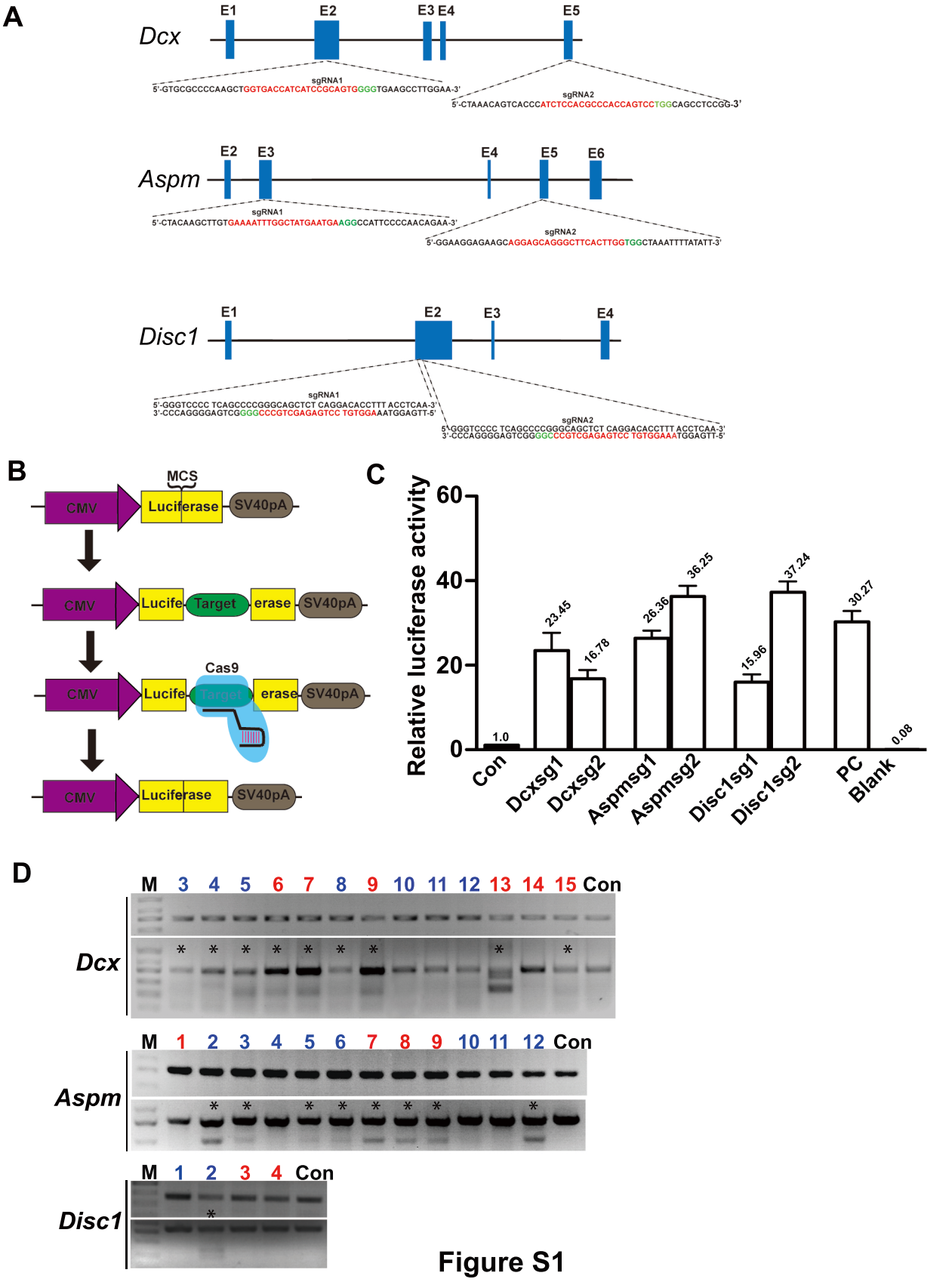

A
Dcx
E1
E2
E3 E4
E5
sgRNA1
5'-GTGCGCCCCAAGCTGGTGACCATCATCCGCAGTGGGGTGAAGCCTTGGAA-3'
sgRNA2
5'-CTAAACAGTCACCCATCTCCACGCCCACCAGTCCTGGCAGCCTCCGG-3'
Aspm
E2
E3
E4
E5
E6
sgRNA1
5'-CTACAAGCTTGTGAAAATTTGGCTATGAATGAAGGCCATTCCCCAACAGAA-3'
sgRNA2
5'-GGAAGGAGAAGCAGGAGCAGGGCTTCACTTGGTGGCTAAATTTTATATT-3'
Disc1
E1
E2
E3
E4
sgRNA1
5'-GGGTCCCC TCAGCCCCGGGGCAGCTCT CAGGACACCTTT ACCTCAA-3'
3'-CCCAGGGGAGTCGGGGCCCGTCGAGAGTCC TGTGGAAATGGAGTT-5'
sgRNA2
5'-GGGTCCCC TCAGCCCCGGGGCAGCTCT CAGGACACCTTT ACCTCAA-3'
3'-CCCAGGGGAGTCGGGGCCCGTCGAGAGTCC TGTGGAAATGGAGTT-5'
B
MCS
CMV
Luciferase
SV40pA
Lucife
Target
erase
Cas9
C
Relative luciferase activity
60
40
20
0
1.0
23.45
16.78
26.36
36.25
15.96
37.24
30.27
0.08
Con
Dcxsg1
Dcxsg2
Aspmsg1
Aspmsg2
Disc1sg1
Disc1sg2
PC
Blank
D
M 3 4 5 6 7 8 9 10 11 12 13 14 15 Con
Dcx
M 1 2 3 4 5 6 7 8 9 10 11 12 Con
Aspm
M 1 2 3 4 Con
Disc1

Figure S1

E

Dcx	GCGCCCCAAGCTGGTGACCATCATCCGCAGTGGGGTGAAGCCTTGGAA	(WT)
#3	GCGCCCCAAGCTGGTGACCATCATCCGCAGTGGGGTGAAGCCTTGGAA	(+1,16/20)
	GCGCCCCAAGCTGGTGACCATCATCC : : : : : GGGGTGAAGCCTTGGAA	(-5, 2/20)
#4	GCGCCCCAAGCTGGTGACCA : : : : : : : AGTGGGGTGAAGCCTTGGAA	(-8, 18/20)
#5	GCGCCCCAAGCTGGTGACCATCATCCGC : : : : GGGTGAAGCCTTGGAA	(-4, 11/20)
	GCGCC : : : : : : : : : GA : : : : : : : : : : : : TGGGGTGAAGCCTTGGAA	(-23, 5/20)
#6	GCGCCCCAAGCTGGTGACCATCATCCGCAGTGGGGTGAAGCCTTGGAA	(+1,16/20)
	GCGCCCCAAGCT : GGTGAAGCCTTGGAA	(-21, 2/20)
#7	GCGCCCCAAGCTGGTGACCATCATCC : : : : : GGGGTGAAGCCTTGGAA	(-5, 9/20)
	GCGCCCC : AAGCCTTGGAA	(-30, 8/20)
	GCGCCCCAAGCTGGTGACCATCATCCGCAGTGGGGTGAAGCCTTGGAA	(+1, 2/20)
#8	GCGCCCCAAGCTGGTGACCATCATCCGCAGTGGGGTGAAGCCTTGGAA	(+1, 15/20)
	GCGCCCCAAGCT : GGTGAAGCCTTGGAA	(-21, 2/20)
#9	GCGCCCCAAGCTGGTGACCATCATCC : : : : : GGGGTGAAGCCTTGGAA	(-5, 10/20)
	GCGCCCCAAGC : GGGGTGAAGCCTTGGAA	(-20, 7/20)
	GCGCCCC : AAGCCTTGGAA	(-30, 2/20)
#13	GCGCCCCAAGCT : GGTGAAGCCTTGGAA	(-21, 7/20)
	: GAAGCCTTGGAA	(-98, 7/20)
	GCGCCCCAAGCTGGTGACCATCATCCGCAGTGGGGTGAAGCCTTGGAA	(+1, 3/20)
#15	GCGCCCCAAGCTGGTGACCATCATCCGCAGTGGGGTGAAGCCTTGGAA	(+1, 9/20)
	GCGCCCCAAGC : TGGGGTGAAGCCTTGGAA	(-19, 6/20)
Aspm	GGAAGGAGAAGCAGGAGCAGGGCTTCACTTGGTGGCTAAATTTTATATT	(WT)
#2	GGAAGGAGAAGCAGGAGCAGGG : : : : : CTTGGTGGCTAAATTTTATATT	(-5, 12/20)
	GGAAGGAGAAGCAGGAGCAG : : : : : : : : : : : : : : : GGCTAAATTTTATATT	(-13, 7/20)
#3	GGAAGGAGAAGCAGGAGCAGGG : : : : : CTTGGTGGCTAAATTTTATATT	(-5, 10/20)
	GGAAGGAGAAGCAGGAGCAGGGCTT : : CTTGGTGGCTAAATTTTATATT	(-2, 6/20)
#5	GGAAGGAGAAGCAGGAGCAGGG : : : : : CTTGGTGGCTAAATTTTATATT	(-5, 8/20)
	GGAAGGAGAAGCAGGAGCAGGGCTTC : : : TGGTGGCTAAATTTTATATT	(-3, 8/20)
	GGAAGGAGAAGCAGGAGCAGGGCTT : :CTTGGTGGCTAAA TTTTATATT	(-2, 3/20)
Disc1	AAGCTTGAGGTAAAGGTGTCCTGAGAGCTGCCCGGGGCTGAGGGGACC	(WT)
#2	AAGCTTGAGGTAAAGGTGTCCTGAGAGC : : : CCGGGGCTGAGGGGACC	(-3, 11/20)
	AAGCTTGAGGTAAAGGTGTCCTGAGAGCTGCCCGGGGCTGAGGGGACC	(+1, 4/20)

F

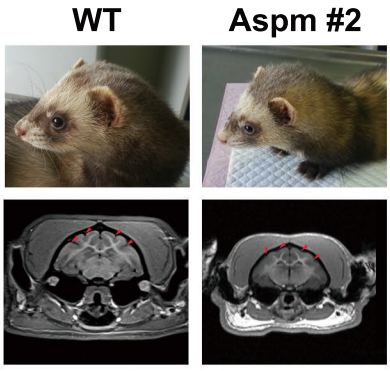
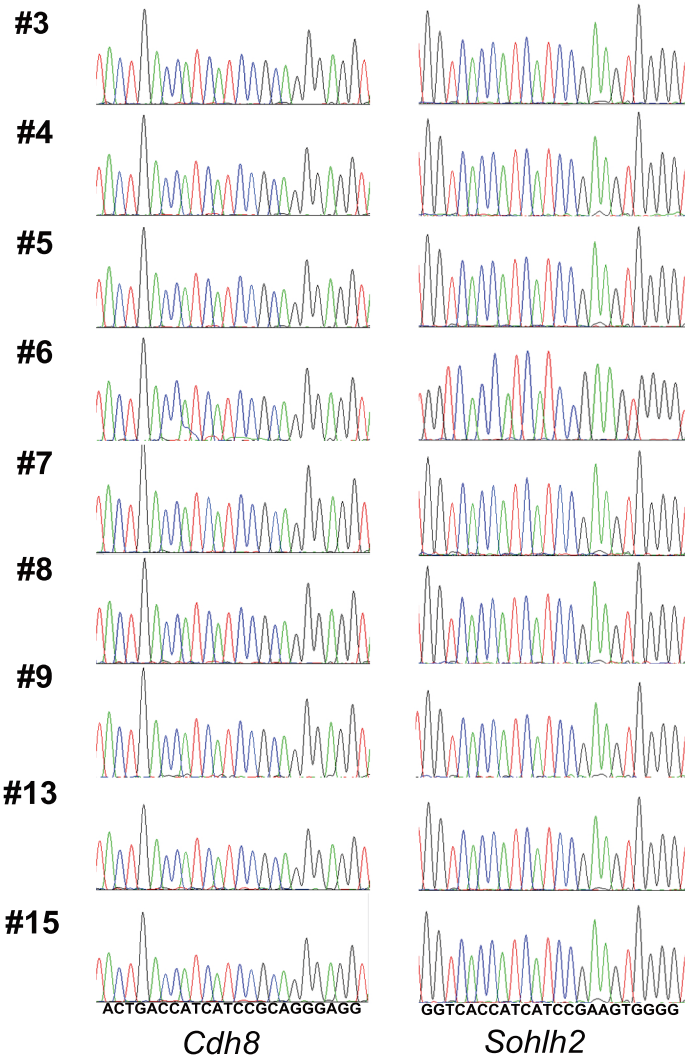


Figure S1

A



B

Cdh8	CCCCCACCCAGGGACTACTGACCATCATCCGCAGGGAGGTATTGAAATGGG	WT
	CCCCCACCCAGGGACTACTGACCATCATCCGCAGGGAGGTATTGAAATGGG	*Dcx* #1 (20/20)
	CCCCCACCCAGGGACTACTGACCATCATCCGCAGGGAGGTATTGAAATGGG	*Dcx* #2 (20/20)
	CCCCCACCCAGGGACTACTGACCATCATCCGCAGGGAGGTATTGAAATGGG	*Dcx* #4 (20/20)
	CCCCCACCCAGGGACTACTGACCATCATCCGCAGGGAGGTATTGAAATGGG	*Dcx* #7 (20/20)
	CCCCCACCCAGGGACTACTGACCATCATCCGCAGGGAGGTATTGAAATGGG	*Dcx* #9 (20/20)
	CCCCCACCCAGGGACTACTGACCATCATCCGCAGGGAGGTATTGAAATGGG	*Dcx* #15 (20/20)
Sohlh2	TCATCCGGCCCAAGCTGGTCACCATCATCCGAAGTGGGGTGAAGCCACGGA	WT
	TCATCCGGCCCAAGCTGGTCACCATCATCCGAAGTGGGGTGAAGCCACGGA	*Dcx* #1 (20/20)
	TCATCCGGCCCAAGCTGGTCACCATCATCCGAAGTGGGGTGAAGCCACGGA	*Dcx* #2 (20/20)
	TCATCCGGCCCAAGCTGGTCACCATCATCCGAAGTGGGGTGAAGCCACGGA	*Dcx* #4 (20/20)
	TCATCCGGCCCAAGCTGGTCACCATCATCCGAAGTGGGGTGAAGCCACGGA	*Dcx* #7 (20/20)
	TCATCCGGCCCAAGCTGGTCACCATCATCCGAAGTGGGGTGAAGCCACGGA	*Dcx* #9 (20/20)
	TCATCCGGCCCAAGCTGGTCACCATCATCCGAAGTGGGGTGAAGCCACGGA	*Dcx* #15 (20/20)

Figure S2

Table S1A. Summary of embryo microinjection of Cas9 mRNA and sgRNAs

Gene	Injected Embryos	Embryos for ET	Surrogates (Pregnancies)	Fetuses	Knockout Fetuses(Dead), Knockout Rate	Survived Knockout Fetuses Female/Male
Dcx	124	117	10(5)	15	11(2), 73.3%	5/4
Aspm	64	64	4(2)	12	8(0), 66.7%	3/5
Disc1	18	18	1(1)	4	1(0), 25%	0/1

Table S1B. Oligonucleotides used for cloning the DNA segments of targeted genes

Gene Segment	**Direction**	**Sequence (5' to 3')**
Aspm exon3(a540e3)	F	CACCGGAAAATTTGGCTATGAATGA
	R	AAACTCATTCATAGCCAAATTTTCC
Aspm exon3(a740e5)	F	CACCGAGCAGGAGCAGGGCTTCACT
	R	AAACAGTGAAGCCCTGCTCCTGCTC
Dcx exon2 (*dcx660*e2)	F	CGAGGTGAGTTGTTAGAGAGACAG
	R	CTCAGACATATGGTCCATTGCTTG
Dcx exon5 (*dcx450*e4)	F	CAGAGCCCTGTAATTGGAAG
	R	CTTGATGCATTGCTGAAGTAG
Disc1 exon2 (d430e2)	F	TCCAAGTTGGCACCACACTG
	R	GAGAGAGAAGTCGAGGGTGTTC
Disc1 exon2 (d430e2)	F	CCAAGTTGGCACCACACTG
	R	GAGAGAGAAGTCGAGGGTGT

Table S1C. Oligonucleotides used for cloning sgRNA expression vector

Gene Segment	Direction	Sequence (5' to 3')
AspmsgRNA1	F	CACCGGAAAATTTGGCTATGAATGA
	R	AAACTCATTCATAGCCAAATTTTCC
AspmsgRNA2	F	CACCGAGCAGGAGCAGGGCTTCACT
	R	AAACAGTGAAGCCCTGCTCCTGCTC
DcxsgRNA1	F	AAACAGTGAAGCCCTGCTCCTGCTC
	R	CACCGGGTGACCATCATCCGCAGTG
DcxsgRNA2	F	CACCGATCTCCACGCCCACCAGTCC
	R	AAACGGACTGGTGGGCGTGGAGATC
Disc1sgRNA1	F	CACCGAGGTGTCCTGAGAGCTGCCC
	R	AAACGGGCAGCTCTCAGGACACCTC
Disc1sgRNA2	F	CACCGAAGGTGTCCTGAGAGCTGCC
	R	AAACGGCAGCTCTCAGGACACCTTC

Table S1D. Oligonucleotides used for in vitro transcription

Gene Segment	Direction	Sequence (5' to 3')
T7-*Dcx*-sgRNA1	F	ttaatacgactcactataggGGTGACCATCATCCGCAGTG
T7-*Aspm*-sgRNA2	F	ttaatacgactcactataggAGGAGCAGGGCTTCACTTGG
T7-*Disc1*-sgRNA2	F	ttaatacgactcactataggAAGGTGTCCTGAGAGCTGCC
T7-sgRNA	R	AAACAGTGAAGCCCTGCTCCTGCTC
T7-Cas9	F	ttaatacgactcactataggGGAGAATGGACTATAAGGAC CACGAC
T7-Cas9	R	GCGAGCTCTAGGAATTCTTAC

Table S1E. Oligonucleotides used for T7EN1 assay

Gene Segment	Direction	Sequence (5' to 3')
Dcx	F	CGAGGTGAGTTGTTAGAGAGACAG
	R	CTCAGACATATGGTCCATTGCTTG
Aspm	F	CACCGAGCAGGAGCAGGGCTTCACT
	R	AAACAGTGAAGCCCTGCTCCTGCTC
Disc1	F	TCCAAGTTGGCACCACACTG
	R	GAGAGAGAAGTCGAGGGTGTTC

Table S1F. Oligonucleotides used for off-target assay

Gene Segment	Direction	Sequence (5' to 3')
Cdh8	F	GCCTCTTAAACTCAAATTCATC
	R	CAGGTATATGCTACACTCATAATG
Sohlh2	F	CATCGGTGATGTCAGTGAGAAC
	R	CCCTTCAAGAAACTGGAGTACAC

SUPPLEMENTARY INFORMATION

EXPERIMENTAL PROCEDURES

Animals

All animal procedures were performed according to protocol approved in advance by the Committee on Animal Care at the Institute of Biophysics, Chinese Academy of Sciences. Health Domestic Ferret (*Mustela putorius furo*) female, ranging in age from 1 to 3 years and having body weights of 1.2 to 2.0 kg, male ranging in age from 2 to 4 years and having body weights of 1.5 to 2.5 kg, were selected for use in this study. Female ferrets were in oestrus when delivered and non-vasectomized males were proven breeders. Eight vasectomized male ferrets were prepared 4 months in advance. After vasectomy, each male was mated repeatedly before a sperm count was carried out to confirm the sterile state of the animal. Sterility, as inferred by aspermia and the lack of pregnancy after three sequential matings with an oestrous ferret, was confirmed within 3 months of vasectomy in all eight males. All animals were housed under controlled temperature (20–22°C) and long day light cycles (16 h light: 8 h dark).

Superovulation of ferrets

For superovulation, female ferrets were intraperitoneally injected (i.p.) with 200-300IU PMSG (Pregnant Mare Serum Gonadotropin), followed by sequential 20 times injections of 5IU rhFSH (recombinant human follitropin alfa, GONAL-F, Merck Serono) (interval 12h) 48 h later, then injected 200-300IU hCG (human chorionic gonadotropin) (Merck Serono). Each female was mated with one male or with two males in succession immediately after hCG treatment. Oocytes or embryos were flushed and harvested from the oviducts or uterine horns with HCZB medium.

Plasmids

To validate the efficiency of sgRNA selected as described, we used the UCATM CRISPR/Cas9 kits (Biocytogen, Cat. BCG-DX-001). Following the kit instruction, ~500 bp genomic fragments containing sgRNA target sequence were PCR amplified and placed between the luciferase fragments, which is linearized with blunt end. The DNA

segments of targeted genes (*Aspm, Dcx, Disc1*) were amplified and sequenced. Primers for amplifying these segments were listed in Table S1B. sgRNA targets were selected by an online design tool Cas9 design (http://crispr.mit.edu/ and http://cas9.cbi.pku.edu.cn/). The sgRNA expression vectors were constructed as described [1]. In brief, the sgRNA oligos (Supplementary information, Table S1C) were phosphorylated and annealed; the vector px330 (Addgene, 42230) and the annealed sgRNA oligos for each targeting site were digested with BbsI and ligated.

In Vitro Transcription

T7 promoter was added to Cas9 coding region by PCR amplification using primer Cas9 F and R (Supplementary information, Table S1D). T7-Cas9 PCR product was gel-purified and used as the template for in vitro transcription (IVT) using mMESSAGE mMACHINE T7 ULTRA kit (Life Technologies). T7 promoter was added to sgRNAs template by PCR amplification using primer listed in Table S1D. The T7-sgRNA PCR product was gel-purified and used as the template for IVT using MEGAshortscript T7 kit (Life Technologies). Both the Cas9 mRNA and the sgRNAs were purified using MEGAclear kit (Life Technologies) and eluted in RNase-free water.

Hek293T cell culture and SSA reporter Assay

HEK293T cells (ATCC, CRL-3216) were cultured in DMEM/high glucose (HyClone) with 10% FBS, penicillin (100 U/ml) and streptomycin (100 mg/ml); $3x10^4$ cells per well were plated in 96 well plate. Cells were grown to ~ 80% confluence and transfected using Lipofectamine 2000 (Life Technologies) with 20ng/well of pUCA-target plasmids and 80ng/well of Cas9 and sgRNA expression plasmids. The activity of luciferase was examined 24h after transfection with GloMax-Multi Detection System (Promega) follow the manufacturer's instruction.

Cas9/sgRNA Injection of One-Cell Embryos

The zygotes were injected with a mixture of Cas9 mRNA (20ng/μl) and sgRNAs (30ng/μl). Microinjections were performed in the cytoplasm of zygotes using a Piezo-

drill micromanipulator (Origio, PIEZO-8-15). The injected zygotes were cultured in HCZB medium at 38.5°C in 5% CO_2 for 3h.

Embryo transfer

A pseudopregnant state was achieved in recipient females mated with vasectomized males 12–24h after embryo donors were mated. ~ 15-18 injected embryos were transferred into the oviduct of the matched recipient ferret. The earliest pregnancy diagnosis was performed by touch about 21 days after the embryo transfer.

T7EN1 Cleavage Assay and Sequencing

Samples were collected and the genomic DNA was extracted by AxyPrep Multisource Genomic DNA Miniprep Kit (Axygen, Cat.AP-MN-MS-GDNA-50) according to the manufacturer's instruction. T7EN1 cleavage assay was performed as described[2]. Briefly, targeted fragments were amplified by TransTaq DNA Polymerase High Fidelity (Transgene, Cat. AP131-02) from extracted genomic DNA, and purified with PCR cleanup kit (Axygen, Cat. AP-PCR- 50). Purified PCR product was denatured and re-annealed in NEBuffer 2 (NEB) using a thermocycler. Hybridized PCR products (300ng) were digested with T7EN1 (NEB, Cat. M0302L) for 30 min and separated by 2.0% agarose gel. PCR products with mutations detected by T7EN1 cleavage assay were sub-cloned into pMD-19T vector (Takara, D104A). For each sample, colonies were picked up randomly and sequenced by M13-47 primer. Primers for amplifying *Aspm, Dcx, and Disc1* targeted fragments and T7EN1 cleavage assay are listed in Table S1E.

Off-target Analysis

All potential off-target sites with homology to the 23 bp sequence (sgRNA+PAM) were retrieved by blastn in NCBI, allowing for ungapped alignments with up to four mismatches in the sgRNA target sequence as described [3-6]. The potential off-target sites were amplified using tail genomic DNA as templates. PCR products were sub-cloned into pMD-19T vector (Takara, D104A). For each sample, colonies were picked up randomly and sequenced by M13-47 primer. The primers for amplifying the off-target sites are listed in Table S1F.

SUPPLEMENTARY FIGURE LEGEND

Figure S1. sgRNA:Cas9-Mediated Modification of *Dcx*, *Aspm and Disc1* in ferrets. (A) Schematic diagram of sgRNAs targeting at *Dcx*, *Disc1* and *Aspm* loci. The Protospacer-adjacent motif (PAM) is labeled in green. sgRNA targeting sites are highlighted in red. (B) Luciferase assay to identify the efficiency of sgRNA:CAS9 system. Plasmid contains 5' and 3' Luciferase fragments that share ~800bp under ubiquitous CMV promoter, ~500 bp genomic fragment containing the target sequence was placed between luciferase fragments of pUCALucplasmid with stop codon following the first share fragment. The resulting target plasmid was cotransfected with pX330 plasmids expressing sgRNA and hCas9 into HEK293T cells. When the target sequence was digested by sgRNA guided CAS9 endonuclease, the homology dependent repair (HDR); homologous recombination (HR) or single strand annealing (SSA) took place and reconstituted the luciferase expression cassette. (C) Relative luciferase activity for the efficiency of sgRNA: Cas9-mediated cleavage of *Dcx, Disc1* and *Aspm*. The recombination assay shows luciferase activity with and without cleavage of the target site by the Cas9/sgRNA system in 293T cells. The luciferase activity increased by ~23.45 and 16.78 folds in the presence of Cas9 with *Dcx*-sgRNA1 and *Dcx*-sgRNA2, respectively. 26.36 and 36.25 fold increase with *Disc1*-sgRNA1 and *Disc1*-sgRNA2, and 15.96 and 37.24 fold increase with *Asmp*-sgRNA1 and *Aspm*-sgRNA2. Control group (Cas9 + empty vector). Error bars indicate SD, n = 8. sg1: sgRNA1; sg2, sgRNA2; Con, control; PC, positive control. (D) Detection of sgRNA: Cas9-mediated modification of *Dcx, Disc1* and *Aspm* by PCR and T7EN1 cleavage assay. M: marker; Con, control. Sample with cleavage bands were marked with an asterisk "*". Female ferrets were marked in red and male ferrets were marked in blue. (E) The sequence of nine mutant alleles from *Dcx* #3, #4, #5, #6, #7, #8, #9, #13 and #15; three mutant alleles from *Aspm* #2, #3 and #5; one mutant allele from *Disc1* #2. The PAM sequences are underlined and highlighted in green; the targeting sequences in red; the mutations in blue, lower case; deletions (-), and insertions (+). N/N indicates positive colonies out of total sequenced. (F) MRI reveals the microcephalic brain of founder #2 after gene modification of *Aspm*. Red arrowheads indicate normal brain folds in wild type (left panel) but not in *Aspm* mutated ferret (right panel).

Figure S2. Off-target analysis of ferrets with Dcx mutations. (A) Sequences of PCR products from *Dcx* ferrets #3, #4, #5, #6, #7, #8, #9, #13 and #15 show no mutations in both potential off-target loci (*Cdh8* and *Sohlh2*). (B) 20 TA clones of *Cdh8* and *Sohlh2* PCR products were analyzed by DNA sequencing. The PAM sequences are in green; the targeting sequences are in red. N/N indicates positive colonies out of total sequenced. *Dcx* ferrets #1, #2, #4, #7, #9 and #15 were analyzed.

SUPPLEMENTARY TABLE

Table S1. Summary of embryo microinjection and oligonucleotides. (A) Summary of embryo microinjection of Cas9 mRNA and sgRNAs. (B) Oligonucleotides used for cloning the DNA segments of targeted genes. (C) Oligonucleotides used for cloning sgRNA expression vector. (D) Oligonucleotides used for in vitro transcription. (E) Oligonucleotides used for T7EN1 assay. (F) Oligonucleotides used for off-target assay.

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