Metabolism	UCHL1; HK2; GCK; GPI; HK1

TABLE C-continued

CELLULAR FUNCTION	GENES
Aminosugars Metabolism	NQO1; HK2; GCK; HK1
Arachidonic Acid Metabolism	PRDX6; GRN; YWHAZ; CYP1B1
Circadian Rhythm Signaling	CSNK1E; CREB1; ATF4; NR1D1
Coagulation System	BDKRB1; F2R; SERPINE1; F3
Dopamine Receptor Signaling	PPP2R1A; PPP2CA; PPP1CC; PPP2R5C
Glutathione Metabolism	IDH2; GSTP1; ANPEP; IDH1
Glycerolipid Metabolism	ALDH1A1; GPAM; SPHK1; SPHK2
Linoleic Acid Metabolism	PRDX6; GRN; YWHAZ; CYP1B1
Methionine Metabolism	DNMT1; DNMT3B; AHCY; DNMT3A
Pyruvate Metabolism	GLO1; ALDH1A1; PKM2; LDHA
Arginine and Proline Metabolism	ALDH1A1; NOS3; NOS2A
Eicosanoid Signaling	PRDX6; GRN; YWHAZ
Fructose and Mannose Metabolism	HK2; GCK; HK1
Galactose Metabolism	HK2; GCK; HK1
Stilbene, Coumarine and Lignin Biosynthesis	PRDX6; PRDX1; TYR
Antigen Presentation Pathway	CALR; B2M
Biosynthesis of Steroids	NQO1; DHCR7
Butanoate Metabolism	ALDH1A1; NLGN1
Citrate Cycle	IDH2; IDH1
Fatty Acid Metabolism	ALDH1A1; CYP1B1
Glycerophospholipid Metabolism	PRDX6; CHKA
Histidine Metabolism	PRMT5; ALDH1A1
Inositol Metabolism	ERO1L; APEX1
Metabolism of Xenobiotics by Cytochrome p450	GSTP1; CYP1B1
Methane Metabolism	PRDX6; PRDX1
Phenylalanine Metabolism	PRDX6; PRDX1
Propanoate Metabolism	ALDH1A1; LDHA
Selenoamino Acid Metabolism	PRMT5; AHCY
Sphingolipid Metabolism	SPHK1; SPHK2
Aminophosphonate Metabolism	PRMT5
Androgen and Estrogen Metabolism	PRMT5
Ascorbate and Aldarate Metabolism	ALDH1A1
Bile Acid Biosynthesis	ALDH1A1
Cysteine Metabolism	LDHA
Fatty Acid Biosynthesis	FASN
Glutamate Receptor Signaling	GNB2L1
NRF2-mediated Oxidative Stress Response	PRDX1
Pentose Phosphate Pathway	GPI
Pentose and Glucuronate Interconversions	UCHL1
Retinol Metabolism	ALDH1A1
Riboflavin Metabolism	TYR
Tyrosine Metabolism	PRMT5, TYR
Ubiquinone Biosynthesis	PRMT5
Valine, Leucine and Isoleucine Degradation	ALDH1A1
Glycine, Serine and Threonine Metabolism	CHKA
Lysine Degradation	ALDH1A1
Pain/Taste	TRPM5; TRPA1
Pain	TRPM7; TRPC5; TRPC6; TRPC1; Cnr1; cnr2; Grk2; Trpa1; Pomc; Cgrp; Crf; Pka; Era; Nr2b; TRPM5; Prkaca; Prkacb; Prkar1a; Prkar2a
Mitochondrial Function	AIF; CytC; SMAC (Diablo); Aifm-1; Aifm-2
Developmental Neurology	BMP-4; Chordin (Chrd); Noggin (Nog); WNT (Wnt2; Wnt2b; Wnt3a; Wnt4; Wnt5a; Wnt6; Wnt7b; Wnt8b; Wnt9a; Wnt9b; Wnt10a; Wnt10b; Wnt16); beta-catenin; Dkk-1; Frizzled related proteins; Otx-2; Gbx2; FGF-8; Reelin; Dab1; unc-86 (Pou4f1 or Brn3a); Numb; Reln

[0197] Embodiments of the invention also relate to methods and compositions related to knocking out genes, amplifying genes and repairing particular mutations associated with DNA repeat instability and neurological disorders (Robert D. Wells, Tetsuo Ashizawa, Genetic Instabilities and Neurological Diseases, Second Edition, Academic Press, Oct. 13, 2011-Medical). Specific aspects of tandem repeat sequences have been found to be responsible for more than twenty human diseases (New insights into repeat instability: role of RNA-DNA hybrids. McIvor E I, Polak U, Napierala M. RNA Biol. 2010 September-October; 7(5):551-8). The CRiISPR-Cas system may be harnessed to correct these defects of genomic instability.

[0198] A further aspect of the invention relates to utilizing the CRISPR-Cas system for correcting defects in the EMP2A and EMP2B genes that have been identified to be associated with Lafora disease. Lafora disease is an autosomal recessive condition which is characterized by progressive myoclonus epilepsy which may start as epileptic seizures in adolescence. A few cases of the disease may be caused by mutations in genes yet to be identified. The disease causes seizures, muscle spasms, difficulty walking, dementia, and eventually death. There is currently no therapy that has proven effective against disease progression. Other genetic abnormalities associated with epilepsy may also be targeted by the CRISPR-Cas sytem and the underlying genetics is further described in Genetics of Epilepsy and Genetic Epilepsies, edited by Giuliano Avanzini, Jeffrey L. Noebels, Mariani Foundation Paediatric Neurology:20; 2009).

[0199] In yet another aspect of the invention, the CRISPR-Cas system may be used to correct ocular defects that arise from several genetic mutations further described in Genetic Diseases of the Eye, Second Edition, edited by Elias I. Traboulsi, Oxford University Press, 2012.

[0200] Several further aspects of the invention relate to correcting defects associated with a wide range of genetic diseases which are further described on the website of the National Institutes of Health under the topic subsection Genetic Disorders (website at health.nih.gov/topic/GeneticDisorders). The genetic brain diseases may include but are not limited to Adrenoleukodystrophy, Agenesis of the Corpus Callosum, Aicardi Syndrome, Alpers' Disease, Alzheimer's Disease, Barth Syndrome, Batten Disease, CADASIL, Cerebellar Degeneration, Fabry's Disease, Gerstmann-Straussler-Scheinker Disease, Huntington's Disease and other Triplet Repeat Disorders, Leigh's Disease, Lesch-

Nyhan Syndrome, Menkes Disease, Mitochondrial Myopathies and NINDS Colpocephaly. These diseases are further described on the website of the National Institutes of Health under the subsection Genetic Brain Disorders.

[0201] In some embodiments, the condition may be neoplasia. In some embodiments, where the condition is neoplasia, the genes to be targeted are any of those listed in Table A (in this case PTEN asn so forth). In some embodiments, the condition may be Age-related Macular Degeneration. In some embodiments, the condition may be a Schizophrenic Disorder. In some embodiments, the condition may be a Trinucleotide Repeat Disorder. In some embodiments, the condition may be Fragile X Syndrome. In some embodiments, the condition may be a Secretase Related Disorder. In some embodiments, the condition may be a Prion—related disorder. In some embodiments, the condition may be ALS. In some embodiments, the condition may be a drug addiction. In some embodiments, the condition may be Autism. In some embodiments, the condition may be Alzheimer's Disease. In some embodiments, the condition may be inflammation. In some embodiments, the condition may be Parkinson's Disease.

[0202] Examples of proteins associated with Parkinson's disease include but are not limited to α-synuclein, DJ-1, LRRK2, PINK1, Parkin, UCHL1, Synphilin-1, and NURR1.

[0203] Examples of addiction-related proteins may include ABAT for example.

[0204] Examples of inflammation-related proteins may include the monocyte chemoattractant protein-1 (MCP1) encoded by the Ccr2 gene, the C-C chemokine receptor type 5 (CCR5) encoded by the Ccr5 gene, the IgG receptor IIB (FCGR2b, also termed CD32) encoded by the Fcgr2b gene, or the Fc epsilon R1g (FCER1g) protein encoded by the Fcer1g gene, for example.

[0205] Examples of cardiovascular diseases associated proteins may include IL1B (interleukin 1, beta), XDH (xanthine dehydrogenase), TP53 (tumor protein p53), PTGIS (prostaglandin 12 (prostacyclin) synthase), MB (myoglobin), IL4 (interleukin 4), ANGPT1 (angiopoietin 1), ABCG8 (ATP-binding cassette, sub-family G (WHITE), member 8), or CTSK (cathepsin K), for example.

[0206] Examples of Alzheimer's disease associated proteins may include the very low density lipoprotein receptor protein (VLDLR) encoded by the VLDLR gene, the ubiquitin-like modifier activating enzyme 1 (UBA1) encoded by the UBA1 gene, or the NEDD8-activating enzyme E1 catalytic subunit protein (UBEIC) encoded by the UBA3 gene, for example.

[0207] Examples of proteins associated Autism Spectrum Disorder may include the benzodiazapine receptor (peripheral) associated protein 1 (BZRAP1) encoded by the BZRAP1 gene, the AF4/FMR2 family member 2 protein (AFF2) encoded by the AFF2 gene (also termed MFR2), the fragile X mental retardation autosomal homolog 1 protein (FXR1) encoded by the FXR1 gene, or the fragile X mental retardation autosomal homolog 2 protein (FXR2) encoded by the FXR2 gene, for example.

[0208] Examples of proteins associated Macular Degeneration may include the ATP-binding cassette, sub-family A (ABC1) member 4 protein (ABCA4) encoded by the ABCR gene, the apolipoprotein E protein (APOE) encoded by the APOE gene, or the chemokine (C-C motif) Ligand 2 protein (CCL2) encoded by the CCL2 gene, for example.

[0209] Examples of proteins associated Schizophrenia may include NRG1, ErbB4, CPLX1, TPH1, TPH2, NRXN1, GSK3A, BDNF, DISC1, GSK3B, and combinations thereof.

[0210] Examples of proteins involved in tumor suppression may include ATM (ataxia telangiectasia mutated), ATR (ataxia telangiectasia and Rad3 related), EGFR (epidermal growth factor receptor), ERBB2 (v-erb-b2 erythroblastic leukemia viral oncogene homolog 2), ERBB3 (v-erb-b2 erythroblastic leukemia viral oncogene homolog 3), ERBB4 (v-erb-b2 erythroblastic leukemia viral oncogene homolog 4), Notch 1, Notch2, Notch 3, or Notch 4, for example.

[0211] Examples of proteins associated with a secretase disorder may include PSENEN (presenilin enhancer 2 homolog (*C. elegans*)), CTSB (cathepsin B), PSEN1 (presenilin 1), APP (amyloid beta (A4) precursor protein), APH1B (anterior pharynx defective 1 homolog B (*C. elegans*)), PSEN2 (presenilin 2 (Alzheimer disease 4)), or BACE1 (beta-site APP-cleaving enzyme 1), for example.

[0212] Examples of proteins associated with Amyotrophic Lateral Sclerosis may include SOD1 (superoxide dismutase 1), ALS2 (amyotrophic lateral sclerosis 2), FUS (fused in sarcoma), TARDBP (TAR DNA binding protein), VAGFA (vascular endothelial growth factor A), VAGFB (vascular endothelial growth factor B), and VAGFC (vascular endothelial growth factor C), and any combination thereof.

[0213] Examples of proteins associated with prion diseases may include SOD1 (superoxide dismutase 1), ALS2 (amyotrophic lateral sclerosis 2), FUS (fused in sarcoma), TARDBP (TAR DNA binding protein), VAGFA (vascular endothelial growth factor A), VAGFB (vascular endothelial growth factor B), and VAGFC (vascular endothelial growth factor C), and any combination thereof.

[0214] Examples of proteins related to neurodegenerative conditions in prion disorders may include A2M (Alpha-2-Macroglobulin), AATF (Apoptosis antagonizing transcription factor), ACPP (Acid phosphatase prostate), ACTA2 (Actin alpha 2 smooth muscle aorta), ADAM22 (ADAM metallopeptidase domain), ADORA3 (Adenosine A3 receptor), or ADRA1D (Alpha-1D adrenergic receptor for Alpha-1D adrenoreceptor), for example.

[0215] Examples of proteins associated with Immunodeficiency may include A2M [alpha-2-macroglobulin]; AANAT [arylalkylamine N-acetyltransferase]; ABCA1 [ATP-binding cassette, sub-family A (ABC1), member 1]; ABCA2 [ATP-binding cassette, sub-family A (ABC1), member 2]; or ABCA3 [ATP-binding cassette, sub-family A (ABC1), member 3]; for example.

[0216] Examples of proteins associated with Trinucleotide Repeat Disorders include AR (androgen receptor), FMR1 (fragile X mental retardation 1), HTT (huntingtin), or DMPK (dystrophia myotonica-protein kinase), FXN (frataxin), ATXN2 (ataxin 2), for example.

[0217] Examples of proteins associated with Neurotransmission Disorders include SST (somatostatin), NOS1 (nitric oxide synthase 1 (neuronal)), ADRA2A (adrenergic, alpha-2A-, receptor), ADRA2C (adrenergic, alpha-2C-, receptor), TACR1 (tachykinin receptor 1), or HTR2c (5-hydroxytryptamine (serotonin) receptor 2C), for example.

[0218] Examples of neurodevelopmental-associated sequences include A2BP1 [ataxin 2-binding protein 1], AADAT [aminoadipate aminotransferase], AANAT [arylalkylamine N-acetyltransferase], ABAT [4-aminobutyrate aminotransferase], ABCA1 [ATP-binding cassette, sub-fam-

ily A (ABC1), member 1], or ABCA13 [ATP-binding cassette, sub-family A (ABC1), member 13], for example.

[0219] Further examples of preferred conditions treatable with the present system include may be selected from: Aicardi-Goutieres Syndrome; Alexander Disease; Allan-Herndon-Dudley Syndrome; POLG-Related Disorders; Alpha-Mannosidosis (Type II and III); Alstrom Syndrome; Angelman; Syndrome; Ataxia-Telangiectasia; Neuronal Ceroid-Lipofuscinoses; Beta-Thalassemia; Bilateral Optic Atrophy and (Infantile) Optic Atrophy Type 1; Retinoblastoma (bilateral); Canavan Disease; Cerebrooculofacioskeletal Syndrome 1 [COFS1]; Cerebrotendinous Xanthomatosis; Cornelia de Lange Syndrome; MAPT-Related Disorders; Genetic Prion Diseases; Dravet Syndrome; Early-Onset Familial Alzheimer Disease; Friedreich Ataxia [FRDA]; Fryns Syndrome; Fucosidosis; Fukuyama Congenital Muscular Dystrophy; Galactosialidosis; Gaucher Disease; Organic Acidemias; Hemophagocytic Lymphohistiocytosis; Hutchinson-Gilford Progeria Syndrome; Mucolipidosis II; Infantile Free Sialic Acid Storage Disease; PLA2G6-Associated Neurodegeneration; Jervell and Lange-Nielsen Syndrome; Junctional Epidermolysis Bullosa; Huntington Disease; Krabbe Disease (Infantile); Mitochondrial DNA-Associated Leigh Syndrome and NARP; Lesch-Nyhan Syndrome; LIS1-Associated Lissencephaly; Lowe Syndrome; Maple Syrup Urine Disease; MECP2 Duplication Syndrome; ATP7A-Related Copper Transport Disorders; LAMA2-Related Muscular Dystrophy; Arylsulfatase A Deficiency; Mucopolysaccharidosis Types I, II or III; Peroxisome Biogenesis Disorders, Zellweger Syndrome Spectrum; Neurodegeneration with Brain Iron Accumulation Disorders; Acid Sphingomyelinase Deficiency; Niemann-Pick Disease Type C; Glycine Encephalopathy; ARX-Related Disorders; Urea Cycle Disorders; COL1A1/2-Related Osteogenesis Imperfecta; Mitochondrial DNA Deletion Syndromes; PLP1-Related Disorders; Perry Syndrome; Phelan-McDermid Syndrome; Glycogen Storage Disease Type II (Pompe Disease) (Infantile); MAPT-Related Disorders; MECP2-Related Disorders; Rhizomelic Chondrodysplasia Punctata Type 1; Roberts Syndrome; Sandhoff Disease; Schindler Disease—Type 1; Adenosine Deaminase Deficiency; Smith-Lemli-Opitz Syndrome; Spinal Muscular Atrophy; Infantile-Onset Spinocerebellar Ataxia; Hexosaminidase A Deficiency; Thanatophoric Dysplasia Type 1; Collagen Type VI-Related Disorders; Usher Syndrome Type I; Congenital Muscular Dystrophy; Wolf-Hirschhorn Syndrome; Lysosomal Acid Lipase Deficiency; and Xeroderma Pigmentosum.

[0220] As will be apparent, it is envisaged that the present system can be used to target any polynucleotide sequence of interest. Some examples of conditions or diseases that might be usefully treated using the present system are included in the Tables above and examples of genes currently associated with those conditions are also provided there. However, the genes exemplified are not exhaustive.

EXAMPLES

[0221] The following examples are given for the purpose of illustrating various embodiments of the invention and are not meant to limit the present invention in any fashion. The present examples, along with the methods described herein are presently representative of preferred embodiments, are exemplary, and are not intended as limitations on the scope of the invention. Changes therein and other uses which are encompassed within the spirit of the invention as defined by the scope of the claims will occur to those skilled in the art.

Example 1: Improvement of the Cas9 System for In Vivo Application

[0222] Applicants conducted a Metagenomic search for a Cas9 with small molecular weight. Most Cas9 homologs are fairly large. For example the SpCas9 is around 1368aa long, which is too large to be easily packaged into viral vectors for delivery.

[0223] Through computational analysis, Applicants found that in the bacterial strain *Campylobacter*, there are two Cas9 proteins with less than 1000 amino acids. The sequence for one Cas9 from *Campylobacter jejuni* is presented below. At this length, CjCas9 can be easily packaged into AAV, lentiviruses, Adenoviruses, and other viral vectors for robust delivery into primary cells and in vivo in animal models. In a preferred embodiment of the invention, the Cas9 protein from *S. aureus* is used.

```
>Campylobacter jejuni Cas9 (CjCas9)
MARILAFDIGISSIGWAFSENDELKDCGVRIFTKVENPKTGESLALPRRL

ARSARKRLARRKARLNHLKHLIANEFKLNYEDYQSFDESLAKAYKGSLIS

PYELRFRALNELLSKQDFARVILHIAKRRGYDDIKNSDDKEKGAILKAIK

QNEEKLANYQSVGEYILYKEYFQKFKENSKEFTNVRNKKESYERCIAQSF

LKDELKLIFKKQREFGFSFSKKFEEEVLSVAFYKRALKDFSHLVGNCSFF

TDEKRAPKNSPLAFMFVALTRIINLLNNLKYTEGILYTKDDLNALLNEVL

KNGTLTYKQTKKLLGLSDDYEFKGEKGTYFIEFKKYKEFIKALGEHNLSQ

DDLNEIAKDITLIKDEIKLKKALAKYDLNQNQIDSLSKLEFKDHLNISFK

ALKLVTPLMLEGKKYDEACNELNLKVAINEDKKDFLPAFNETYYKDEVTN

PVVLRAIKEYRKVLNALLKKYGKVHKINIELAREVGKNHSQRAKIEKEQN

ENYKAKKDAELECEKLGLKINSKNILKLRLFKEQKEFCAYSGEKIKISDL

QDEKMLEIDHIYPYSRSFDDSYMNKNLVFTKQNQEKLNQTPFEAFGNDSA

KWQKIEVLAKNLPTKKQKRILDKNYKDKEQKNFKDRNLNDTRYIARLVLN

YTKDYLDFLPLSDDENTKLNDTQKGSKVEAKSGMLTSALRHTWGFSAKDR

NNHLHHAIDAVIIAYANNSIVKAFSDFKKEQESNSAELYAKKISELDYKN

KRKFFEPFSGFRQKVLDKIDEIFVSKPERKKPSGALHEETFRKEEEFYQS

YGGKEGVLKALELGKIRKVNGKIVKNGDMFRVDIFKHKKTNKFYAVPIYT

MDFALKVLPNKAVARSKKGEIKDWILMDENYEECFSLYKDSLILIQTKDM

QEPEFVYYNAFTSSTVSLIVSKHDNKFETLSKNQKILFKNANEKEVIAKS

IGIQNILKVFEKYIVSALGEVTKAEFRQREDFKK.
```

[0224] The putative tracrRNA element for this CjCas9 is:

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TATAATCTCATAAGAAATTTAAAAAGGGACTAAAATAAAGAGTTTGCGGG

ACTCTGCGGGGTTACAATCCCCTAAAACCGCTTTTAAAATT
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[0225] The Direct Repeat sequence is:

ATTTTACCATAAAGAAATTTAAAAAGGGACTAAAAC

[0226] An example of a chimeric guideRNA for CjCas9 is:

NNNNNNNNNNNNNNNNNNNNGUUUUAGUCCCGAAAGGGACUAAAAUAAAG

AGUUUGCGGGACUCUGCGGGGUUACAAUCCCCUAAAACCGCUUUU

Example 2: Cas9 Optimization

[0227] For enhanced function or to develop new functions, Applicants generate chimeric Cas9 proteins by combining fragments from different Cas9 homologs. For example, two example chimeric Cas9 proteins:

[0228] For example, Applicants fused the N-term of St1Cas9 (fragment from this protein is in bold) with C-term of SpCas9 (fragment from this protein is underlined).

>St1(N)Sp(C)Cas9

MSDLVLGLDIGIGSVGVGILNKVTGEIIHKNSRIFPAAQAENNLVRRTNR

QGRRLARRKKHRRVRLNRLFEESGLITDFTKISINLNPYQLRVKGLTDEL

SNEELFIALKNMVKHRGISYLDDASDDGNSSVGDYAQWKENSKQLETKIP

GQIQLERYQTYGQLRGDFTVEKDGKKHRLINVFPTSNYRSEALRILQTQQ

EFNPQITDEFINRYLEILTGKRKYYHGPGNEKSRTDYGRYRTSGETLDNI

FGILIGKCTFYPDEFRAAKASYTAQEFNLLNDLNNLTVPTETKKLSKEQK

NQIINYVKNEKAMGPAKLFKYIAKLLSCDVADIKGYRIDKSGKAEIHTFE

AYRKMKTLETLDIEQMDRETLDKLAYVLTLNTEREGIQEALEHEFADGSF

SQKQVDELVQFRKANSSIFGKGWHNFSVKLMMELIPELYETSEEQMTILT

RLGKQKTTSSSNKTKYIDEKLLTEEIYNPVVAKSVRQAIKIVNAAIKEYG

DFDNIVIEMARENQTTQKGQKNSRERMKRIEEGIKELGSQILKEHPVENT

QLQNEKLYLYYLQNGRDMYVDQELDINRLSDYDVDHIVPQSFLKDDSIDN

KVLTRSDKNRGKSDNVPSEEVVKKMKNYWRQLLNAKLITQRKFDNLTKAE

RGGLSELDKAGFIKRQLVETRQITKHVAQILDSRMNTKYDENDKLIREVK

VITLIKSKLVSDFRKDFQFYKVREINNYHHAHDAYLNAVVGTALIKKYPK

LESEFVYGDYKVYDVRKMIAKSEQEIGKATAKYFFYSNIMNFFKTEITLA

NGEIRKRPLIETNGETGEIVWDKGRDFATVRKVLSMPQVNIVKKTEVQTG

GFSKESILPKRNSDKLIARKKDWDPKKYGGFDSPTVAYSVLVVAKVEKGK

SKKLKSVKELLGITIMERSSFEKNPIDFLEAKGYKEVKKDLIIKLPKYSL

FELENGRKRMLASAGELQKGNELALPSKYVNFLYLASHYEKLKGSPEDNE

QKQLFVEQHKHYLDEIIEQISEFSKRVILADANLDKVLSAYNKHRDKPIR

EQAENIIHLFTLTNLGAPAAFKYFDTTIDRKRYTSTKEVLDIHQSITGLY

ETRIDLSQLGGD

>Sp(N)St1(C)Cas9

MDKKYSIGLDIGTNSVGWAVITDEYKVPSKKFKVLGNTDRHSIKKNLIGA

LLFDSGETAEATRLKRTARRRYTRRKNRICYLQEIFSNEMAKVDDSFFHR

-continued

LEESFLVEEDKKHERHPIFGNIVDEVAYHEKYPTIYHLRKKLVDSTDKAD

LRLIYLALAHMIKFRGHFLIEGDLNPDNSDVDKLFIQLVQTYNQLFEENP

INASGVDAKAILSARLSKSRRLENLIAQLPGEKKNGLFGNLIAISLGLTP

NFKSNFDLAEDAKLQLSKDTYDDDLDNLLAQIGDQYADLFLAAKNLSDAI

LLSDILRVNTEITKAPLSASMIKRYDEHHQDLTLLKALVRQQLPEKYKEI

FFDQSKNGYAGYIDGGASQEFTYKFIKPILEKMDGTEELLVKLNREDLLR

KQRTFDNGSIPHQIHLGELHAILRRQEDFYPFLKDNREKIEKILTFRIPY

YVGPLARGNSRFAWMTRKSEETITPWNFEEVVDKGASAQSFIERMTNFDK

NLPNEKVLPKHSLLYEYFTVYNELTKVKYVTEGMRKPAFLSGEQKKAIVD

LLFKTNRKAVTVKQLKEDYFKKIECFDSVEISGVEDRFNASLGTYHDLLK

IIKDKDFLDNEENEDILEDIVLTLTLFEDREMIEERLKTYAHLFDDKNMK

QLKRRRYTGWGRLSRKLINGIRDKQSGKTILDFLKSDGFANRNFMQLIHD

DSLTFKEDIQKAQVSGQGDSLHEHIANLAGSPAIKKGILQTVKVVDELVK

VMGRHKPENIVIEMARE**TNEDDEKKAIQKIQKANKDEKDAAMLKAANQYN**

GKAELPHSVFHGHKQLATKIRLWHQQGERCLYTGKTISHIDLINNSNQFE

VDHILPLSITFDDSLANKVLVYATANQEKGQRTPYQALDSMDDAWSFREL

KAFVRESKTLSNKKKEYLLTEEDISKFDVRKKFIERNLVDTRYASRVVLN

ALQEHFRAHKIDTKVSVVRGQFTSQLRRHWGIEKTRDTYHHHAVDALIIA

ASSQLNLWKKQKNTLVSYSEDQLLDIETGELISDDEYKESVFKAPYQHFV

DTIKSKEFEDSILFSYQVDSKFNRKISDATIYATRQAKVGKDKADETYVL

GKIKDIYTQDGYDAFMKIYKKDKSKFLMYRHDPQTFEKVIEPELENYPNK

QINEKGKEVPCNPFLKYKEEHGYIRKYSKKGNGPEIKSLKYYDSKLGNHI

DITPKDSNNKVVLQSVSPWRADVYFNKTTGKYEILGLKYADLQFEKGTGT

YKISQEKYNDIKKKEGVDSDSEFKFTLYKNDLLLVKDTETKEQQLFRFLS

RTMPKQKIHYVELKPYDKQKFEGGEALIKVLGNVANSGQCKKGLGKSNIS

IYKVRTDVLGNQHIIKNEGDKPKLDF

[0229] The benefit of making chimeric Cas9 include:

[0230] a. reduce toxicity

[0231] b. improve expression in eukaryotic cells

[0232] c. enhance specificity

[0233] d. reduce molecular weight of protein, make protein smaller by combining the smallest domains from different Cas9 homologs.

[0234] e. Altering the PAM sequence requirement

Example 3: Utilization of Cas9 as a Generic DNA Binding Protein

[0235] Applicants used Cas9 as a generic DNA binding protein by mutating the two catalytic domains (D10 and H840) responsible for cleaving both strands of the DNA target. In order to upregulate gene transcription at a target locus Applicants fused the transcriptional activation domain (VP64) to Cas9 (FIG. **5**). Applicants hypothesized that it would be important to see strong nuclear localization of the Cas9-VP64 fusion protein because transcription factor activation strength is a function of time spent at the target. Therefore, Applicants cloned a set of Cas9-VP64-GFP con-

structs, transfected them into 293 cells and assessed their localization under a fluorescent microscope 12 hours post-transfection (FIG. **8**).

[0236] The same constructs were cloned as a 2A-GFP rather than a direct fusion in order to functionally test the constructs without a bulky GFP present to interfere. Applicants elected to target the Sox2 locus with the Cas9 transactivator because it could be useful for cellular reprogram and the locus has already been validated as a target for TALE-TF mediated transcriptional activation. For the Sox2 locus Applicants chose eight targets near the transcriptional start site (TSS). Each target was 20 bp long with a neighboring NGG protspacer adjacent motif (PAM) (FIG. **12**). Each Cas9-VP64 construct was co-transfected with each PCR generated chimeric crispr RNA (chiRNA) in 293 cells. 72 hours post transfection the transcriptional activation was assessed using RT-qPCR (FIG. **6**).

[0237] To further optimize the transcriptional activator, Applicants titrated the ratio of chiRNA (Sox2.1 and Sox2.5) to Cas9 (NLS-VP64-NLS-hSpCas9-NLS-VP64-NLS), transfected into 293 cells, and quantified using RT-qPCR (FIG. **11**). These results indicate that Cas9 can be used as a generic DNA binding domain to upregulate gene transcription at a target locus.

[0238] Applicants designed a second generation of constructs. (Table 1 below).

pLenti-EF1a-GFP-2A-6xHis-NLS-VP64-NLS-hSpCsn1(D10A, H840A)-NLS
pLenti-EF1a-GFP-2A-6xHis-NLS-VP64-NLS-hSpCsn1(D10A, H840A)
pLenti-EF1a-GFP-2A-6xHis-NLS-VP64-NLS-NLS-hSpCsn1(D10A,H840A)
pLenti-EF1a-GFP-2A-6xHis-NLS-hSpCsn1(D10A, H840A)-NLS
pLenti-EF1a-GFP-2A-6xHis-NLS-hSpCsn1(D10A, H840A)
pLenti-EF1a-GFP-2A-6xHis-NLS-NLS-hSpCsn1(D10A, H840A)

[0239] Applicants use these constructs to assess transcriptional activation (VP64 fused constructs) and repression (Cas9 only) by RT-qPCR. Applicants assess the cellular localization of each construct using anti-His antibody, nuclease activity using a Surveyor nuclease assay, and DNA binding affinity using a gel shift assay. In a preferred embodiment of the invention, the gel shift assay is an EMSA gel shift assay.

Example 4: Cas9 Repressor

[0240] It has been shown previously that dCas9 can be used as a generic DNA binding domain to repress gene expression. Applicants report an improved dCas9 design as well as dCas9 fusions to the repressor domains KRAB and SID4x. From the plasmid library created for modulating transcription using Cas9 in Table 2 below, and FIG. **15**, the following repressor plasmids were functionally characterized by qPCR: pXRP27, pXRP28, pXRP29, pXRP48, pXRP49, pXRP50, pXRP51, pXRP52, pXRP53, pXRP56, pXRP58, pXRP59, pXRP61, and pXRP62.

[0241] Each dCas9 repressor plasmid was co-transfected with two guide RNAs targeted to the coding strand of the beta-catenin gene (gRNAs were designed by my collaborator at the Broad, Joseph Rosenbluh). RNA was isolated 72 hours after transfection and gene expression was quantified by RT-qPCR (FIG. **16**). The endogenous control gene was GAPDH. Two validated shRNAs were used as positive controls. Negative controls were certain plasmids transfected without gRNA, these are denoted as "pXRP ##control" in FIG. **16**. The plasmids pXRP28, pXRP29, pXRP48, and pXRP49 could repress the beta-catenin gene when using the specified targeting strategy. These plasmids correspond to dCas9 without a functional domain (pXRP28 and pXRP28) and dCas9 fused to SID4x (pXRP48 and pXRP49).

TABLE 2

pXRP024-pLenti2-EF1a-VP64-NLS-FLAG-Linker-dCas9-NLS-gLuc-2A-GFP-WPRE
pXRP025-pLenti2-EF1a-VP64-NLS-GGGGS$_3$Linker-dCas9-NLS-gLuc-2A-GFP-WPRE
pXRP026-pLenti2-EF1a-VP64-NLS-EAAAK$_3$Linker-dCas9-NLS-gLuc-2A-GFP-WPRE
pXRP027-pLenti2-EF1a-NLS-FLAG-Linker-dCas9-NLS-gLuc-2A-GFP-WPRE
pXRP028-pLenti2-EF1a-NLS-GGGGS$_3$Linker-dCas9-NLS-gLuc-2A-GFP-WPRE
pXRP029-pLenti2-EF1a-NLS-EAAAK$_3$Linker-dCas9-NLS-gLuc-2A-GFP-WPRE
pXRP030-pLenti2-pSV40-VP64-NLS-FLAG-Linker-dCas9-NLS-gLuc-2A-GFP-WPRE
pXRP031-pLenti2-pPGK-VP64-NLS-FLAG-Linker-dCas9-NLS-gLuc-2A-GFP-WPRE
pXRP032-pLenti2-LTR-VP64-NLS-FLAG-Linker-dCas9-NLS-gLuc-2A-GFP-WPRE
pXRP033-pLenti2-pSV40-VP64-NLS-GGGGS$_3$Linker-dCas9-NLS-gLuc-2A-GFP-WPRE
pXRP034-pLenti2-pPGK-VP64-NLS-GGGGS$_3$Linker-dCas9-NLS-gLuc-2A-GFP-WPRE
pXRP035-pLenti2-LTR-VP64-NLS-GGGGS$_3$Linker-dCas9-NLS-gLuc-2A-GFP-WPRE
pXRP036-pLenti2-pSV40-VP64-NLS-EAAAK$_3$Linker-dCas9-NLS-gLuc-2A-GFP-WPRE
pXRP037-pLenti2-pPGK-VP64-NLS-EAAAK$_3$Linker-dCas9-NLS-gLuc-2A-GFP-WPRE
pXRP038-pLenti2-LTR-VP64-NLS-EAAAK$_3$Linker-dCas9-NLS-gLuc-2A-GFP-WPRE
pXRP048-pLenti2-EF1a-SID4x-NLS-FLAG-Linker-dCas9-NLS-gLuc-2A-GFP-WPRE
pXRP049-pLenti2-EF1a-SID4X-NLS-GGGGS$_3$Linker-dCas9-NLS-gLuc-2A-GFP-WPRE
pXRP050-pLenti2-EF1a-SID4X-NLS-EAAAK$_3$Linker-dCas9-NLS-gLuc-2A-GFP-WPRE
pXRP051-pLenti2-EF1a-KRAB-NLS-FLAG-Linker-dCas9-NLS-gLuc-2 A-GFP-WPRE
pXRP052-pLenti2-EF1a-KRAB-NLS-GGGGS$_3$Linker-dCas9-NLS-gLuc-2A-GFP-WPRE
pXRP053-pLenti2-EF1a-KRAB-NLS-EAAAK$_3$Linker-dCas9-NLS-gLuc-2A-GFP-WPRE
pXRP054-pLenti2-EF1a-dCas9-Linker-FLAG-NLS-VP64-gLuc-2A-GFP-WPRE
pXRP055-pLenti2-EF1a-dCas9-Linker-FLAG-NLS-SID4X-gLuc-2A-GFP-WPRE
pXRP056-pLenti2-EF1a-dCas9-Linker-FLAG-NLS-KRAB-gLuc-2A-GFP-WPRE
pXRP057-pLenti2-EF1a-dCas9-GGGGGS$_3$-NLS-VP64-gLuc-2A-GFP-WPRE
pXRP058-pLenti2-EF1a-dCas9-GGGGGS$_3$-NLS-SID4X-gLuc-2A-GFP-WPRE
pXRP059-pLenti2-EF1a-dCas9-GGGGGS$_3$-NLS-KRAB-gLuc-2A-GFP-WPRE
pXRP060-pLenti2-EF1a-dCas9-EAAAK$_3$-NLS-VP64-gLuc-2A-GFP-WPRE
pXRP061-pLenti2-EF1a-dCas9-EAAAK$_3$-NLS-SID4X-gLuc-2A-GFP-WPRE
pXRP062-pLenti2-EF1a-dCas9-EAAAK$_3$-NLS-KRAB-gLuc-2A-GFP-WPRE
pXRP024-pLenti2-EF1a-VP64-NLS-FLAG-Linker-Cas9-NLS-gLuc-2A-GFP-WPRE
pXRP025-pLenti2-EF1a-VP64-NLS-GGGGS$_3$Linker-Cas9-NLS-gLuc-2A-GFP-WPRE
pXRP026-pLenti2-EF1a-VP64-NLS-EAAAK$_3$Linker-Cas9-NLS-gLuc-2A-GFP-WPRE

TABLE 2-continued

pXRP027-pLenti2-EF1a-NLS-FLAG-Linker-Cas9-NLS-gLuc-2A-GFP-WPRE
pXRP028-pLenti2-EF1a-NLS-GGGGS$_3$Linker-Cas9-NLS-gLuc-2A-GFP-WPRE
pXRP029-pLenti2-EF1a-NLS-EAAAK$_3$Linker-Cas9-NLS-gLuc-2A-GFP-WPRE
pXRP030-pLenti2-pSV40-VP64-NLS-FLAG-Linker-Cas9-NLS-gLuc-2A-GFP-WPRE
pXRP031-pLenti2-pPGK-VP64-NLS-FLAG-Linker-Cas9-NLS-gLuc-2A-GFP-WPRE
pXRP032-pLenti2-LTR-VP64-NLS-FLAG-Linker-Cas9-NLS-gLuc-2A-GFP-WPRE
pXRP033-pLenti2-pSV40-VP64-NLS-GGGGS$_3$Linker-Cas9-NLS-gLuc-2A-GFP-WPRE
pXRP034-pLenti2-pPGK-VP64-NLS-GGGGS$_3$Linker-Cas9-NLS-gLuc-2A-GFP-WPRE
pXRP035-pLenti2-LTR-VP64-NLS-GGGGS$_3$Linker-Cas9-NLS-gLuc-2A-GFP-WPRE
pXRP036-pLenti2-pSV40-VP64-NLS-EAAAK$_3$Linker-Cas9-NLS-gLuc-2A-GFP-WPRE
pXRP037-pLenti2-pPGK-VP64-NLS-EAAAK$_3$Linker-Cas9-NLS-gLuc-2A-GFP-WPRE
pXRP038-pLenti2-LTR-VP64-NLS-EAAAK$_3$Linker-Cas9-NLS-gLuc-2A-GFP-WPRE
pXRP048-pLenti2-EF1a-SID4x-NLS-FLAG-Linker-Cas9-NLS-gLuc-2A-GFP-WPRE
pXRP049-pLenti2-EF1a-SID4X-NLS-GGGGS$_3$Linker-Cas9-NLS-gLuc-2A-GFP-WPRE
pXRP050-pLenti2-EF1a-SID4X-NLS-EAAAK$_3$Linker-Cas9-NLS-gLuc-2A-GFP-WPRE
pXRP051-pLenti2-EF1a-KRAB-NLS-FLAG-Linker-Cas9-NLS-gLuc-2A-GFP-WPRE
pXRP052-pLenti2-EF1a-KRAB-NLS-GGGGS$_3$Linker-Cas9-NLS-gLuc-2A-GFP-WPRE
pXRP053-pLenti2-EF1a-KRAB-NLS-EAAAK$_3$Linker-Cas9-NLS-gLuc-2A-GFP-WPRE
pXRP054-pLenti2-EF1a-Cas9-Linker-FLAG-NLS-VP64-gLuc-2A-GFP-WPRE
pXRP055-pLenti2-EF1a-Cas9-Linker-FLAG-NLS-SID4X-gLuc-2A-GFP-WPRE
pXRP056-pLenti2-EF1a-Cas9-Linker-FLAG-NLS-KRAB-gLuc-2A-GFP-WPRE
pXRP057-pLenti2-EF1a-Cas9-GGGGGS$_3$-NLS-VP64-gLuc-2A-GFP-WPRE
pXRP058-pLenti2-EF1a-Cas9-GGGGGS$_3$-NLS-SID4X-gLuc-2A-GFP-WPRE
pXRP059-pLenti2-EF1a-Cas9-GGGGGS$_3$-NLS-KRAB-gLuc-2A-GFP-WPRE
pXRP060-pLenti2-EF1a-Cas9-EAAAK$_3$-NLS-VP64-gLuc-2A-GFP-WPRE
pXRP061-pLenti2-EF1a-Cas9-EAAAK$_3$-NLS-SID4X-gLuc-2A-GFP-WPRE
pXRP062-pLenti2-EF1a-Cas9-EAAAK$_3$-NLS-KRAB-gLuc-2A-GFP-WPRE

Example 5: Cas9 Transcriptional Modulator and Nuclear Localization

[0242] Cas9 Transcriptional Modulator. Applicants set out to turn the Cas9/gRNA CRISPR system into a generalized DNA binding system in which Applicants can execute functions beyond DNA cleavage. Whereby, fusing functional domains onto a catalytically inactive Cas9, dCas9, Applicants can impart novel functions, such as transcriptional activation/repression, methylation/demethylation, or chromatin modifications. To accomplish this goal Applicants made a catalytically inactive Cas9 mutant by changing two residues essential for nuclease activity, D10 and H840, to alanine. It has been shown previously that by mutating these two residues the nuclease activity of Cas9 is abolished while maintaining the ability to bind target DNA. The functional domains Applicants decided to focus on to test Applicants' hypothesis are the transcriptional activator VP64 and the transcriptional repressors SID and KRAB.

[0243] dCas9 Nuclear localization. Applicants hypothesized that the most effective dCas9 transcriptional modulator would be strongly localized to the nucleus where it would have its greatest influence on transcription. Moreover, any residual dCas9 in the cytoplasm could have unwanted effects. In previous work Applicants determined that wild-type Cas9 does not localize into the nucleus without including multiple nuclear localization signals (NLSs). Because multiple NLS sequences were required Applicants reasoned that it is difficult to get Cas9 into the nucleus and any additional domain that is fused to Cas9 could disrupt the nuclear localization. Therefore, Applicants built five dCas9-VP64-GFP fusion constructs to optimize the position and number of NLS sequences. A second version of each construct was made for functional testing, and these constructs were not directly fused to GFP. A schematic of these designs is shown in FIG. **7**. These constructs were cloned into a plenti2 backbone under the expression of the human EF1α promoter. The WPRE element was also added for more robust protein expression. Each construct was transfected into HEK 293FT cells using Lipofectame 2000 and imaged 24 hours post-transfection (FIG. **8**). FIG. **8** shows that the best nuclear localization is obtained when the fusion proteins have NLS sequences on both the N- and C-term of the fusion protein. The highest observed nuclear localization occurred in the construct with four NLS elements.

[0244] To more robustly understand the influence of NLS elements on dCas9 Applicants made 16 dCas9-GFP fusions by adding the same alpha importin NLS sequence on either the N- or C-term looking at zero to three tandem repeats. The same cloning strategy was used as explained previously. Each construct was transfected into HEK 293FT cells using Lipofectame 2000 and imaged 24 hours post-transfection (FIG. **9**). Notably, the number of NLS elements does not directly correlate with the extent of nuclear localization. Adding an NLS on the C-term has a greater influence on nuclear localization than adding on the N-term. For future applications it is more favorable to have smaller proteins. Therefore, the optimal NLS architecture for dCas9 was having one NLS on the N-term and C-term (pXRPNLS006).

[0245] One potential confounding factor of using a GFP fusion to determine nuclear localization is that the GFP itself could have a large influence on where the protein localizes in the cell. Therefore, Applicants determined that a good approach would be to include a 6×His tag to dCas9, transfect into 293FT cells, and then stain with an anti-6×His antibody. Applicants made six versions utilizing this idea (FIG. **10**). Three were constructed for transcriptional activation (VP64 fusions), and the other three were for transcriptional repression (no functional domain). These constructs were also made so Applicants could purify the recombinant protein using a nickel column. There are many applications of the 6×His-dCas9 constructs, such as, to perform an electromobility gel shift assay to show that mutated Cas9 can indeed bind its DNA target. The repression constructs (pXRP013, pXRP014, pXRP015) were transfected in HEK 293 FT cells and were immunostained and imaged after 24 hours (FIG. **11**). Under this specific context, dCas9 requires two NLS signals on the N-term to be localized into the nucleus. Interestingly, one NLS on each terminus did not cause dCas9 to be strongly nuclear localized. Taken together, what this data suggests is that there is no generalized rule that can be

made to conclusively say that a dCas9 fusion protein will be localized into the nucleus. The construct of interest must be validated in the system it will be used to determine if it is in fact localized to the nucleus.

[0246] Cas9 Transcriptional Modulator Functional Testing. Applicants functionally tested the dCas9-VP64 protein by targeting the Sox2 locus and quantifying transcriptional activation by RT-qPCR. Eight DNA target sites were chosen to span the promoter of Sox2 (FIG. **12**). The gRNA expression cassettes were cloned into a pAAV-U6-gRNA/trcrRNA backbone plasmid, pA6. The plasmid utilizes the type IIs enzyme Bbsi to reveals sticky ends for annealed oligo ligation. So for each target all one needs is two complimentary oligos with the appropriate sticky ends.

[0247] Each dCas9 containing construct was co-transfected with pA6 plasmids into HEK 293FT cells using Lipofectame 2000. 72 hours post-transfection total RNA was extracted from the cells. 1 ug of RNA was reverse transcribed into cDNA (qScript Supermix) in a 40 ul reaction. 2 ul of reaction product was added into a single 20 ul TaqMan assay qPCR reaction. Each experiment was performed in biological and technical triplicates. No RT control and no template control reactions showed no amplification. The results of this experiment are shown in FIG. **13**. Constructs that do not show strong nuclear localization, pXRP02 and pXRP04, result in no activation. For the construct that did show strong nuclear localization, pXRP08, moderate activation was observed. Statistically significant activation was observed in the case of gRNAs Sox2.4 and Sox2.5. Of the 6×His-dCas9 constructs (pXRP011, pXRP013, pXRP015) were also tested using the same Sox2 targets (FIG. **14**). Compared to no gRNA mock controls Applicants could repress or activate Sox2.

[0248] To further optimize the dCas9 transcriptional activator and repressor Applicants built 31 constructs to explore different linkers, functional domains, and N- and C-term fusions. The list of constructs and the critical elements are shown in FIG. **15**. Three linkers were tested and the first was an unusual linker that was published by Shen and collegues (Shen et al. 2013, *Cell Research paper from Xingxu Huang's lab: Cell Research* 23, 720-723 (May 2013)) where they showed it drastically improved nuclear localization and nuclease activity of Cas9. The second and third linkers were very common linkers known to be flexible (GGGGS3) and rigid (EAAAK3). The functional domains tested were VP64 for activation and SID4x and KRAB for repression. dCas9 without a functional domain was also made to test repression. The promoters tested were human EF1a, human pPGK, SV40, and the lack of a promoter where expression is driven by the viral LTR.

[0249] All transcriptional activator constructs were targeted to the Sox2 locus using a combination of two gRNAs, hSox2.1 and hSox2.4. The dCas9 activator constructs were co-transfected with the gRNAs into HEK 293FT cells and analyzed by qPCR after 72 hours (FIG. **16**).

[0250] A luciferase Sox2 reporter assay was adopted to simplify the methods associated for determining levels of activation. The assay works by co-transfecting in three plasmids, 1) dCas9, 2) gRNA, 3) Sox2 responsive element driving luciferase. So the level of luciferase is proportional to the amount of Sox2. The Sox2 responsive plasmid was purchised from BioCat. The Sox2 locus was targeted using different combinations of gRNAs and the constructs pXRPO24 and pXRPO25. A "No dCas9" control was used to determine the basal level of activation. The results shown in the top panel of FIG. **11** suggest that 3-fold activation could be reached compared to "No dCas9" and "No gRNA" controls.

[0251] A similar luciferase assay was adopted to test dCas9 transcriptional repression. The candidate gene Applicants chose to repress was beta-catenin and the targets were located near the 5' end of the gene body on the non-coding strand. The first experiment utilizing this assay compared the repressor pXRPNLS006 to the "dCas9" repressor published by Qi and collegues (Qi et al. 2003, Cell paper from Wendell Lim's lab:

[0252] http://www.sciencedirect.com/science/article/pii/S0092867413002110). The luciferase assay results (bottom panel of FIG. **11**) show that 3-fold repression can be reached and the pXRPNLS006 plasmid reported here works significantly better than the current standard, "dCas9".

Example 6: Cas9 transcriptional activator in murine and human cell lines

[0253] Applicants previously demonstrated the capacity for CRISPR/Cas9 to act as a transcriptional activator. In this example Applicants further test this capability in a murine cell line. Applicants also examined the unguided basal activity of the dCas9-VP64 (double mutant D10A H840A Cas9 fused to the VP64 transcriptional activation domain). Applicants delivered the Cas9 activator system to mouse cells and, separately, characterized the unguided effects of dCas9-VP64 for several human genes.

[0254] Mouse Cell Line Experiments: Applicants selected the locus for the mouse gene Neurog2 for testing dCas9-VP64 transcriptional activation in Neuro 2A cells. Applicants also selected 7 sgRNA targeting sequences from a region 300 bp upstream to 100 bp downstream of the annotated Neurog2 transcriptional start site. These guide sequences were cloned by the golden gate method into the px362 plasmid containing the U6 promoter upstream of a chimeric sgRNA cloning backbone.

[0255] Neuro 2A cells were cultured in medium consisting of a 1:1 ratio of D5 (100:5:1 DMEM high glucose w/Glutamax and sodium pyruvate: fetal bovine serum:

[0256] penecillin/stretomycin). For transfection, 120K cells in 0.5 mL culture medium were plated in each well of a 24 well plate. After 22 h in culture, each well of Neuro 2A cells was transfected with 1 μg of DNA using 5 μL of Lipofectamine following the recommended steps of the manufacturer (LIfe Technologies). For all dCas9-VP64+sgRNA samples, 0.5 ug dCas9-VP64 plasmid and 0.5 ug sgRNA expression plasmid were used. For control samples, plasmid masses replaced GFP expressing plasmid as necessary. 4 h after transfection, the medium was replaced with 1 mL culture medium. 48 h after transfection, RNA was purified using the Macherey Nagel Nucleospin 96 RNA kit. Reverse transcription was performed with qScript cDNA supermix according to the manufacturer's protocol. qPCR was performed using Taqman probes and Taqman Fast Advanced Master Mix from Life Technologies on a Roche LightCycler 480 II real-time PCR machine.

[0257] Human Cell Line Experiments: Applicants selected 8 previously available sgRNA sequences for testing in 293FT cells using Applicants' pXRP057 dCas9-VP64 activator construct. Guide sequences were cloned by the golden gate method into the px362 plasmid containing the U6 promoter upstream of a chimeric sgRNA cloning backbone. [00235]293FT cells were cultured in D10 medium (100:10:1 ratio of DMEM high glucose w/Glutamax and sodium pyruvate: fetal bovine serum: 100×HEPES solution). For transfection, 100K cells in 0.5 mL culture medium were plated in each well of a 24 well plate. After 22 h in culture, each well of 293FT cells was transfected with 1 μg of DNA using 5 μL of Lipofectamine following the recommended steps of the manufacturer (LIfe Technologies). For all dCas9-VP64+sgRNA samples, 0.5 ug dCas9-VP64 plasmid and 0.5 ug sgRNA expression plasmid were used. For control samples, including those analyzed for unguided transcriptional effects, plasmid masses were replaced by GFP expressing plasmid as necessary. 4 h after transfection, the medium was replaced with 1 mL culture medium. 48 h after transfection, RNA was purified using the Macherey Nagel Nucleospin 96 RNA kit. Reverse transcription was performed with qScript cDNA supermix according to the manufacturer's protocol. qPCR was performed using Taqman probes and Taqman Fast Advanced Master Mix from Life Technologies on a Roche LightCycler 480 II real-time PCR machine.

[0258] Experimental results for Mouse Cell Line Experiments: As shown in FIG. **20**, 6 out of 7 sgRNAs targeted to the mouse Neurog2 locus were able to induce upregulation of Neurog2 mRNA as measured by qPCR. With these results, Applicants demonstrated the utility of the Cas9 activator system for gene modulation in a mouse cellular model. The 7 sgRNa sequences are listed below:

sgRNA	SEQUENCE
sgRNA0001	TGGTTCAGTGGCTGCGTGTC
sgRNA0002	TGTTTTCTTGGTGGTATATA
sgRNA0003	ATACGATGAAAAGAATAAGC
sgRNA0004	GGGGGAGAGGGACTAAAGAA
sgRNA0005	GGGCGGGGGAAGGGTAGGTG
sgRNA0006	ATTAGATAAAGGGGGGACGG
sgRNA0007	CGGCTTTAACTGGAGTGCCT

[0259] Experimental results for Human Cell Line Experiments: As shown in FIG. **21**, sgRNAs targeted to several human genes (ASCL1, MYOD1, VEGFA, and NTF3) successfully induced upregulation of the desired mRNA using Applicants pXRP057 dCas9-VP64 construct (Konermann et al., Optical control of mammalian endogenous transcription and epigenetic states. Nature. 2013 Aug. 22; 500(7463):472-6). Applicants showed that samples transfected with the dCas9-VP64 construct, but without an sgRNA had significant changes in expression level for several genes and further characterization is to be carried out (FIG. **22**). The PAM counts (~100 to +100 bp of TSS) are indicated below:

Gene	Basal Fold Change	NGG + NAG counts
Neurog2	0.60	213
VEGFA	1.25	211
NTF3	0.94	203
ASCL1	4.19	235
MYOD1	0.90	204

Example 7: Further Studies on the Cas9 Modulator-Activator and Repressor Data

[0260] Applicants carried out studies to further develop the dCas9 activator (pXRP57) and repressor (pXRP48) (FIG. **23**). Applicants cloned plasmids pXRP57 and pXRP48 (Konermann et al., Optical control of mammalian endogenous transcription and epigenetic states. Nature. 2013 Aug. 22; 500(7463):472-6). The Cas9 activator sequence is indicated in FIG. **24** and the Cas9 repressor sequence is indicated in FIG. **25**. Additional data was collected for the activator (FIG. **26**), and repressor (FIG. **27**). A list of guide RNAs used in the experiment is listed below:

Gene	guide RNA
hSox2-1	GTGGCTGGCAGGCTGGCTCT
hSox2-2	GGCCTCCCCCGCGCGGCCGG
hSox2-3	GCCCCCTTTCATGCAAAACC
hSox2-4	GACAGCCCCCGTCACATGGA
hSox2-5	GGCAGGCGAGGAGGGGGAGG
hSox2-6	GGCGGGGCCTCCCGCGCCGC
hSox2-7	GCTGCCGGGTTTTGCATGAA
hSox2-8	GGGGCTGTCAGGGAATAAAT
hKlf4-1	GAGAGAACGAACGTGTCTGC
hKlf4-2	GAGGGTCACTCGGCGGCTCC
hKlf4-3	GCGCGCTCCACACAACTCAC
hKlf4-4	GGGGCTGTGGCCGGGGCGGT
hKlf4-5	GGCGACCGCGACAGTGGTGG
hKlf4-6	GCAAAAATAGACAATCAGCA
hKlf4-7	GAAGGATCTCGGCCAATTTG
hKlf4-8	GTGGGGGCCCAGAAGGTCCT
hNanog-1	GCCACGGCCTCCCAATTTAC
hNanog-2	GGAATATGGTTCAACAGGAA
hNanog-3	GCTGCAGAGTAACCCAGACT
hNanog-4	GCCTTGGTGAGACTGGTAGA
hNanog-5	GATTAACTGAGAATTCACAA
hNanog-6	GTGTGCCCGCCAGGAGGGGT
hNanog-7	GTTGCCTGCATAATAACATG
hNanog-8	GGAGGAAAAAATTTAAGAGG

-continued

Gene	guide RNA
hOct4-1	GGACCGGGATTGTCCAGCCA
hOct4-2	GAGTGATAAGACACCCGCTT
hOct4-3	GCAGCTGGCCATTGTGCTTA
hOct4-4	GGAGAGGGGGTCAAGCACCT
hOct4-5	GCGGGTTGGGAGTTGAAAGT
hOct4-6	GCTCCAGCCTCCTAAGTGGC
hOct4-7	GGAGGTGGGGGGAGAAACTG
hOct4-8	GGTGAAATGAGGGCTTGCGA

Example 8: CRISPR Cas9 Activator System with the Light-Inducibility of the LITE System

[0261] Applicants combined the RNA guidance of the CRISPR/Cas9 activator system with the light-inducibility of the LITE system. An inducible expression system based on CRISPR/Cas9 may make the inducible screen of many gene targets much faster and easier than previously possible, while also providing the opportunity to easily multiplex different targets by combining sgRNAs targeted to disparate genetic loci.

[0262] Materials and methods: The CasLITE system consists of 3 components: a dCas9-CIB1 fusion protein, the CRY2 PHR-VP64 construct, and an sgRNA guide sequence. Applicants synthesized 3 different versions of dCas9-CIB1: (1) dCas9-GS-NLS-CIB1 comprising the double mutant (D10A H840A) dCas9 from our pXRP057 plasmid, a glycine serine linker, SV40 nuclear localization signal, and CIB1 from LITE1.0. (2) dCas9-GS-NLS-NLS-CIB1 comprising the double mutant dCas9, glycine serine linker, 2 SV40 NLS sequences, and CIB1 from LITE1.0. (3) dCas9-GS-CIB1(mNLS d318-334) comprising the double mutant dCas9, glycine serine linker from LITE2.0, and CIB1 from LITE2.0. Vectors used in the practice of the invention are illustrated in FIGS. **28**, **29**, **30**.

[0263] Applicants selected sgRNA sequences targeted to ASCL1 and MYOD1, previously validated with a constitutive dCas9-VP64 activator, for testing the CasLITE systems in human cells. Guide sequences were cloned by the golden gate method into the px362 plasmid containing the U6 promoter upstream of a chimeric sgRNA cloning backbone.

[0264] 293FT cells were cultured in D10 medium without HEPES (10:1 ratio of DMEM high glucose w/Glutamax and sodium pyruvate: fetal bovine serum). For transfection, 100K cells in 0.5 mL culture medium were plated in each well of a 24 well plate. After 22 h in culture, each well of 293FT cells was transfected with 1.5 ug of DNA using 7.5 uL of Lipofectamine following the recommended steps of the manufacturer (Life Technologies). For all CasLITE+ sgRNA samples, 0.5 ug dCas9-CIB1 plasmid, 0.5 ug of CRY2 PHR-VP64 plasmid (LITE1.0 or LITE2.0 to match CIB1), and 0.5 ug sgRNA expression plasmid were used. Experiments with 4 sgRNAs used equal masses of each of 4 sgRNAs totaling 0.5 ug. For control samples, plasmid masses were replaced by GFP expressing plasmid as necessary. 4 h after transfection, the medium was replaced with 1 mL culture medium. 48 h after transfection excitation was started on light stimulated samples using 5 mW/cm2 475 nm blue light. 12 h after starting stimulation, all samples, including all controls, were harvested for RNA using the Macherey Nagel Nucleospin 96 RNA kit. Reverse transcription was performed with qScript cDNA supermix according to the manufacturer's protocol. qPCR was performed using Taqman probes and Taqman Fast Advanced Master Mix from Life Technologies on a Roche LightCycler 480 II real-time PCR machine.

[0265] Results: CasLITE constructs exhibited varying levels of light-inducible transcriptional activation (FIG. **31**). Though the initial functionality of the system appears to be modest, calling for repeated experimentation, these results suggest the possibility for light-inducible RNA-guided transcriptional activation.

Example 9: Cas9 Mouse Model and Validation of Mouse Embryonic Stem Cell Line

[0266] Applicants validated a mouse embryonic stem cell line that was modified to include the Cre-dependent Cas9 cassette (pCM2) into the Rosa26 locus (FIG. **32**) and obtained genotyping results for germ line transmitted founders (FIG. **33**).

[0267] To validate that the Cas9 cassette was functional, the modified mESC line was electroporated with the designated constructs in FIG. **32**. The mESC line was used for blastocyst injection into C57BL/6J recipients (performed by the DCM at MIT). Six chimeric progeny were produced and then backcrossed to the C57BL/6J background. Successful germ line transmission was identified on the basis of agouti coat color. Two of the six chimerics produced germ line transmitted pups. Genomic DNA was extracted from tissue from the Cas9 mice and Rosa26 short arm genotyping PCR was performed (FIG. **33**). A 1.5 kb band is the correct size for correctly inserted transgenes into the Rosa26 locus. These results demonstrate the successful germ line transmission of the Cre-dependent Cas9 transgene in the Rosa26 locus.

[0268] The benefit of the Rosa26 Cre-dependent Cas9 knockin line is that it can be crossed to any Cre driver line to enable Cas9 expression. Moreover, Cas9 can be activated by the delivery of Cre by viral vector or other delivery means. This enables cell type, tissue, and developmental specificity of Cas9 expression. An additional benefit is that the mouse can be used for isolation of primary cells. This would be particularly useful in the case of a primary cell type that is not amenable to the delivery of nucleic acids or cannot be cultured long enough for the utilization of viral vectors.

Example 10: RNA-Targeted CRISPR-Based Platform for Mammalian Epigenome Engineering

[0269] Applicants develop an RNA-targeted CRISPR-based platform for mammalian epigenome engineering. Two RNA-targeted CRISPR-mediated immune systems, CRISPR/Cmr loci, were recently characterized from *Pyrococcus furiosus* and *Sulfolobus solfataricus*. Based on protein homology these CRISPR/Cmr loci are part of a much larger family that exists within a diversity of bacterial and archaeal genomes. Similar to RNAi and Cas9 the interference mechanism of a CRISPR/Cmr locus is based on the homology of a short stretch of nucleotides and is thus amenable to genome-wide screens. Moreover, the homology

region to the target site is significantly increased making the potential for specificity much greater.

[0270] Applicants conduct a metagenomic analysis of the CRISPR/Cmr family, development of an in vitro assay system to identify functional loci, and characterization of the specificity and capacity for multiplexing in mammalian cells. A site-specific RNA-targeted CRISPR-based genome engineering platform (FIG. **34**) can replace the many utilities of RNAi and provide a system for perturbing RNA biology that has never before been possible.

[0271] Preliminary work with the CRISPR/Cmr locus from *P. furiosus*: Based on the extensive knowledge base on the CRISPR/Cmr locus from *P. furiosus* Applicants investigated the feasibility of expressing the Cmr genes within mammalian cells. Genomic DNA from *P. furiosus* was obtained from ATCC and used as a PCR template to amplify the six Cmr genes (Cmr1-6). Applicants also amplified another protein within the locus, Cas6. Cas6 is involved with the processing of crRNA into its mature form. Although only five Cmr genes (Cmr1,2,3,4,6) are essential Applicants considered that Cas6 may aid in troubleshooting if the five Cmr genes happened to be insufficient in the mammalian context.

[0272] Cmr1-6 and Cas6 were cloned into mammalian expression plasmids and transfected into HEK 293FT cells. For easy visualization the cloning plasmid contained an HA tag and a P2A-EGFP sequence. The P2A sequence causes a ribosomal skip resulting in two mature proteins from the same transcript. 72 hours post-transfection EGFP fluorescence was observed for each Cmr expression plasmid (FIG. **35**A), suggesting that Cmr1-6 and Cas6 are successfully being expressed. To validate that the proteins are stable within the mammalian cell environment and are also the correct size Applicants performed a western blot (FIG. **35**B). Within each lane there is a band of the expected size, suggesting that each protein is being expressed and is stable within mammalian cells. The larger second band that appears within each lane may be a result of failure of the P2A sequence to cause a ribosomal skip and thus one protein fusion is created and not two individual protein products. The band located above the predicted size is ~30 kDa larger than the expected size, which corresponds to P2A-EGFP. Applicants will confirm this by immunostaining the same blot using an anti-EGFP antibody and should see two bands per lane, one that is the size of P2A-EGFP and another larger band the size of Cmr-P2A-EGFP. The western blot results also show variable expression for the seven proteins, whereas, Cmr1, Cmr3, and Cmr6 have low expression while Cmr2, Cmr4, and Cmr5 have high expression. Applicants demonstrated that it is indeed possible to express Cmr proteins from *P. furiosus* within mammalian cells.

[0273] In this example, Applicants identify putative RNA-targeted CRISPR/Cmr loci and validate the expression of individual proteins and corresponding crRNAs in mammalian cells. Using a homology-based approach Applicants identify putative RNA-targeted CRISPR/Cmr loci across a diversity of bacterial and archaeal genomes. Cmr proteins and crRNAs from each locus are identified and validated through cloning, transfection into mammalian cells, and detection by western blot and northern blot, respectively. The methodology is as follows:

[0274] 1. Identify RNA-targeted CRISPR loci: Identification of RNA-targeted CRISPR loci from published work is the first step in understanding the essential components of a functional Type III-B CRISPR/Cmr system. Two RNA-targeted loci from two different organisms have been published and characterized to different extents.

[0275] 2. Identify putative RNA-targeted CRISPR loci across a diversity of bacterial and archaeal species: Extensive work has been done towards classifying all known CRISPR loci into phylogenetic groups and has thus resulted in high confidence databases of orthologous proteins. Using the most extensive and manually curated databases Applicants identify putative CRISPR/Cmr loci based on the presence of multiple Cmr protein orthologs. I will pick a subset that spans the diversity of CRISPR/Cmr loci for further molecular characterization in a broad attempt to sample the natural diversity.

[0276] 3. Identify Cmr proteins and crRNAs within CRISPR/Cmr loci: CRISPR/Cmr loci are well studied and many bacterial and archaeal genomes have been extensively annotated so the identification of Cmr proteins should be straightforward in these cases. However, in the absence of good annotation the problem will be more complicated. For this reason Applicants preferentially select CRISPR/Cmr loci from organisms where good annotations are available. If these strategies do not provide comprehensive coverage of the diversity CRISPR/Cmr loci Applicants use a homology-based approach combined with ORF prediction algorithms to identify the Cmr proteins. CRISPR repeat arrays have predictable structures and are located near the Cmr CRISPR associated proteins. The CRISPR array consists of a leader sequence that drives expression of the entire array of repeats and spacers creating a primary transcript that is extensively processed to make mature CRISPR RNAs (crRNA)s. Existing computational tools (CRISPRFinder) will allow for identification of the repeat arrays. Predicting the mature crRNA from each locus may not be as straightforward and may require optimization. There are general features known about mature crRNAs from Type III-B CRISPR/Cmr loci that Applicants use to predict novel crRNAs. All identified mature crRNAs from Type III-B CRISPR loci have a 5' handle that is part of the repeat sequence followed by a spacer. The 5' handle functions in the recognition of the crRNA by the Cmr complex and the spacer guides the Cmr complex to target RNA through Watson-Crick base pairing. Applicants design a panel of likely crRNA architectures that are of different total length and have 5' handles of different length as it is difficult to predict any specific unique features of novel crRNAs a priori.

[0277] 4. Clone Cmr proteins and crRNAs into mammalian expression vectors: Cmr proteins are individually cloned into two different mammalian expression vectors (FIG. **36**). The first vector (V1.0) expresses individual Cmr proteins using a CMV promoter, bovine growth hormone polyA tail sequence, and a WPRE element to maximize protein expression. The second vector (V1.2) uses the same elements as in V1.0 but will include an N-term HA tag and a C-term P2A-EGFP sequence. The HA tag enables protein detection and quantification and the P2A-EGFP enables an easy visual method for detecting expression in mammalian cells. Applicants make both versions because the

impact of adding an HA tag and a P2A peptide on Cmr proteins and how that influences correct formation of the Cmr complex is unknown. crRNAs is expressed using the human U6 promoter, which has been successfully used to express noncoding RNAs utilized for RNAi and DNA-targeted CRISPR/Cas9.

[0278] 5. Validate Cmr protein and crRNA expression: Cmr protein expression is validated by transfecting V1.2 plasmids into HEK 293FT cells and observing EGFP expression. To further validate that the protein is expressed and the correct size Applicants prepare protein lysates from transfected cells and perform a western blot using an anti-HA tag antibody. To validate crRNA expression Applicants purify small RNAs from transfected cells and probe by northern blot.

[0279] Applicants demonstrate RNA cleavage in an in vitro mammalian cell lysate assay using naturally encoded crRNAs. Applicants develop a simple mammalian cell lysate in vitro assay to rapidly validate putative CRISPR/Cmr loci. Cell lysates of mammalian cells transfected with Cmr proteins are prepared and incubated with naturally encoded crRNAs and a corresponding RNA target. Functioning CRISPR/Cmr loci are identified on the bases of correctly sized cleavage products using gel electrophoresis. The methodology is as follows:

[0280] 1. Expression of Cmr proteins in mammalian cells and preparation of cell lysates: Applicants transfect individual expression plasmids for all Cmr proteins of a species into HEK 293FT cells and wait 72 hours for robust protein expression to occur. The cells are lysed, homogenized, and aliquoted for future use. Although not all cells are transfected with each Cmr plasmid, once the lysates are prepared all Cmr proteins will be in the same mixture. This is a beneficial over the live cell model where cotransfection of seven components may be challenging. Moreover, this will give Applicants absolute control over the final concentration of the crRNA and target RNA in the reaction.

[0281] 2. Preparation of crRNAs: For each CRISPR/Cmr locus Applicants make mature crRNAs containing the endogenous spacer. Specifically Applicants utilize the spacer sequence of the crRNA nearest the leader. As was discussed previously Applicants experiment with different crRNA architectures with different 5' ends. A direct method for creating mature crRNA of a defined sequence is in vitro transcription using commercially available T7 transcription kits.

[0282] 3. In vitro transcribe RNA harboring crRNA target sequences: T7 expression plasmids are cloned to uniquely harbor sequences targeted by the endogenous spacer of each CRISPR/Cmr locus. This target will be cloned into the middle of a ~1 kb piece of RNA so that resulting cleavage products will be easily visualized on an agarose gel.

[0283] 4. Perform and optimize in vitro mammalian cell lysate cleavage experiment: Once all of the components are prepared Applicants combine and incubate the cell lysates, individual crRNAs, and target RNA followed by visualization on an agarose gel. Functional CRISPR systems are identified on the basis of cleavage products of the correct size. Certain parameters are kept in mind. Parameters for optimization are Cmr protein identity and concentration, crRNA architecture and concentration. Less significant parameters for optimization are buffer content and concentration, crRNA and RNA stability, reaction time, and RNA visualization.

[0284] Applicants demonstrate and characterize knockdown of endogenous mammalian genes using novel crRNAs. crRNAs are engineered to target endogenous mammalian genes and cotransfected with Cmr expression plasmids into mammalian cells. mRNA and protein knockdown are quantified by quantitative PCR and western blot. The multiplexing capability is investigated by targeting multiple genes at the same time. The specificity is characterized and compared to RNAi using RNA-seq. The methodology is as follows:

[0285] 1. Target crRNAs to endogenous mammalian genes: With functionally validated RNA-targeted CRISPR/Cmr loci Applicants design novel crRNAs targeting three genes that are therapeutically validated RNAi targets, namely, P53, VEGF, and CTNNB1 (Beta-catenin). Applicants pick eight target sites within each gene to help resolve any sequence-specific limitations of the technology. For different CRISPR/Cmr loci the exact target sight may differ by a few bases because of the unique required features of each crRNA but Applicants target a consistent region to aid in comparisons.

[0286] 2. Quantify and characterize mRNA knockdown: Applicants test the CRISPR/Cmr systems in HEK 293FT cells by transfecting mammalian expression plasmids for Cmr protein components and individual crRNAs. Cleaved mRNAs are rapidly degraded in the cell, whereas, the resulting RNA fragments do not have both a 5' cap and a polyA tail. Applicants quantify the extent of mRNA knockdown by performing quantitative PCR. When mRNA knockdown is observed Applicants perform a western blot for the targeted protein.

[0287] In addition to single crRNA transfection experiments Applicants add multiple crRNAs targeted to the same gene. It was observed previously that other transcriptional modulators work synergistically when targeted to multiple sites within the same region. This is also a useful strategy to identify suboptimal CRISPR/Cmr systems that do not function when using single crRNAs but can function overall and just need to be optimized for one reason or another. Applicants also add multiple crRNAs targeting different genes in the same condition to further consider multiplexing in this system. This has broad implications for pathway engineering and genome-scale screens.

[0288] 3. Characterize specificity by RNA-seq: A major motivation of this work is to develop an RNA-targeting platform with greater specificity than RNAi. Inherently there is a greater opportunity for the CRISPR/Cmr system to have better specificity because the extended complementarity to the target site. However, each base pair within the guide may not be necessary or there may be some tolerance to mismatches. To understand these characteristics of the CRISPR/Cmr system Applicants will use an HEK 293T derived cell line that expresses a single copy of EGFP. Applicants target EGFP for knockdown using 24 unique crRNAs. Using an HEK/GFP cell line makes the off-target analysis straightforward because EGFP may be considered an innocuous protein that has no influence on cellular processes so any altered transcripts are likely off-target effects. Results are compared to an siRNA targeting EGFP with

known off-target effects. Due to the extended base pairing capacity of the CRISPR/Cmr complex Applicants expect the off-targets to be reduced. The tolerance to mismatches is also explored and this will be important for future development of the CRISPR/Cmr platform.

[0289] Given that an RNA-targeted CRISPR/Cmr system functions in mammalian cells Applicants initially pursue two applications: genome-scale screens and a catalytically inactive RNA-binding platform. A genome-scale RNAi screen is a powerful method for probing biology. However, high off-target effects severely limit the approach. A platform with higher specificity could either replace RNAi genomic-screens or provide an orthogonal approach that would aid in identifying true positives. An RNA-targeted CRISPR/Cmr platform is amenable to genomic screens because each target is programed by only a few nucleotides, whereby, a complex library could easily be synthesized and delivered.

[0290] A catalytically inactive RNA-binding platform is an enabling technology for the development of effector complexes that aid in the probing of RNA. Applicants identify the catalytically active components of the Cmr complex and mutate the specific residues responsible. This has been done successfully in a number of cases, e.g. this was done for CRISPR/Cas9 to create a nickase and a transcriptional modulator. Utilizing this platform Applicants develop a technology to detect and image RNA transcripts inside the cell. This has applications for understanding the influence of RNA localization on cellular processes and in vivo detection and quantification of RNA. Alternatively Applicants add effector molecules onto an RNA-binding platform towards probing and understanding areas of RNA biology that are not well understood, namely RNA editing and epigenetics.

[0291] While preferred embodiments of the present invention have been shown and described herein, it will be obvious to those skilled in the art that such embodiments are provided by way of example only. Numerous variations, changes, and substitutions will now occur to those skilled in the art without departing from the invention. It should be understood that various alternatives to the embodiments of the invention described herein may be employed in practicing the invention.

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SEQUENCE LISTING

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1. (canceled)

2. A method for editing a eukaryotic cell, comprising delivering an engineered CRISPR-Cas system into the eukaryotic cell, wherein the engineered CRISPR-Cas system comprises:

(a) a Cas9 protein or a polynucleotide encoding the Cas9 protein, wherein the Cas9 protein is *S. pyogenes* Cas9 and is fused with at least one nuclear localization signal (NLS) and at least one heterologous protein domain;

(b) a CRISPR-Cas system chimeric RNA or a polynucleotide encoding the chimeric RNA, wherein the chimeric RNA comprises a guide sequence capable of hybridizing to a target sequence adjacent to a protospacer adjacent motif (PAM) in a genomic locus of interest of the eukaryotic cell, a tracr-mate sequence capable of hybridizing to a tracr sequence, and a tracr sequence comprising 40 or more nucleotides in length;

wherein a CRISPR complex comprising the Cas9 protein and the chimeric RNA is formed in the eukaryotic cell, and wherein the guide sequence directs sequence-specific binding of the CRISPR complex to the target sequence adjacent to the PAM in the genomic locus of interest of the eukaryotic cell.

3. The method of claim **2**, wherein the PAM is NGG.

4. The method of claim **2**, wherein the tracr sequence comprises 50 or more nucleotides in length.

5. The method of claim **2**, wherein the chimeric RNA further comprises a poly-U sequence.

6. The method of claim **2**, wherein the chimeric RNA is encoded by SEQ ID NO:27.

7. The method of claim **2**, wherein the chimeric RNA comprises one or more modified nucleotides.

8. The method of claim **2**, wherein the chimeric RNA comprises one or more methylated nucleotides or nucleotide analogs.

9. The method of claim **2**, wherein the NLS is independently selected from the group consisting of PKKKRKV, KRPAATKKAGQAKKKK, PAAKRVKLD, RQRRNELKRSP, NQSSNFGPMKGGNFGGRSSGPYGGGGQYFAKPRNQGGY, RMRIZFKNKGKDTAELRRRRVEVSVELRKAKKDEQILKRRNV, VSRKRPRP, PPKKARED, PQPKKKPL, SALIKKKKKMAP, DRLRR, PKQKKRK, RKLKKKIKKL, REKKKFLKRR, KRKGDEVDGVDEVAKKKSKK, and RKCLQAGMNLEARKTKK

10. The method of claim **2**, wherein the NLS comprises PKKKRKV.

11. The method of claim **2**, wherein the Cas9 protein is a nickase comprising D10A mutation.

12. The method of claim **2**, wherein the Cas9 protein is a nickase comprising at least one of H840A, N854A, or N863A mutation.

13. The method of claim **2**, wherein the Cas9 protein comprises D10A mutation and at least one of H840A, N854A, or N863A mutation, and wherein the Cas9 protein substantially lacks DNA cleavage activity.

14. The method of claim **2**, wherein the heterologous protein domain is selected from the group consisting of epitope tags, reporter sequences, and protein domains having one or more of the following activities: methylase activity, demethylase activity, transcription activation activity, transcription repression activity, transcription release factor activity, histone modification activity, RNA cleavage activity, or nucleic acid binding activity.

15. The method of claim **2**, wherein the polynucleotide encoding the Cas9 protein is codon-optimized for expression in the eukaryotic cell.

16. The method of claim **2**, wherein the polynucleotide encoding the Cas9 protein comprises a polyadenylation signal.

17. The method of claim **2**, wherein the CRISPR-Cas system is comprised in a liposome for delivery.

18. The method of claim **2**, wherein gene expression associated with the genomic locus of interest is altered in the eukaryotic cell.

19. A method for editing a eukaryotic cell, comprising delivering an engineered CRISPR-Cas system into the eukaryotic cell, wherein the engineered CRISPR-Cas system comprises:

(a) a Cas9 protein or a polynucleotide encoding the Cas9 protein, wherein the Cas9 protein is *S. pyogenes* Cas9 and is fused with at least one nuclear localization signal (NLS) and at least one heterologous protein domain having methylase activity, demethylase activity, transcription activation activity, transcription repression activity, transcription release factor activity, histone modification activity, RNA cleavage activity, or nucleic acid binding activity;

(b) a CRISPR-Cas system chimeric RNA or a polynucleotide encoding the chimeric RNA, wherein the chimeric RNA comprises NNNNNNNNNNNNNNNNNNNNGUUUUAGAGCUAGAAAUAGCAAGUUAAAAUAAGG CUAGUCCGUUAUCA, wherein NNNNNNNNNNNNNNNNNNNN is a guide sequence capable of hybridizing to a target sequence adjacent to a protospacer adjacent motif (PAM) in a genomic locus of interest of the eukaryotic cell;

wherein a CRISPR complex comprising the Cas9 protein and the chimeric RNA is formed in the eukaryotic cell, and wherein the guide sequence directs sequence-specific binding of the CRISPR complex to the target sequence adjacent to the PAM in the genomic locus of interest of the eukaryotic cell.

20. A method for editing a eukaryotic cell, comprising delivering an engineered CRISPR-Cas system into the eukaryotic cell, wherein the engineered CRISPR-Cas system comprises:

(a) a polynucleotide encoding a Cas9 protein, wherein the Cas9 protein is *S. pyogenes* Cas9 and is fused with at least one nuclear localization signal (NLS) and at least one heterologous protein domain having methylase activity, demethylase activity, transcription activation activity, transcription repression activity, transcription release factor activity, histone modification activity, RNA cleavage activity, or nucleic acid binding activity, wherein the polynucleotide is codon-optimized for expression in the eukaryotic cell and comprises a polyadenylation signal;

(b) a CRISPR-Cas system chimeric RNA comprising NNNNNNNNNNNNNNNNNNNNGUUUUAGAGCUAGAAAUAGCAAGUUAAAAUAAGG CUAGUCCGUUAUCA, wherein NNNNNNNNNNNNNNNNNNNN is a guide sequence capable of hybridizing to a target sequence adjacent to a protospacer adjacent motif (PAM) in a genomic locus of interest of the eukaryotic cell;

wherein a CRISPR complex comprising the Cas9 protein and the chimeric RNA is formed in the eukaryotic cell, wherein the guide sequence directs sequence-specific binding of the CRISPR complex to the target sequence adjacent to the PAM in the genomic locus of interest, thereby altering gene expression associated with the genomic locus of interest in the eukaryotic cell.

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