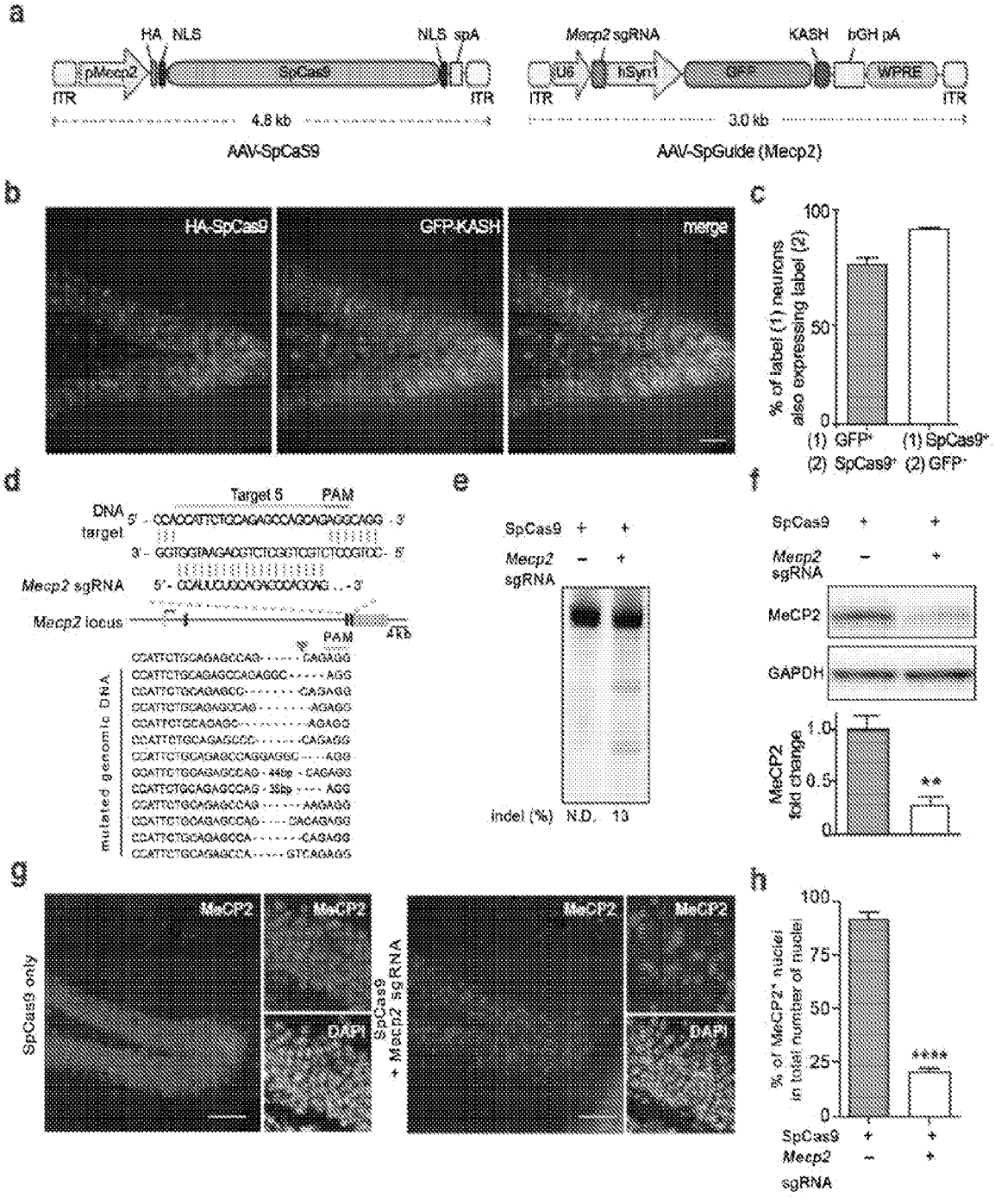

a
HA NLS
NLS spA
pMecp2
SpCas9
ITR
ITR
4.8 kb
AAV-SpCaS9
Mecp2 sgRNA
KASH bGH pA
U6
hSyn1
GFP
WPRE
ITR
ITR
3.0 kb
AAV-SpGuide (Mecp2)
b
HA-SpCas9
GFP-KASH
merge
c
% of label (1) neurons also expressing label (2)
100
50
0
(1) GFP+
(1) SpCas9+
(2) SpCas9+
(2) GFP-
d
Target 5
PAM
DNA
target
Mecp2 sgRNA
Mecp2 locus
PAM
1Kb
mutated genomic DNA
e
SpCas9 + +
Mecp2 sgRNA – +
indel (%) N.D. 13
f
SpCas9 + +
Mecp2 sgRNA – +
MeCP2
GAPDH
MeCP2 fold change
1.0
0.5
0
**
g
SpCas9 only
MeCP2
DAPI
SpCas9 + Mecp2 sgRNA
MeCP2
DAPI
h
% of MeCP2+ nuclei in total number of nuclei
100
75
50
25
0
****
SpCas9 + +
Mecp2 – +
sgRNA

FIG. 1A-H

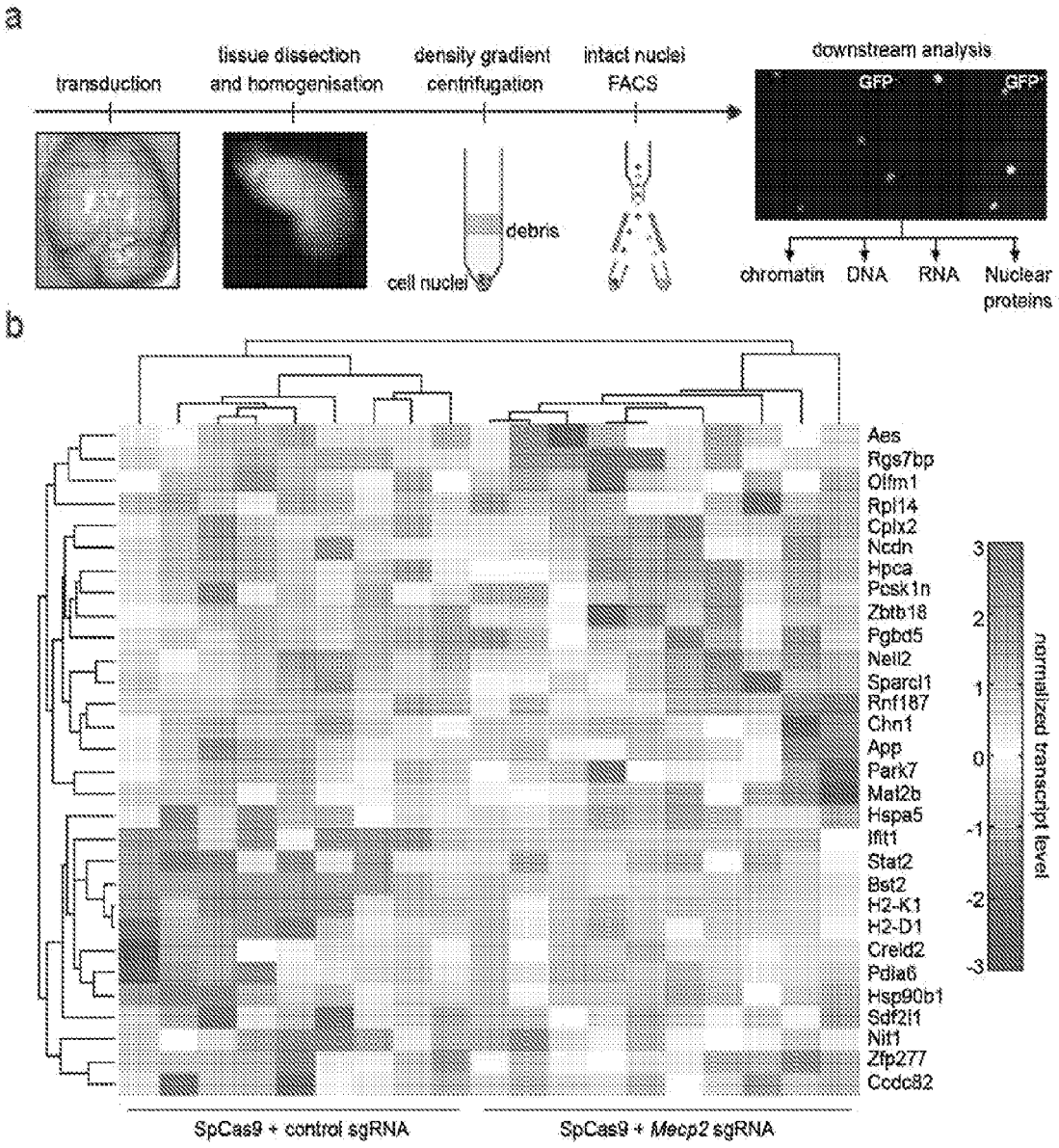

a
transduction
tissue dissection and homogenisation
density gradient centrifugation
intact nuclei FACS
downstream analysis
GFP
GFP
debris
cell nuclei
chromatin
DNA
RNA
Nuclear proteins
b
Aes
Rgs7bp
Olfm1
Rpl14
Cplx2
Ncdn
Hpca
Pcsk1n
Zbtb18
Pgbd5
Nell2
Sparcl1
Rnf187
Chn1
App
Park7
Mal2b
Hspa5
Ifit1
Stat2
Bst2
H2-K1
H2-D1
Creld2
Pdia6
Hsp90b1
Sdf2l1
Nit1
Zfp277
Ccdc82
3
2
1
0
-1
-2
-3
normalized transcript level
SpCas9 + control sgRNA
SpCas9 + Mecp2 sgRNA

FIG. 2A-B

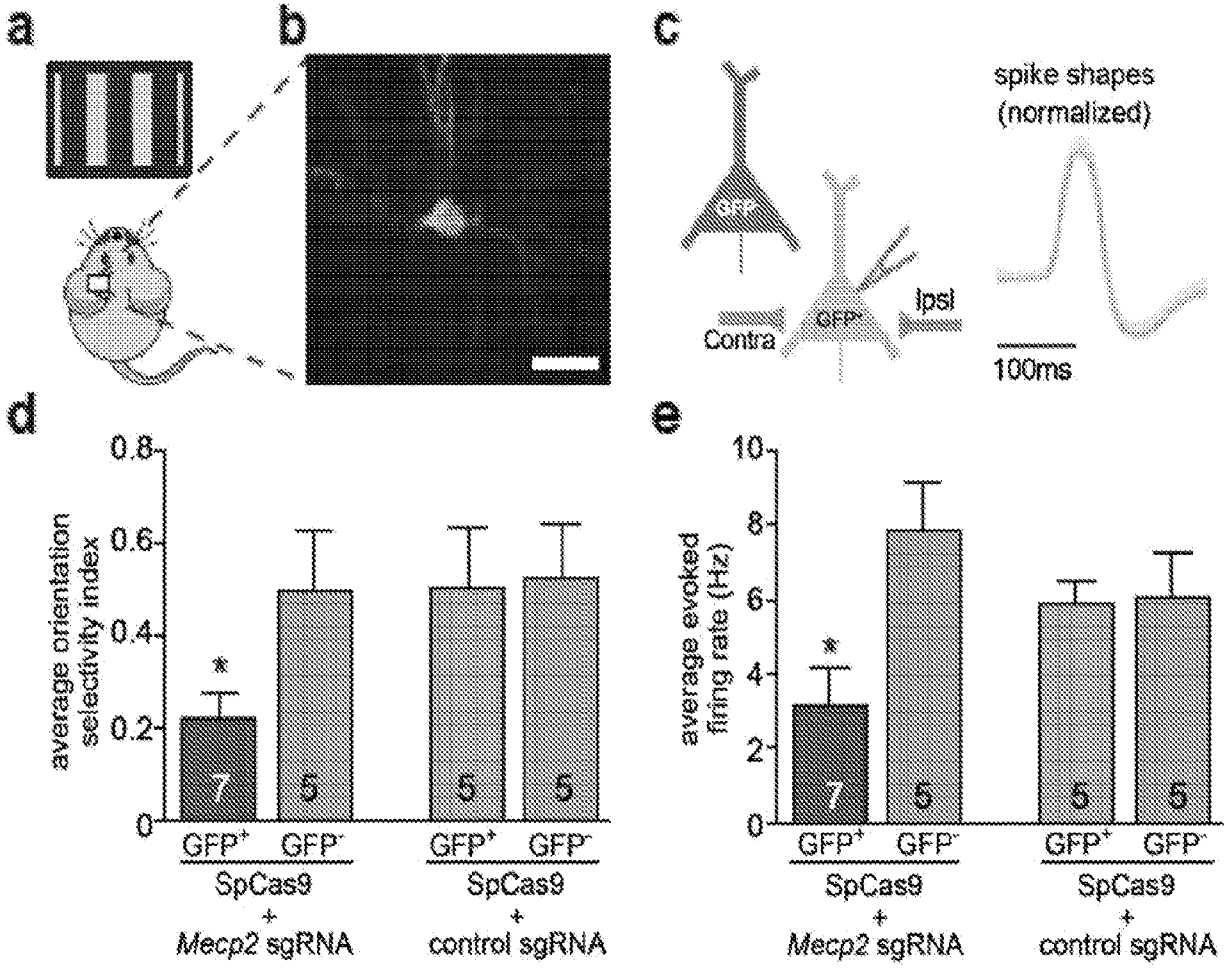

a
b
c
spike shapes
(normalized)
GFP+
GFP-
Contra
Ipsi
100ms
d
e
average orientation selectivity index
0.8
0.6
0.4
0.2
0
*
7
5
5
5
GFP+
GFP-
GFP+
GFP-
SpCas9
+
Mecp2 sgRNA
SpCas9
+
control sgRNA
average evoked firing rate (Hz)
10
8
6
4
2
0
*
7
5
5
5
GFP+
GFP-
GFP+
GFP-
SpCas9
+
Mecp2 sgRNA
SpCas9
+
control sgRNA

FIG. 3A-E

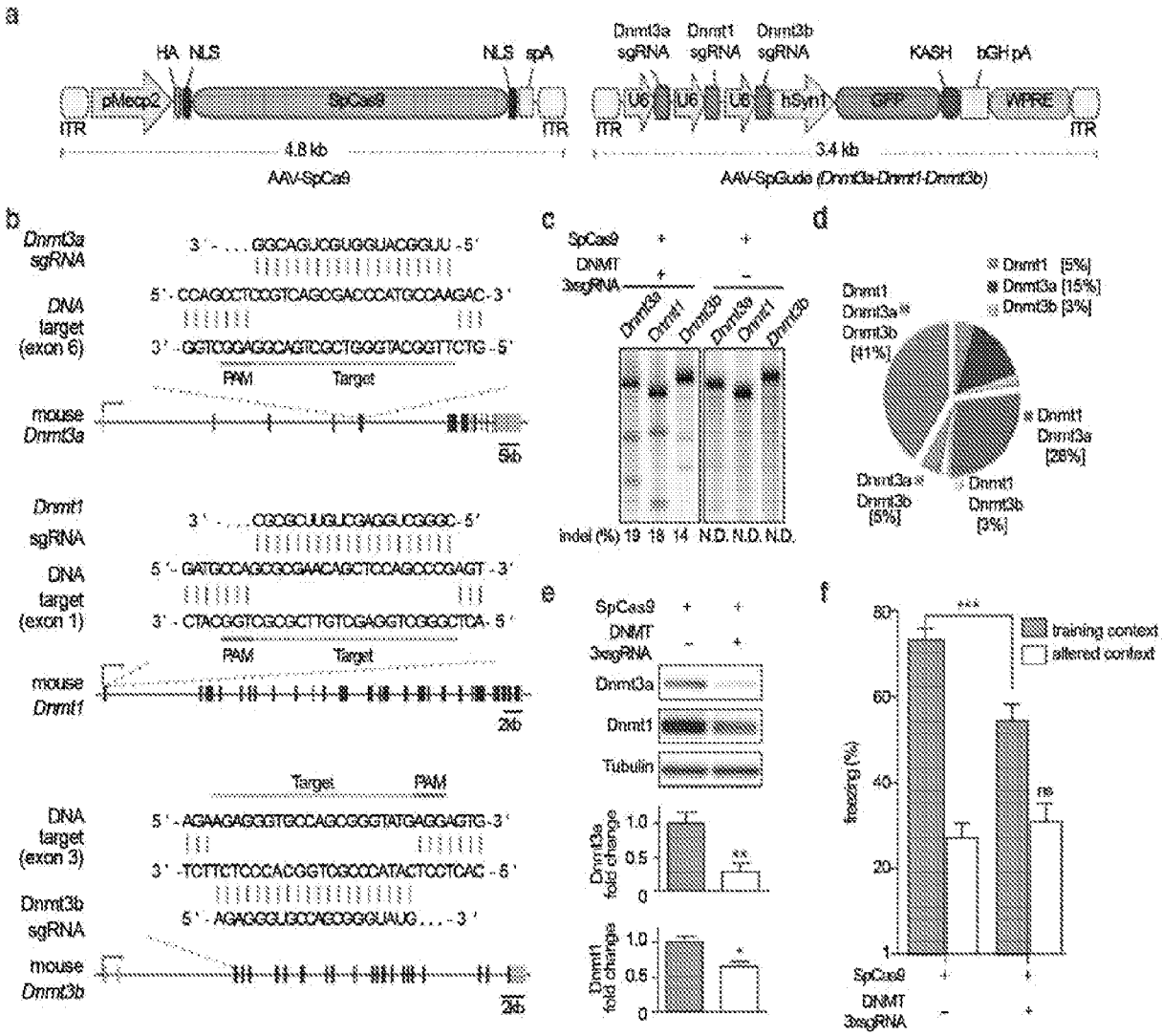

a
HA NLS
NLS spA
pMecp2
SpCas9
ITR
ITR
4.8 kb
AAV-SpCas9
Dnmt3a sgRNA
Dnmt1 sgRNA
Dnmt3b sgRNA
KASH
bGH pA
U6
U6
U6
hSyn1
GFP
WPRE
ITR
ITR
3.4 kb
AAV-SpGuide (Dnmt3a-Dnmt1-Dnmt3b)
b
Dnmt3a sgRNA
3' - . . . GGCAGUCGUGGUACGGUU - 5'
DNA target (exon 6)
5' - CCAGCCTCCGTCAGCGACCCATGCCAAGAC - 3'
3' - GGTCGGAGGCAGTCGCTGGGTACGGTTCTG - 5'
PAM
Target
mouse Dnmt3a
3kb
Dnmt1 sgRNA
3' - . . . CGCGCUUGUCGAGGUCGGGC - 5'
DNA target (exon 1)
5' - GATGCCAGCGCGAACAGCTCCAGCCCGAGT - 3'
3' - CTACGGTCGCGCTTGTCGAGGTCGGGCTCA - 5'
PAM
Target
mouse Dnmt1
2kb
Target
PAM
DNA target (exon 3)
5' - AGAAGAGGGTGCCAGCGGGTATGAGGAGTG - 3'
3' - TCTTCTCCCACGGTCGCCCATACTCCTCAC - 5'
Dnmt3b sgRNA
5' - AGAGGGUGCCAGCGGGUAUG . . . - 3'
mouse Dnmt3b
2kb
c
SpCas9
DNMT 3xsgRNA
Dnmt3a
Dnmt1
Dnmt3b
Dnmt3a Dnmt1
Dnmt3b Dnmt1
indel (%) 19 18 14 N.D. N.D. N.D.
d
Dnmt1 [5%]
Dnmt3a [15%]
Dnmt3b [3%]
Dnmt1 Dnmt3a Dnmt3b [41%]
Dnmt1 Dnmt3a [28%]
Dnmt3a Dnmt3b [5%]
Dnmt1 Dnmt3b [3%]
e
SpCas9
DNMT 3xsgRNA
Dnmt3a
Dnmt1
Tubulin
Dnmt3a fold change
Dnmt1 fold change
f
training context
altered context
freezing (%)
ns
SpCas9
DNMT 3xsgRNA

FIG. 4A-F

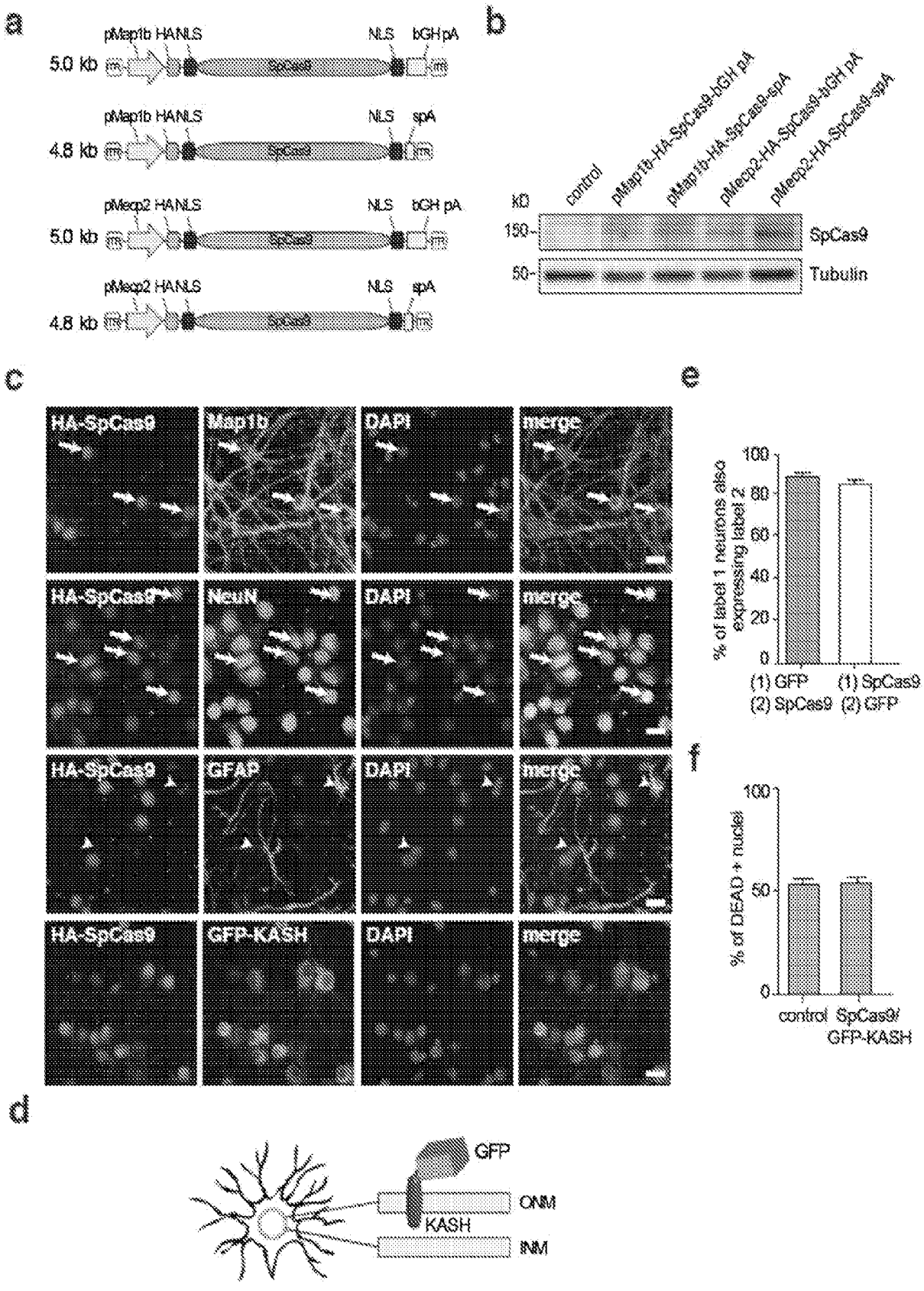

a
pMap1b HA NLS
NLS bGH pA
5.0 kb
SpCas9
pMap1b HA NLS
NLS spA
4.8 kb
SpCas9
pMecp2 HA NLS
NLS bGH pA
5.0 kb
SpCas9
pMecp2 HA NLS
NLS spA
4.8 kb
SpCas9
b
kD
control
pMap1b-HA-SpCas9-bGH pA
pMap1b-HA-SpCas9-spA
pMecp2-HA-SpCas9-bGH pA
pMecp2-HA-SpCas9-spA
150-
SpCas9
50-
Tubulin
c
HA-SpCas9
Map1b
DAPI
merge
HA-SpCas9
NeuN
DAPI
merge
HA-SpCas9
GFAP
DAPI
merge
HA-SpCas9
GFP-KASH
DAPI
merge
e
% of label 1 neurons also expressing label 2
100
80
60
40
20
0
(1) GFP
(2) SpCas9
(1) SpCas9
(2) GFP
f
% of DEAD + nuclei
100
50
0
control
SpCas9/ GFP-KASH
d
GFP
ONM
KASH
INM

FIG. 5A-F

a

	Mecp2 target sequence	PAM
Target 1	CTGGGAGAGGGAGCCCCTCC	AGG
Target 2	AAAGGTGGGAGACACCTCCT	TGG
Target 3	TCCAACCTTCAGGCAAGGTG	GGG
Target 4	AGGAAGTCTGGCCGATCTGC	TGG
Target 5	CCATTCTGCAGAGCCAGCAG	AGG
Target 6	CTCTGAGGCCCTGGAGATCC	TGG

b

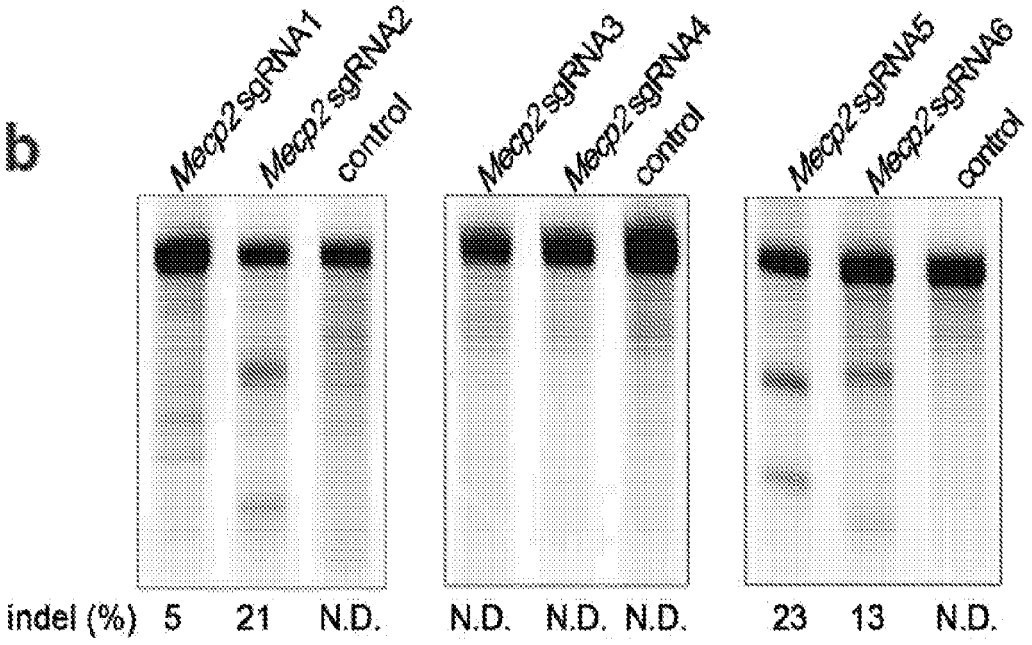


FIG. 6A-B

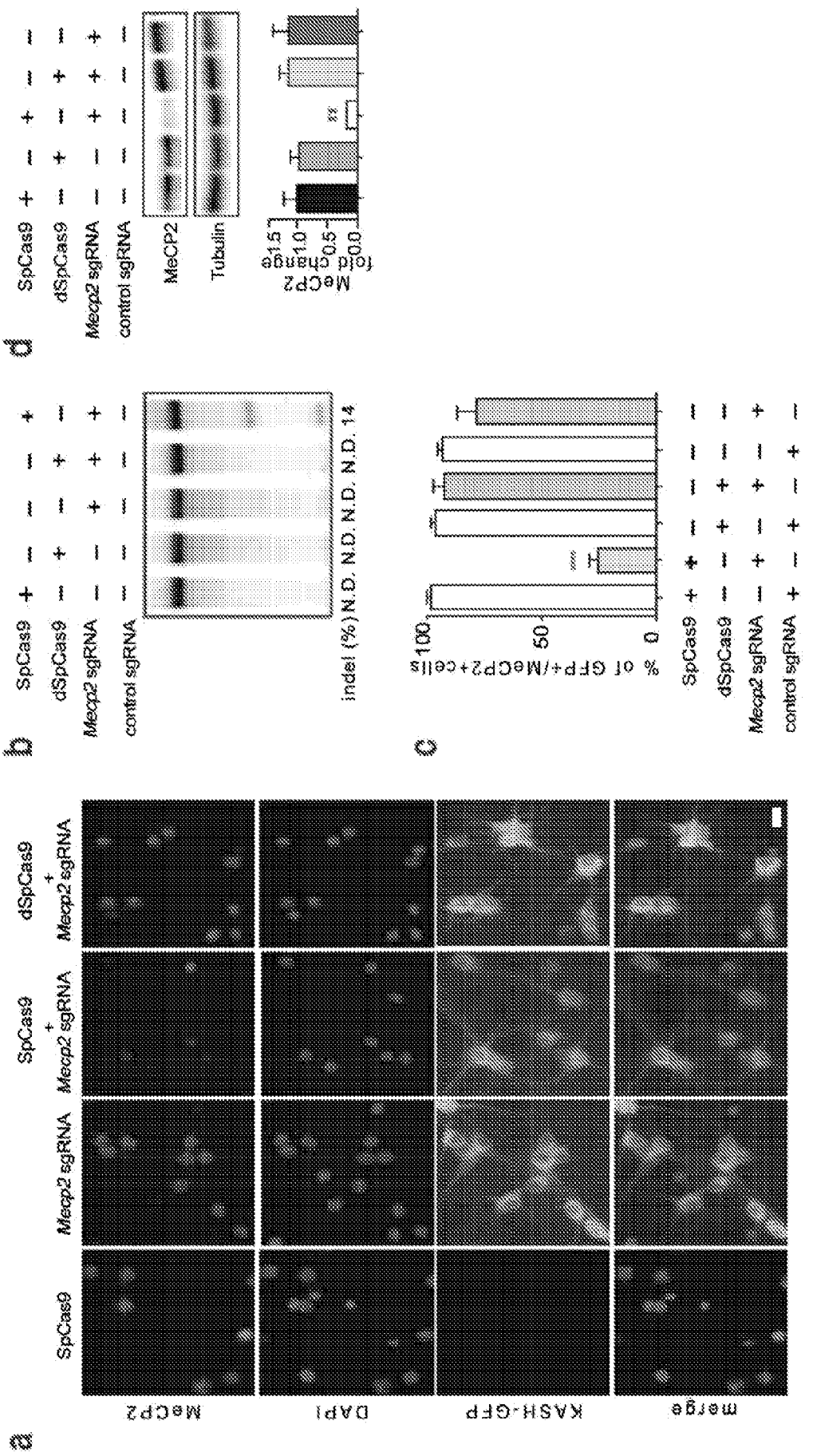

a
SpCas9
Mecp2 sgRNA
SpCas9 + Mecp2 sgRNA
dSpCas9 + Mecp2 sgRNA
MeCP2
DAPI
KASH-GFP
merge
b
SpCas9
dSpCas9
Mecp2 sgRNA
control sgRNA
indel (%) N.D. N.D. N.D. N.D. 14
c
% of GFP+/MeCP2+ cells
100
50
0
SpCas9
dSpCas9
Mecp2 sgRNA
control sgRNA
d
SpCas9
dSpCas9
Mecp2 sgRNA
control sgRNA
MeCP2
Tubulin
MeCP2 fold change
1.5
1.0
0.5
0.0

FIG. 7A-D

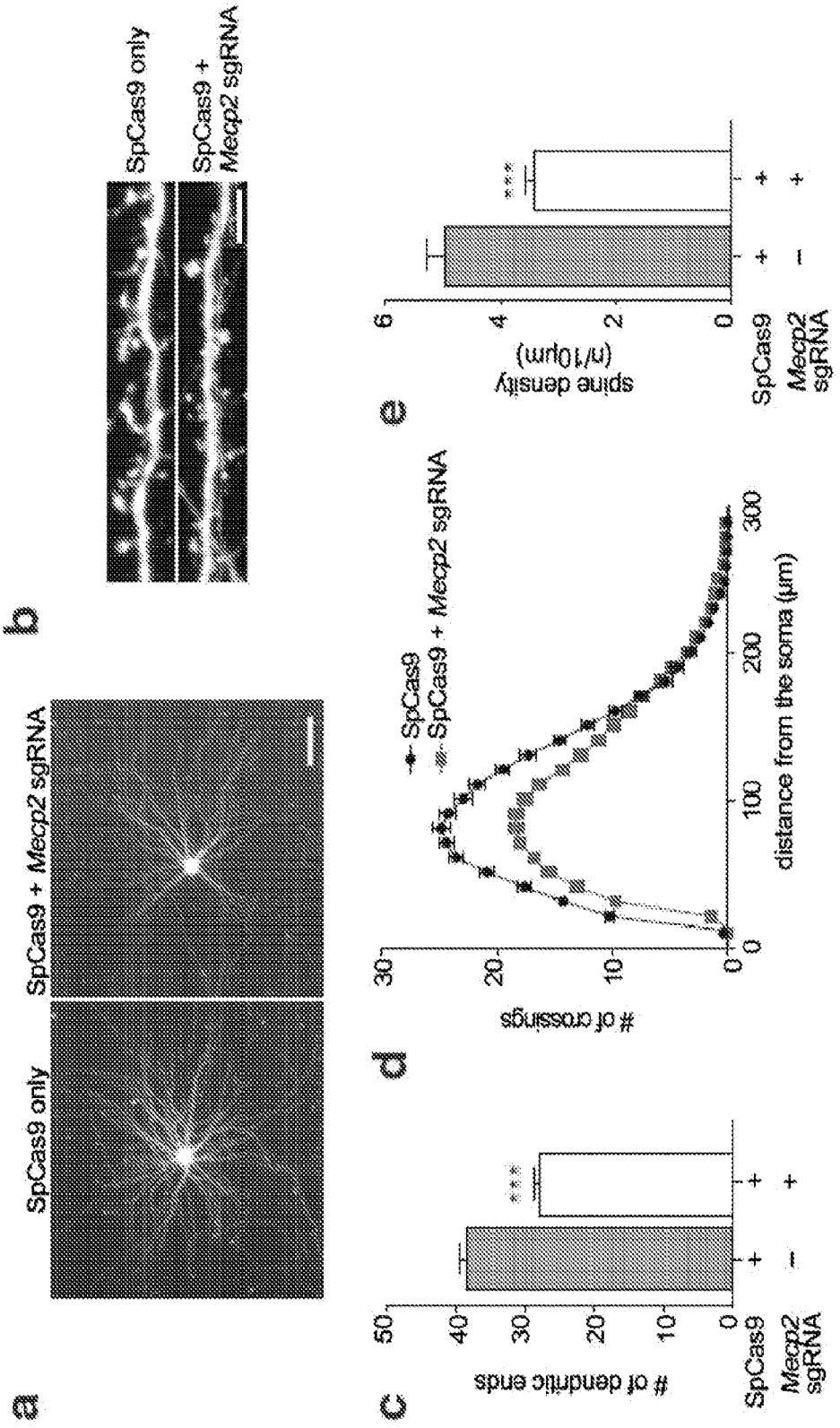

a
SpCas9 only
SpCas9 + Mecp2 sgRNA
b
SpCas9 only
SpCas9 + Mecp2 sgRNA
c
# of dendritic ends
***
SpCas9
Mecp2 sgRNA
d
SpCas9
SpCas9 + Mecp2 sgRNA
# of crossings
distance from the soma (μm)
e
spine density (n/10μm)
***
SpCas9
Mecp2 sgRNA

FIG. 8A-E

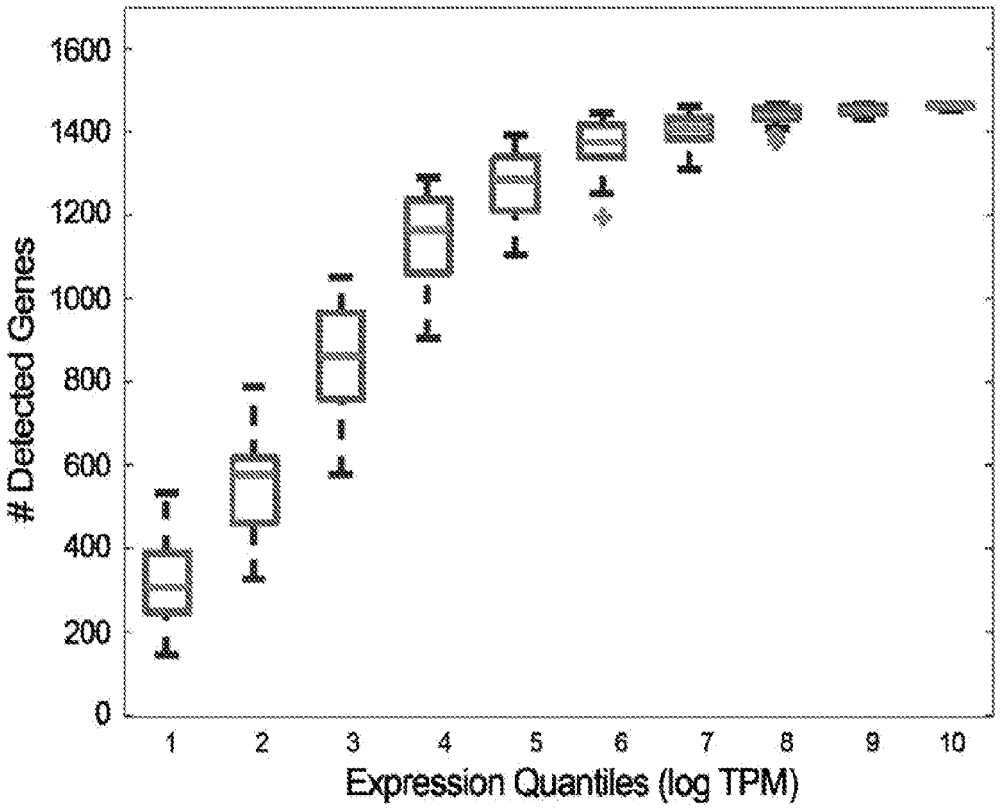

1600
1400
1200
1000
800
600
400
200
0
Detected Genes
1
2
3
4
5
6
7
8
9
10
Expression Quantiles (log TPM)

FIG. 9

a

target	target sequence	PAM
Dnmt3a	TTGGCATGGGTCGCTGACGG	AGG
Dnmt1	CGGGCTGGAGCTGTTCGCGC	TGG
Dnmt3b	AGAGGGTGCCAGCGGGTATG	AGG

b

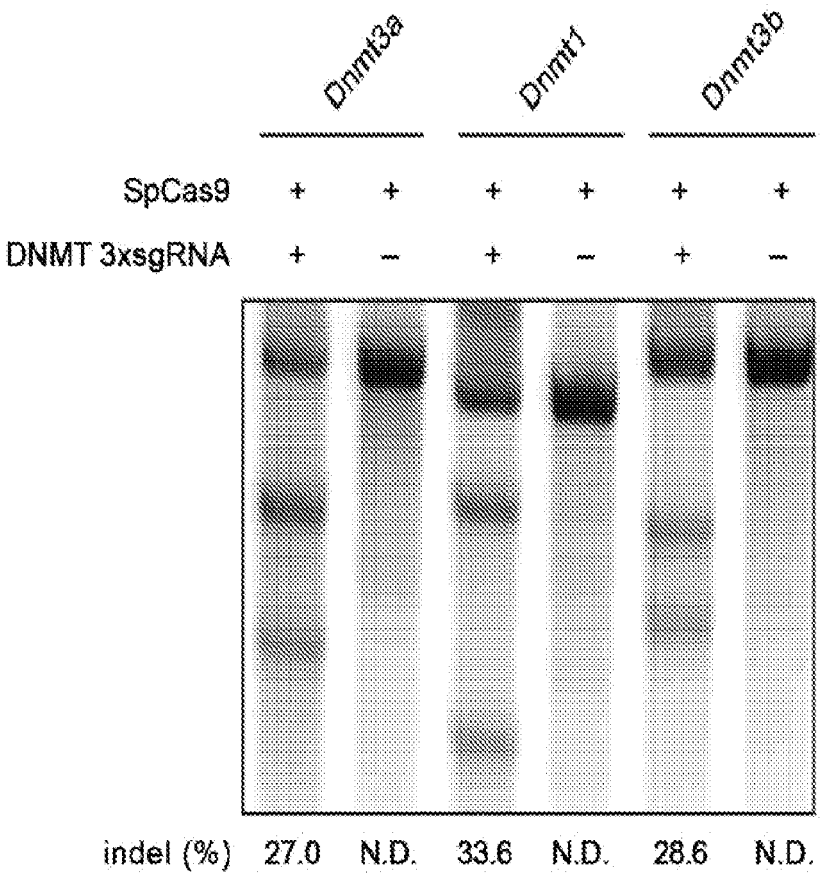


FIG. 10A-B

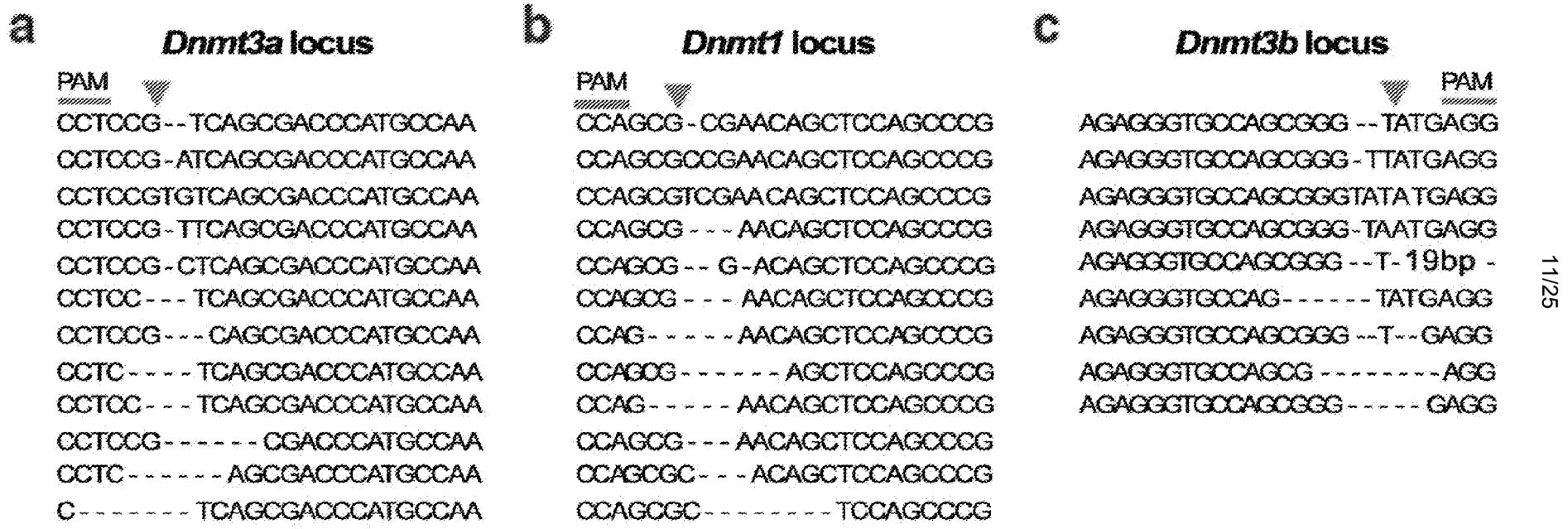

a
Dnmt3a locus
PAM
CCTCCG--TCAGCGACCCATGCCAA
CCTCCG-ATCAGCGACCCATGCCAA
CCTCCGTGTCAGCGACCCATGCCAA
CCTCCG-TTCAGCGACCCATGCCAA
CCTCCG-CTCAGCGACCCATGCCAA
CCTCC---TCAGCGACCCATGCCAA
CCTCCG---CAGCGACCCATGCCAA
CCTC----TCAGCGACCCATGCCAA
CCTCC---TCAGCGACCCATGCCAA
CCTCCG------CGACCCATGCCAA
CCTC------AGCGACCCATGCCAA
C-------TCAGCGACCCATGCCAA
b
Dnmt1 locus
PAM
CCAGCG-CGAACAGCTCCAGCCCG
CCAGCGCCGAACAGCTCCAGCCCG
CCAGCGTCGAACAGCTCCAGCCCG
CCAGCG---AACAGCTCCAGCCCG
CCAGCG--G-ACAGCTCCAGCCCG
CCAGCG---AACAGCTCCAGCCCG
CCAG-----AACAGCTCCAGCCCG
CCAGCG------AGCTCCAGCCCG
CCAG-----AACAGCTCCAGCCCG
CCAGCG---AACAGCTCCAGCCCG
CCAGCGC---ACAGCTCCAGCCCG
CCAGCGC-------TCCAGCCCG
c
Dnmt3b locus
PAM
AGAGGGTGCCAGCGGG--TATGAGG
AGAGGGTGCCAGCGGG-TTATGAGG
AGAGGGTGCCAGCGGGTATATGAGG
AGAGGGTGCCAGCGGG-TAATGAGG
AGAGGGTGCCAGCGGG--T-19bp-
AGAGGGTGCCAG------TATGAGG
AGAGGGTGCCAGCGGG--T--GAGG
AGAGGGTGCCAGCG--------AGG
AGAGGGTGCCAGCGGG-----GAGG

FIG. 11A-C

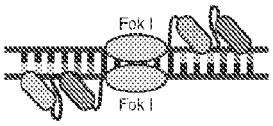
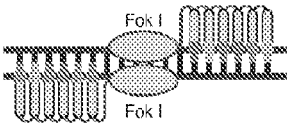
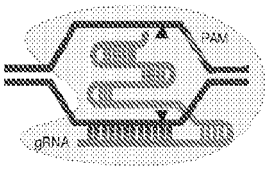
	Zinc Finger Nucleases	TALE Nucleases	CRISPR-Cas9
			
Recognition site	Typically 9 to 18 bp per ZFP, 18 to 36 bp per ZFN pair	Typically 14 to 20 bp per TALE, 28 to 40bp per TALEN	22bp (20bp guide sequence + 2bp PAM sequence); up to 44 bp for double nicking
Specificty	Small number of positional mismatches tolerated	Small number of positional mismatches tolerated	Positional and multiple consecutive mismatches tolerated
Targeting constraints	Difficult to target non-G-rich sequences	5' targeted base must be a T	Targeted sequence must precede a PAM
Ease of engineering	Difficult, may require substantial protein engineering	Moderate, requires complex molecular cloning methods	Easily re-targeted using standard cloning procedures and oligo synthesis
Immunogenicity	Likely low, as ZFs are based on human transcription factor protein scaffold	Unknown, protein derived from the bacteria *Xanthomonas sp.*	Unknown, protein derived from various bacterial species
Ease of *ex vivo* delivery	Relatively easy through methods such as electroporation and lentiviral transduction	Relatively easy through methods such as electroporation and lentiviral transduction	Relatively easy through methods such as electroporation and lentiviral transduction
Ease of *in vivo* delivery	Relatively easy due to small size of ZFN expression casettes, allows use in a variety of viral vectors	Difficult due to the large size of each TALEN and repetitive nature of DNA encoding TALENs, leading to unwanted recombination events	Moderate: The commonly used Cas9 from *S. pyogenes* may be large enough to impose delivery problems, but other, smaller orthologoues exist
Ease of multiplexing	Low	Low	High

FIG. 12

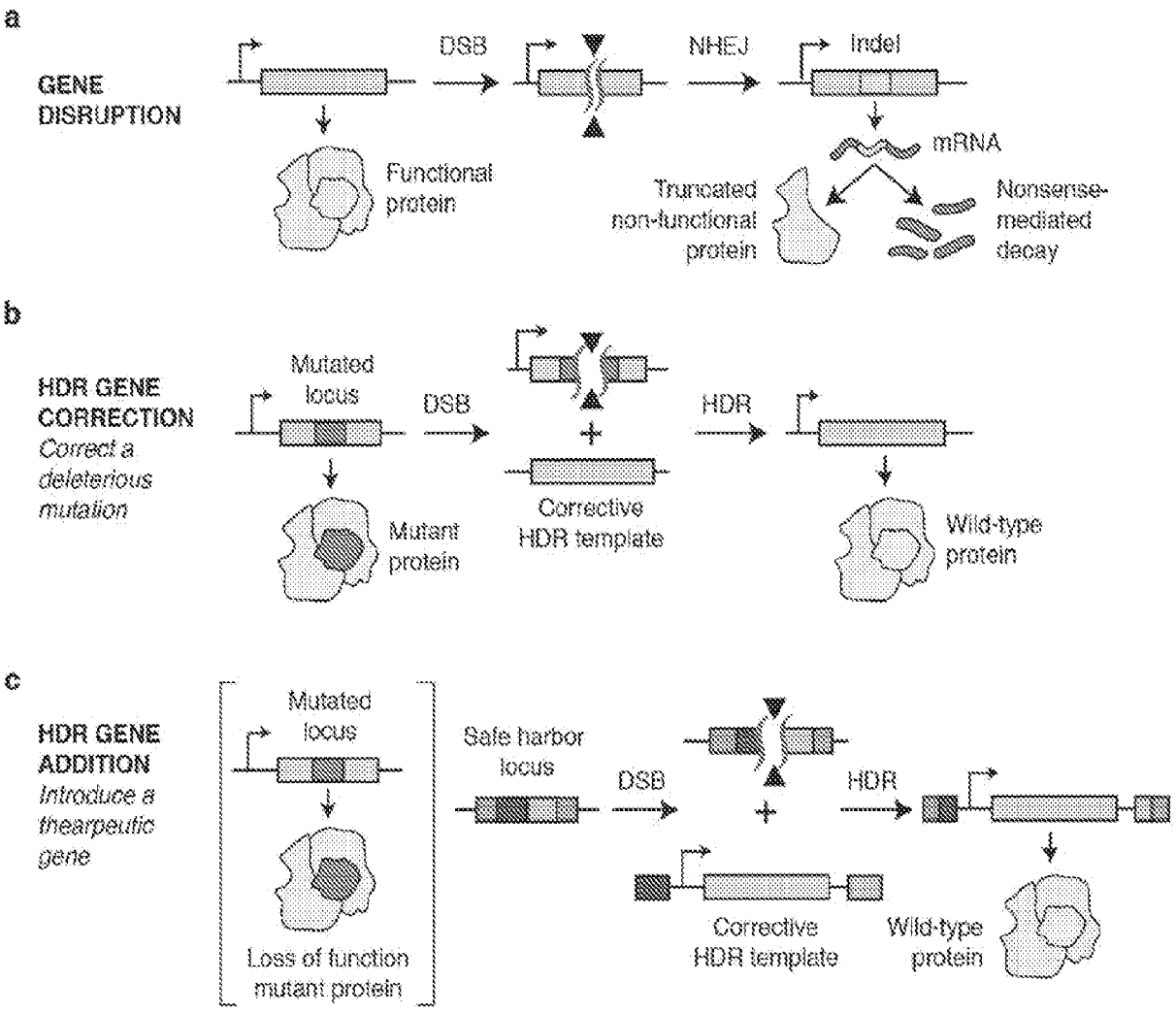

a
GENE DISRUPTION
DSB
NHEJ
Indel
mRNA
Functional protein
Truncated non-functional protein
Nonsense-mediated decay
b
HDR GENE CORRECTION
Correct a deleterious mutation
Mutated locus
DSB
HDR
Mutant protein
Corrective HDR template
Wild-type protein
c
HDR GENE ADDITION
Introduce a thearpeutic gene
Mutated locus
Loss of function mutant protein
Safe harbor locus
DSB
HDR
Corrective HDR template
Wild-type protein

FIG. 13A-C

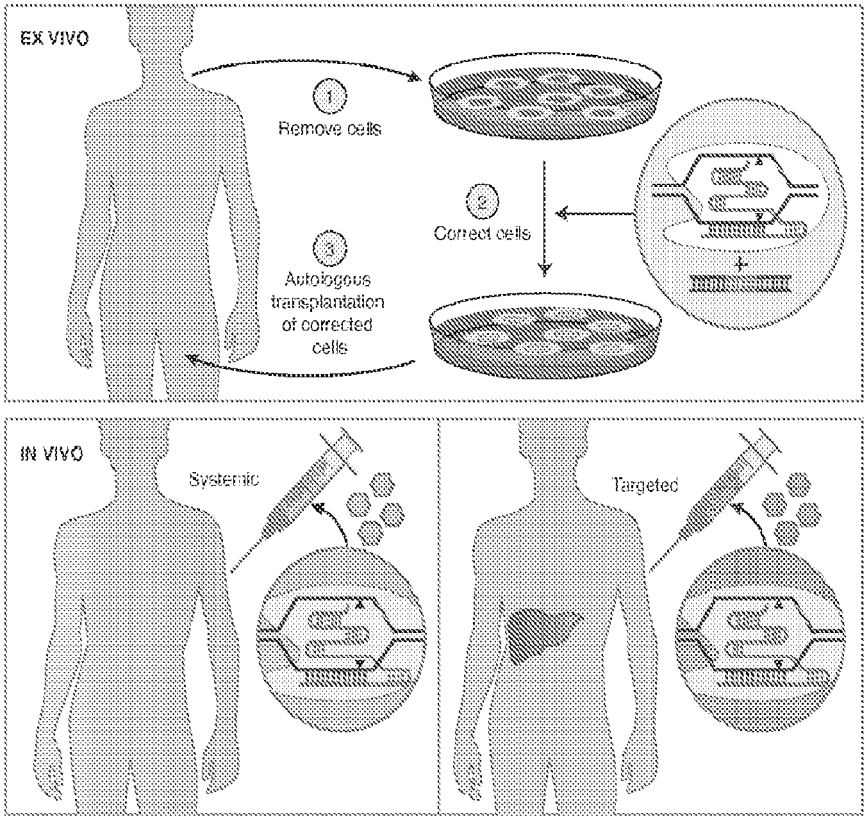
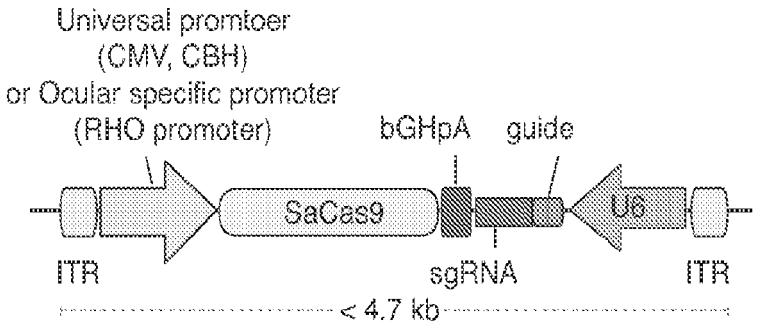

EX VIVO
1
Remove cells
2
Correct cells
3
Autologous transplantation of corrected cells
IN VIVO
Systemic
Targeted

FIG. 14



CMV: cytomegalovirus promoter
CBH: CMV Beta-actin Hybrid promoter
RHO: Rhodopsin promoter

FIG. 15

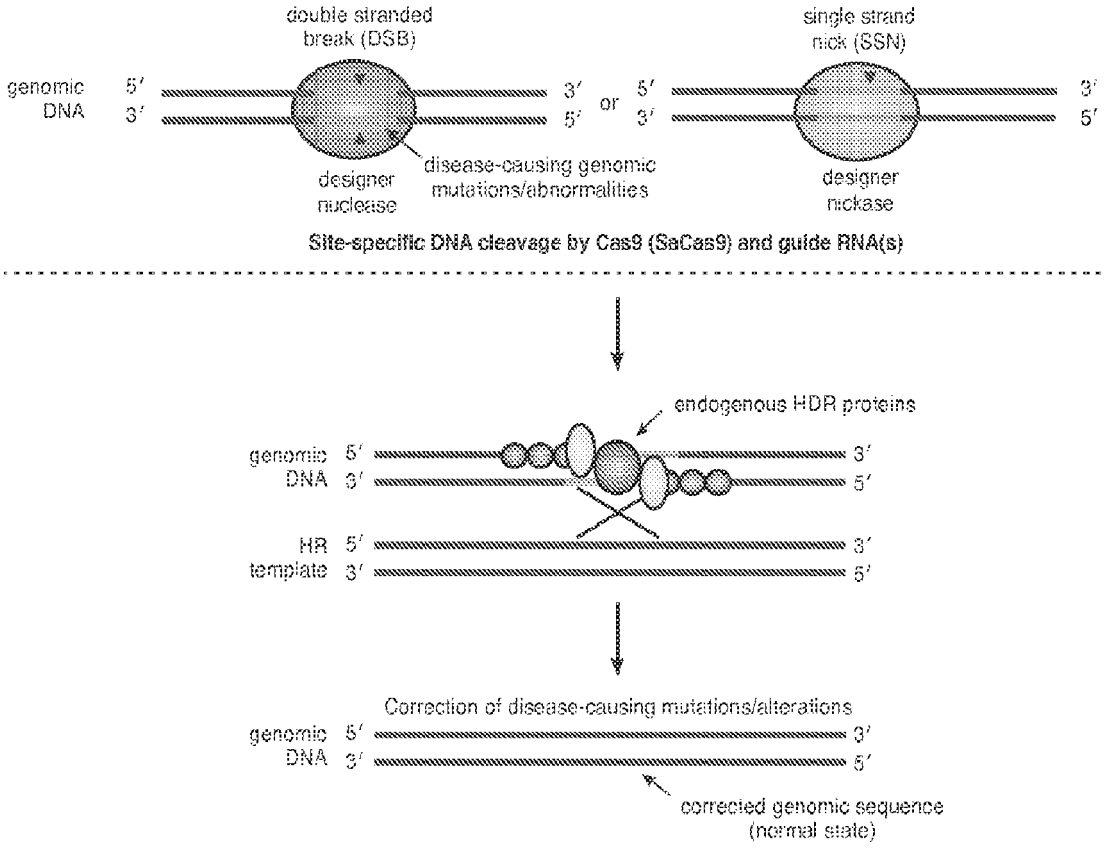

double stranded
break (DSB)
single strand
nick (SSN)
genomic
DNA
5′
3′
3′
5′
or
5′
3′
3′
5′
designer
nuclease
disease-causing genomic
mutations/abnormalities
designer
nickase
Site-specific DNA cleavage by Cas9 (SaCas9) and guide RNA(s)
endogenous HDR proteins
genomic
DNA
HR
template
Correction of disease-causing mutations/alterations
corrected genomic sequence
(normal state)
Homology-directed repair (HDR) by co-delivery of vector encoding
Cas9-sgRNA and Homologous Recombination (HR) template vector
effective genome editing
via Cas9 and HR template
causal mutations or genomic
abnormalities in ocular diseases
correction to normal
genomic sequence
Production and purification of
delivery vehicle
(AAV2 or AAV8 viral particles)
Intravitreal or subretinal injection
into patient's eyes
Gene therapy
Therapeutic correction or relief
of ocular disease phenotype

FIG. 16

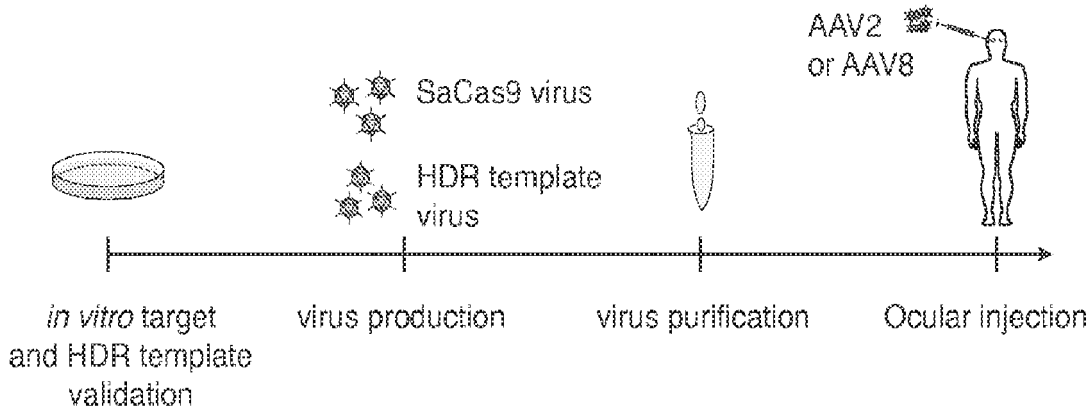
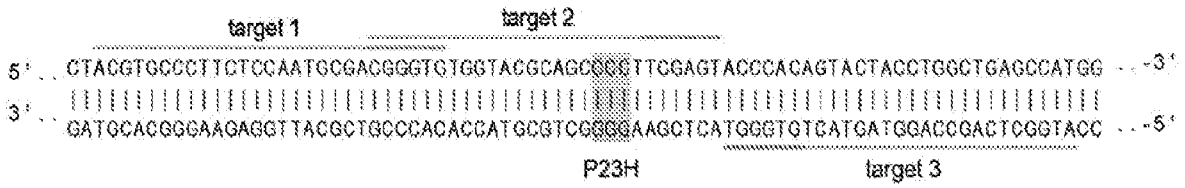

AAV2
or AAV8
SaCas9 virus
HDR template
virus
in vitro target
and HDR template
validation
virus production
virus purification
Ocular injection

FIG. 17

A

human *RHO* locus (allele showing P23H mutation)



B

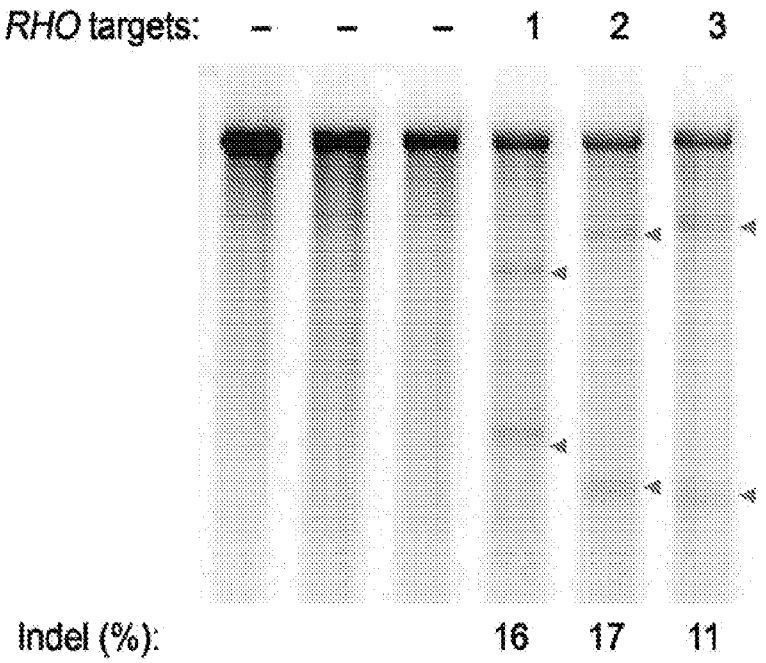


FIG. 18A-B

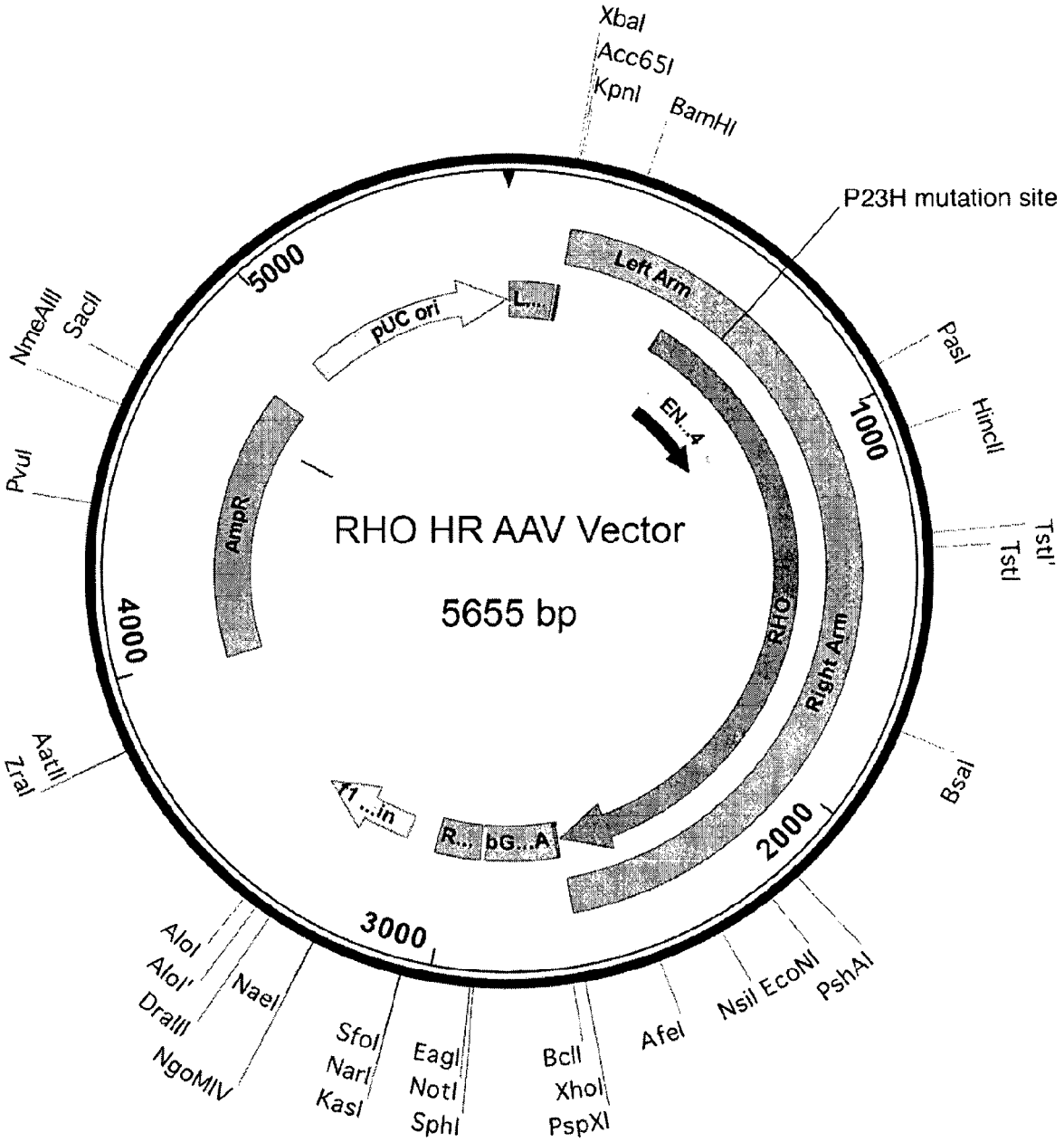

XbaI
Acc65I
KpnI
BamHI
P23H mutation site
Left Arm
pUC ori
L...
EN...4
RHO
Right Arm
AmpR
RHO HR AAV Vector
5655 bp
f1 ...in
R...
bG...A
5000
1000
2000
3000
4000
PasI
HincII
TstI'
TstI
BsaI
PshAI
NsiI EcoNI
AfeI
BclI
XhoI
PspXI
EagI
NotI
SphI
SfoI
NarI
KasI
NaeI
AloI
AloI'
DraIII
NgoMIV
AatII
ZraI
PvuI
NmeAIII
SacII

FIG. 19

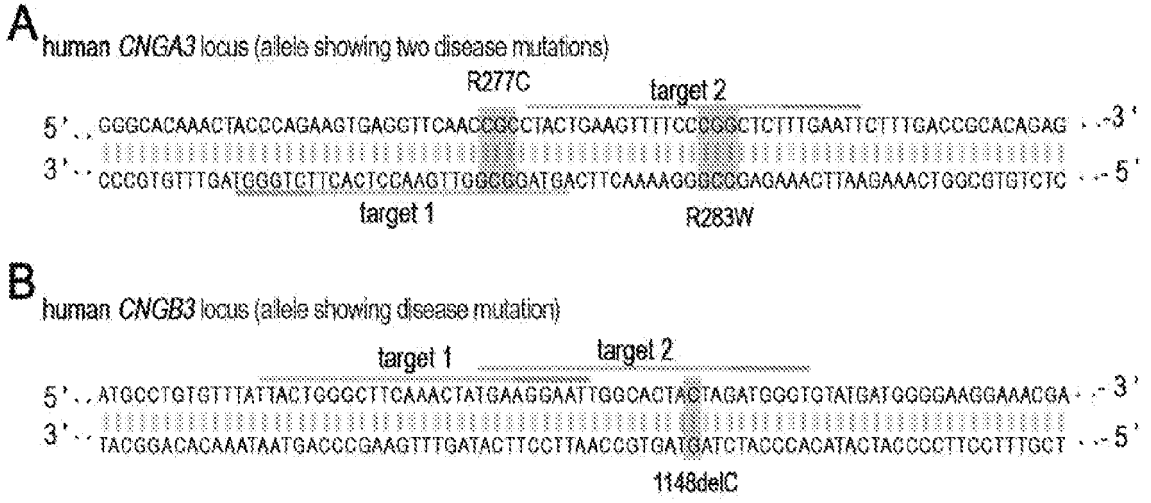

A
human CNGA3 locus (allele showing two disease mutations)
R277C
target 2
5' GGGCACAAACTACCCAGAAGTGACGTTCAACCGCCTACTGAAGTTTTCCCGGCTCTTTGAATTCTTTGACCGCACAGAG 3'
3' CCCGTGTTTGATGGGTCTTCACTCCAAGTTGGCCGATGACTTCAAAAGGGCCGAGAAACTTAAGAAACTGGCGTGTCTC 5'
target 1
R283W
B
human CNGB3 locus (allele showing disease mutation)
target 1
target 2
5' ATGCCTGTGTTTATTACTGGGCTTCAAACTATGAAGGAATTGGCACTACTAGATGGCTGTATGATGGGGAAGGAAACGA 3'
3' TACGGACACAAATAATGACCCGAAGTTTGATACTTCCTTAACCGTGATGATCTACCCACATACTACCCCTTCCTTTGCT 5'
1148delC

FIG. 20A-B

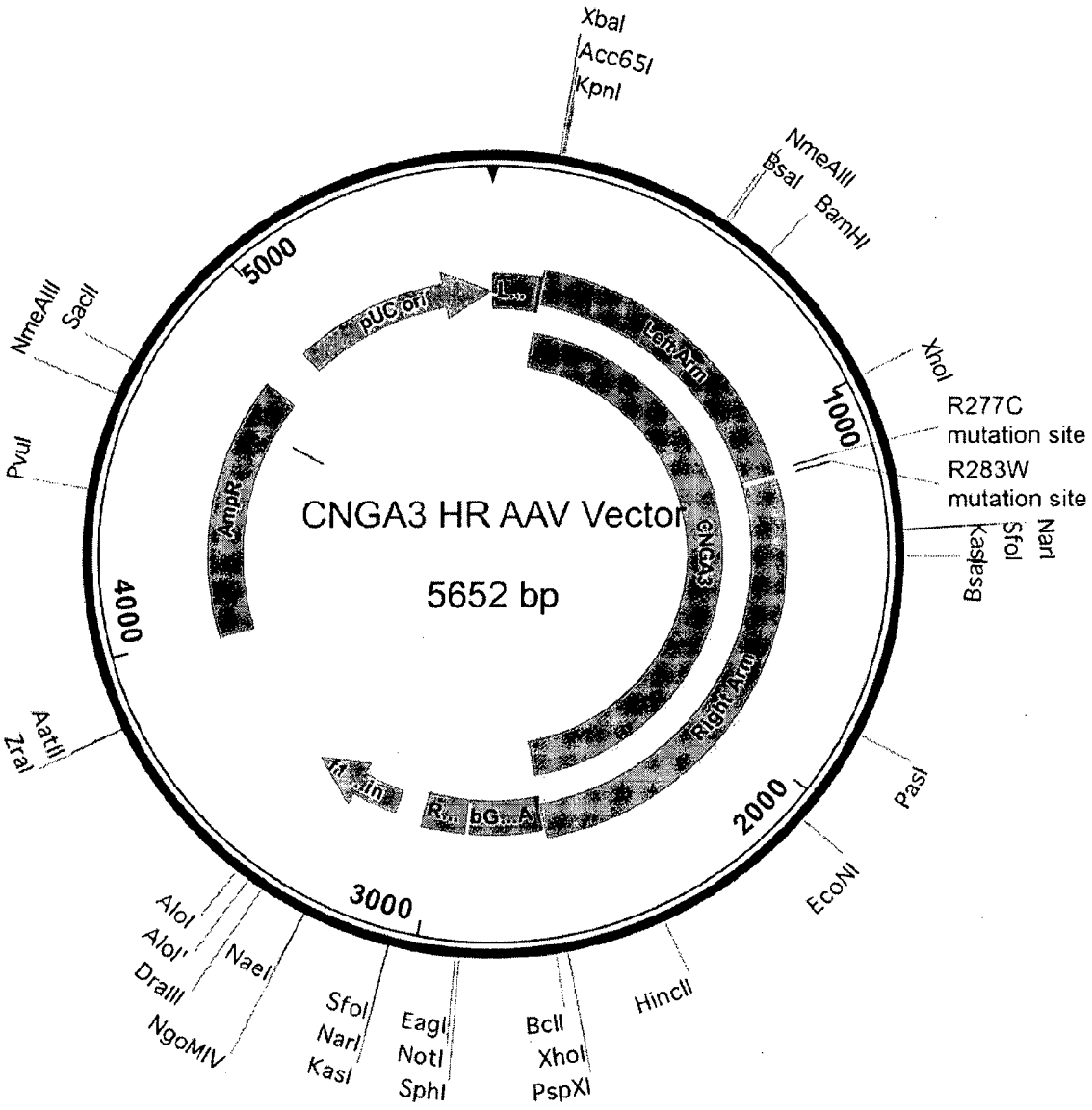

CNGA3 HR AAV Vector
5652 bp
XbaI
Acc65I
KpnI
NmeAIII
BsaI
BamHI
XhoI
R277C
mutation site
R283W
mutation site
NarI
SfoI
KasI
BsaI
PasI
EcoNI
HincII
BclI
XhoI
PspXI
EagI
NotI
SphI
SfoI
NarI
KasI
NaeI
AlwI
AlwI
DraIII
NgoMIV
AatII
ZraI
PvuI
NmeAIII
SacII
5000
1000
2000
3000
4000
pUC ori
AmpR
Left Arm
CNGA3
Right Arm
bG...A

FIG. 21

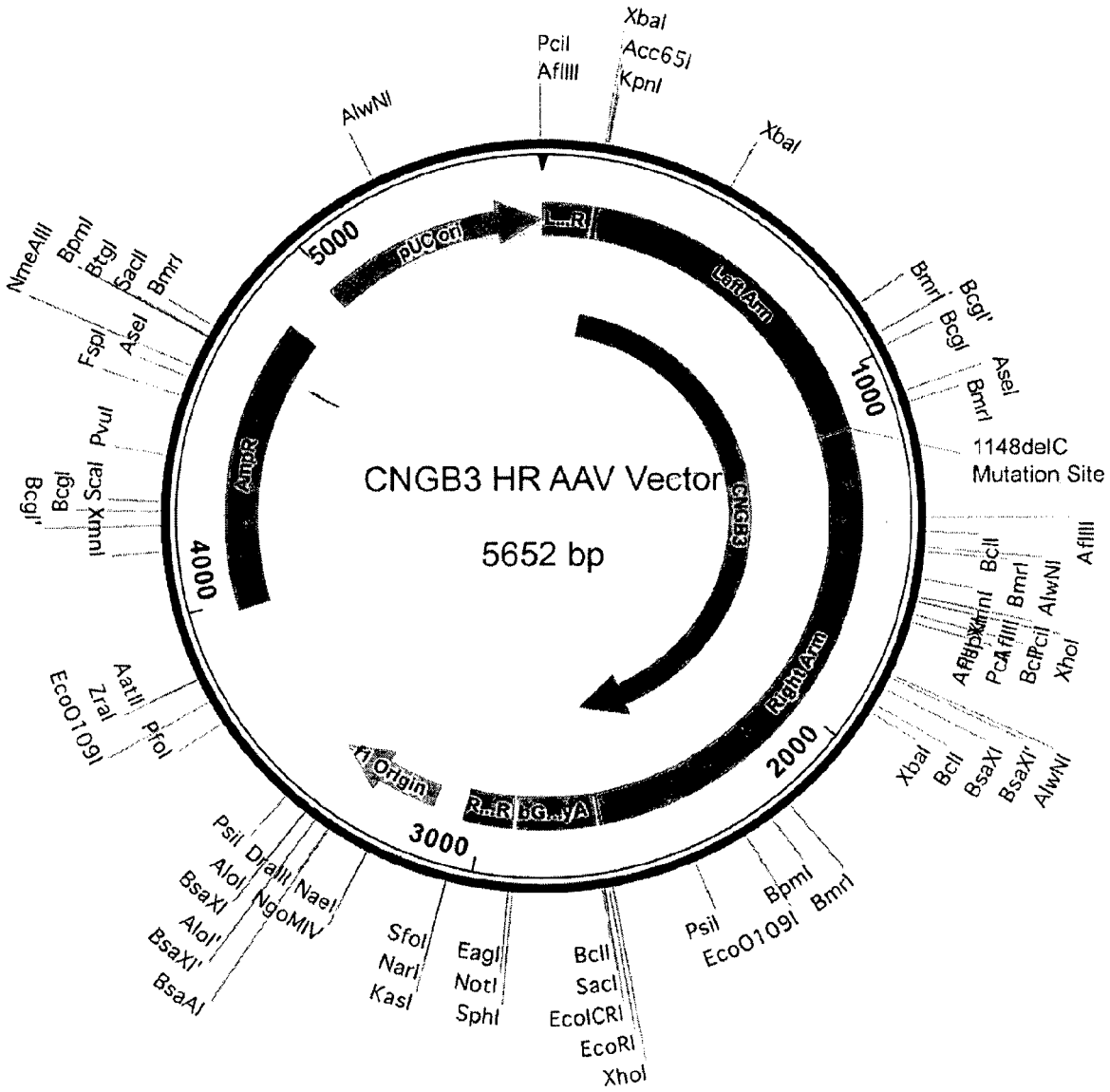

CNGB3 HR AAV Vector
5652 bp
1148delC
Mutation Site
pUC Ori
Left Arm
Right Arm
CNGB3
AmpR
f1 Origin
5000
1000
2000
3000
4000
PciI
AflIII
XbaI
Acc65I
KpnI
AlwNI
NmeAIII
BpmI
BtgI
SacII
BmrI
FspI
AseI
PvuI
ScaI
XmnI
BcgI
AatII
ZraI
Eco0109I
PfoI
PsiI
DraIII
NaeI
AloI
BsaXI
NgoMIV
BsaAI
SfoI
NarI
KasI
EagI
NotI
SphI
BclI
SacI
EcoICRI
EcoRI
XhoI
PsiI
Eco0109I
BpmI
BmrI
BcgI'
AseI
BsaXI'
BclI
AflIII

FIG. 22

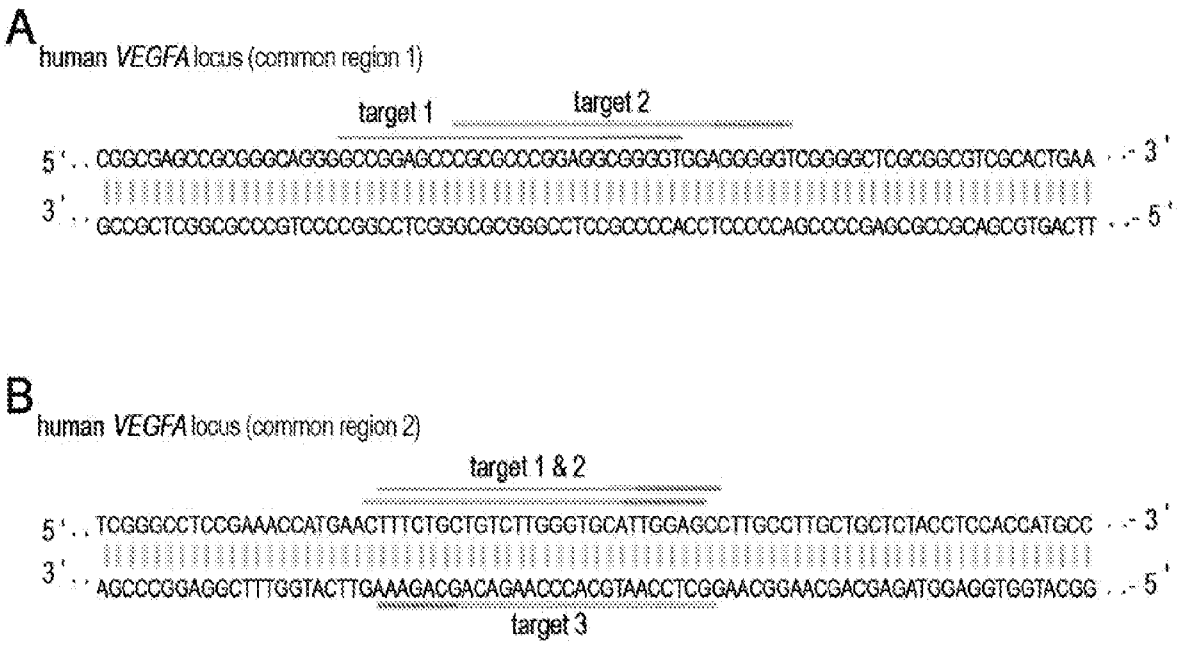
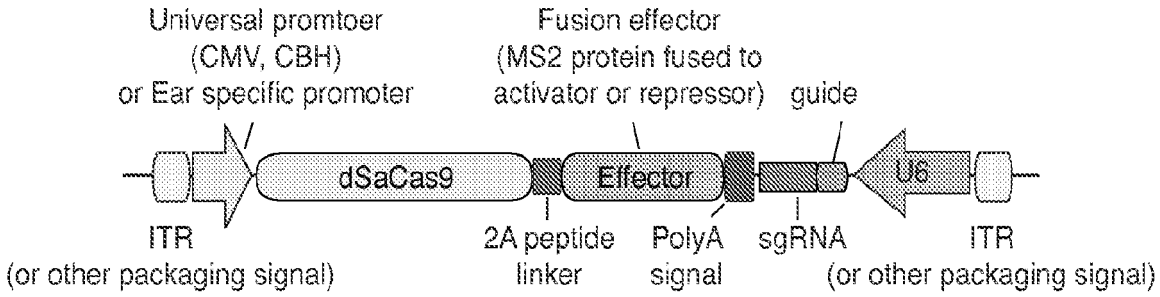

A
human VEGFA locus (common region 1)
target 1
target 2
5' . . CGGCGAGCCGCGGGCAGGGGCCGGAGCCCGCGCCCGGAGGCGGGGTGGAGGGGGTCGGGGCTCGCGGCGTCGCACTGAA . . - 3'
3' . . GCCGCTCGGCGCCCGTCCCCGGCCTCGGGCGCGGGCCTCCGCCCCACCTCCCCCAGCCCCGAGCGCCGCAGCGTGACTT . . - 5'
B
human VEGFA locus (common region 2)
target 1 & 2
5' . . TCGGGCCTCCGAAACCATGAACTTTCTGCTGTCTTGGGTGCATTGGAGCCTTGCCTTGCTGCTCTACCTCCACCATGCC . . - 3'
3' . . AGCCCGGAGGCTTTGGTACTTGAAAGACGACAGAACCCACGTAACCTCGGAACGGAACGACGAGATGGAGGTGGTACGG . . - 5'
target 3

FIG. 23A-B



CMV: cytomegalovirus promoter
CBH: CMV Beta-actin Hybrid promoter

FIG. 24

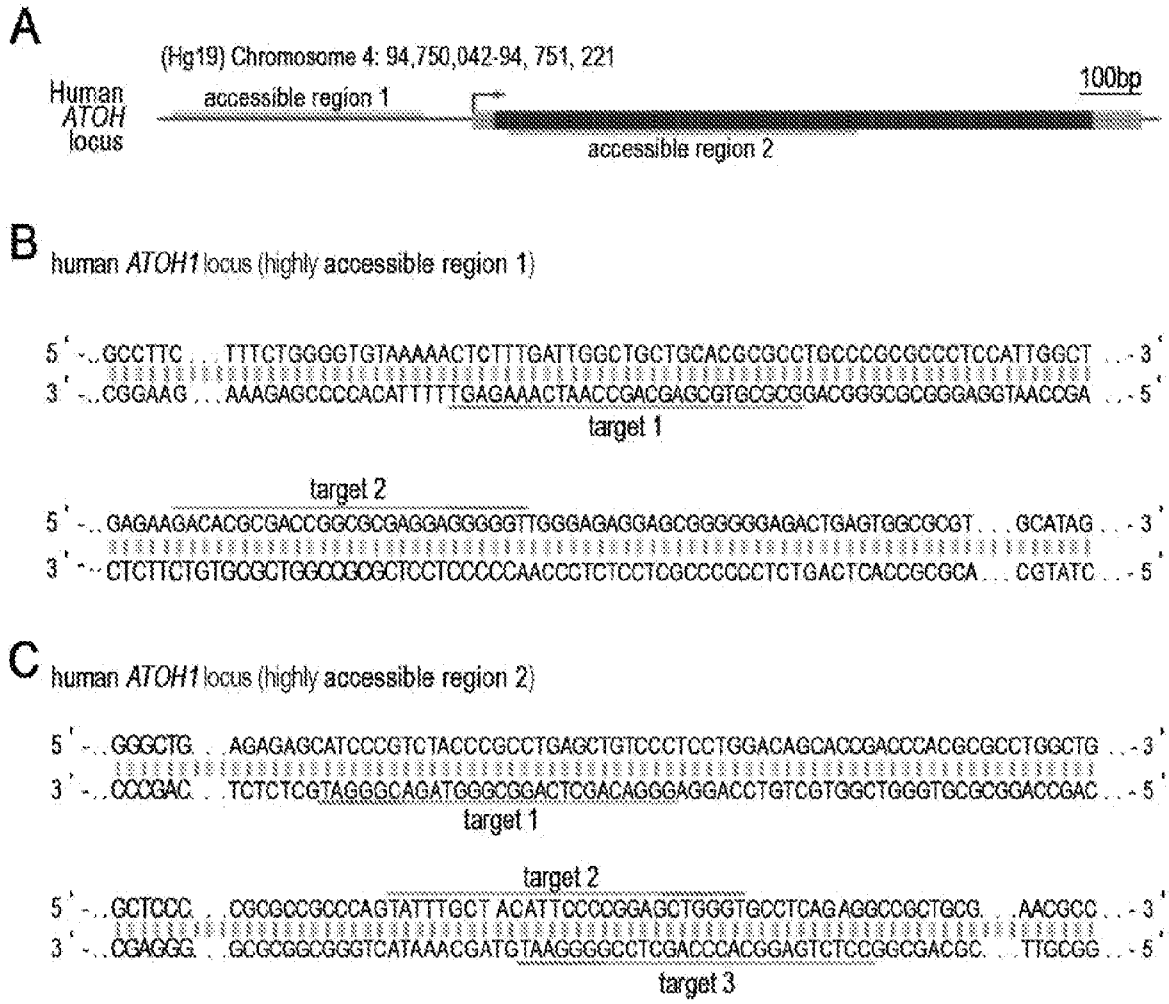

A
(Hg19) Chromosome 4: 94,750,042-94, 751, 221
Human ATOH locus
accessible region 1
100bp
accessible region 2
B
human ATOH1 locus (highly accessible region 1)
5' -..GCCTTC...TTTCTGGGGTGTAAAAACTCTTTGATTGGCTGCTGCACGCGCCTGCCCGCGCCCTCCATTGGCT...-3'
3' -..CGGAAG...AAAGAGCCCCACATTTTTGAGAAACTAACCGACGAGCGTGCGCGGACGGGCGCGGGAGGTAACCGA...-5'
target 1
target 2
5' -...GAGAAGACACGCGACCGGCGCGAGGAGGGGGTTGGGAGAGGAGCGGGGGGAGACTGAGTGGCGCGT...GCATAG...-3'
3' -...CTCTTCTGTGCGCTGGCCGCGCTCCTCCCCCAACCCTCTCCTCGCCCCCCTCTGACTCACCGCGCA...CGTATC...-5'
C
human ATOH1 locus (highly accessible region 2)
5' -..GGGCTG...AGAGAGCATCCCGTCTACCCGCCTGAGCTGTCCCTCCTGGACAGCACCGACCCACGCGCCTGGCTG...-3'
3' -..CCCGAC...TCTCTCGTAGGGCAGATGGGCGGACTCGACAGGGAGGACCTGTCGTGGCTGGGTGCGCGGACCGAC...-5'
target 1
target 2
5' -..GCTCCC...CGCGCCGCCCAGTATTTGCT ACATTCCCCGGAGCTGGGTGCCTCAGAGGCCGCTGCG...AACGCC...-3'
3' -..CGAGGG...GCGCGGCGGGTCATAAACGATGTAAGGGGCCTCGACCCACGGAGTCTCGGGCGACGC...TTGCGG...-5'
target 3

FIG. 25A-C