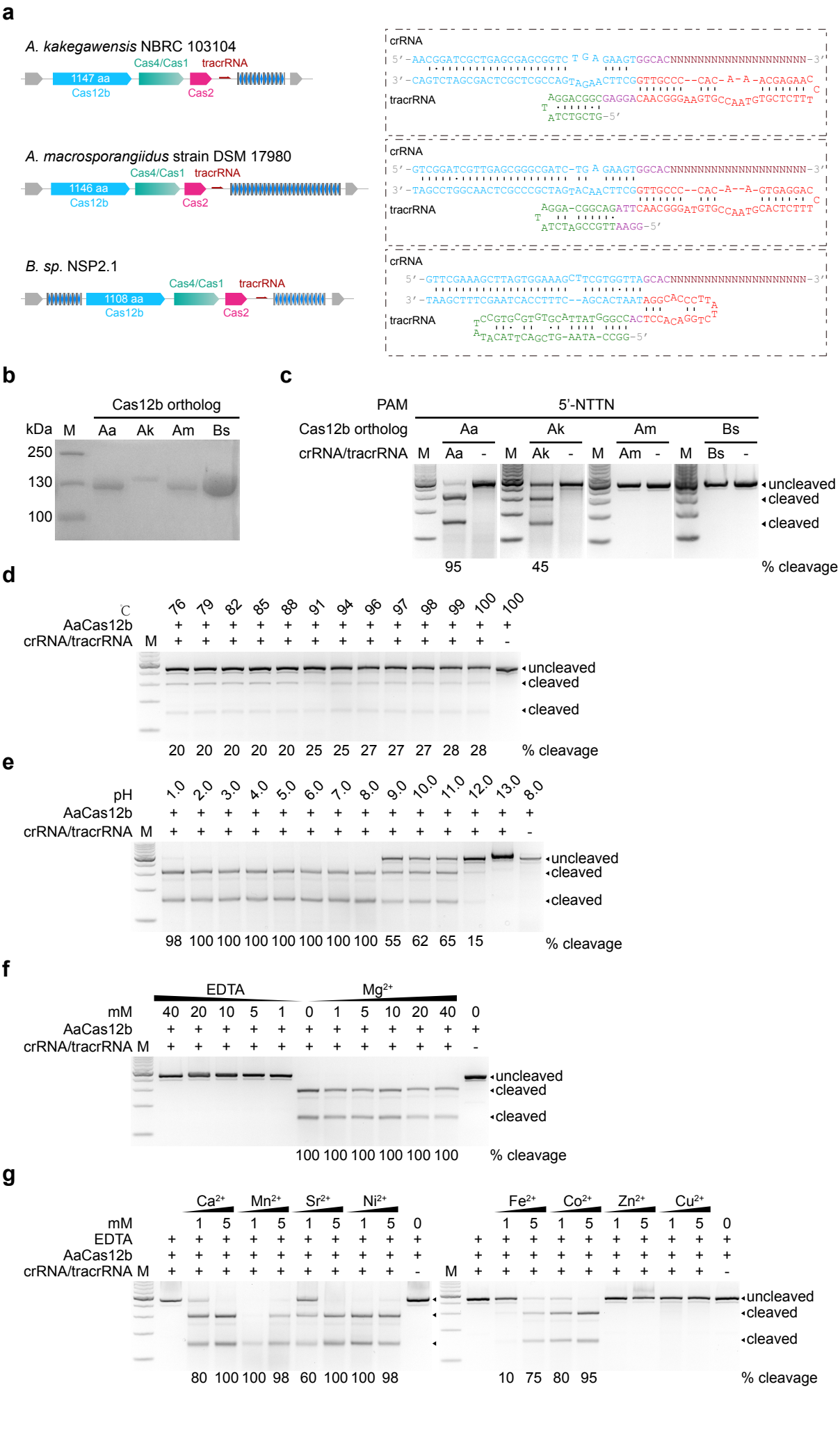
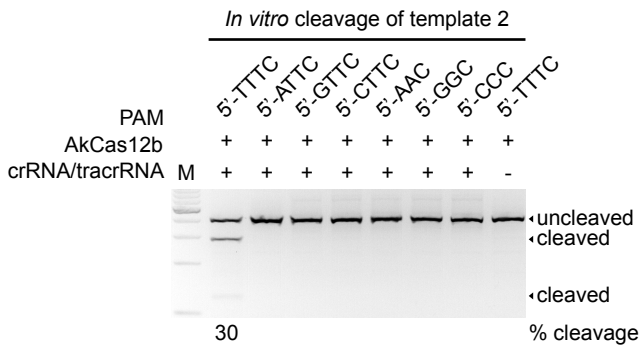
Figure S1


a
A. kakegawensis NBRC 103104
Cas4/Cas1
tracrRNA
1147 aa
Cas12b
Cas2
A. macrosporangiidus strain DSM 17980
1146 aa
B. sp. NSP2.1
1108 aa
crRNA
tracrRNA
b
Cas12b ortholog
kDa M Aa Ak Am Bs
250
130
100
c
PAM
5'-NTTN
Cas12b ortholog
crRNA/tracrRNA
uncleaved
cleaved
95 45
% cleavage
d
°C
AaCas12b
crRNA/tracrRNA
20 20 20 20 20 25 25 27 27 27 28 28
e
pH
98 100 100 100 100 100 100 100 55 62 65 15
f
EDTA
Mg2+
mM
100 100 100 100 100 100
g
Ca2+ Mn2+ Sr2+ Ni2+ Fe2+ Co2+ Zn2+ Cu2+
80 100 100 98 60 100 100 98
10 75 80 95

h


In vitro cleavage of template 2
5'-TTTC
5'-ATTC
5'-GTTC
5'-CTTC
5'-AAC
5'-GGC
5'-CCC
5'-TTTC
PAM
AkCas12b
crRNA/tracrRNA
M
+ + + + + + + +
+ + + + + + + -
uncleaved
cleaved
cleaved
30
% cleavage

i

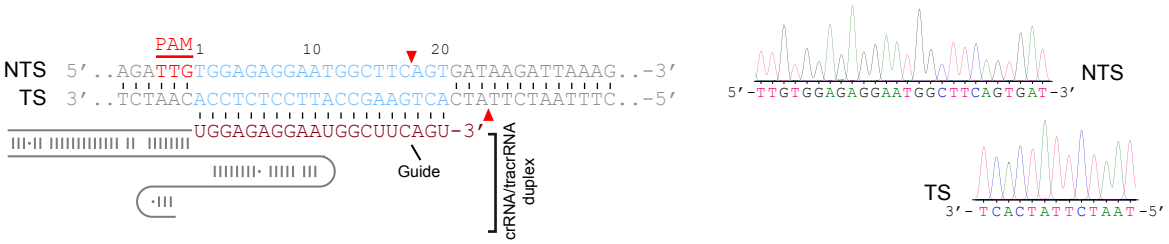
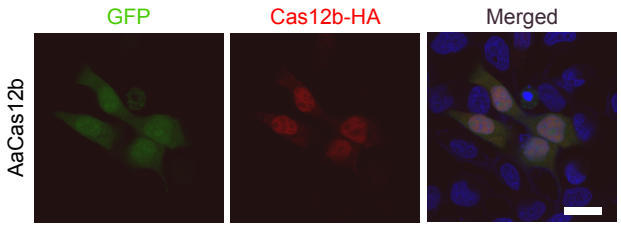

PAM
1
10
20
NTS 5′..AGATTGTGGAGAGGAATGGCTTCAGTGATAAGATTAAAG..-3′
TS 3′..TCTAACACCTCTCCTTACCGAAGTCACTATTCTAATTTC..-5′
UGGAGAGGAAUGGCUUCAGU-3′
Guide
crRNA/tracrRNA duplex
NTS
5′-TTGTGGAGAGGAATGGCTTCAGTGAT-3′
TS
3′-TCACTATTCTAAT-5′

Fig. S1. *In vitro* DNA cleavage assay of Cas12b proteins. a Schematic ilustration of the genomic architecture of CRISPR-Cas12b from *Alicyclobacillus kakegawensis* (NBRC 103104), *Alicyclobacillus macrosporangiidus* (strain DSM 17980), *Bacillus sp. (NSP2.1)* (*left*), and the crRNA/tracrRNA duplex from each strain (*right*). **b** Coomassie blue staining of AaCas12b, AkCas12b, AmCas12b and BsCas12b purified from *E. coli*. **c** *In vitro* cleavage of double-stranded DNAs containing the 5'-NTTN PAMs by the purified AaCas12b, AkCas12b, AmCas12b and BsCas12b proteins and their cognate crRNA/tracrRNA duplexes. The cleavage rate is shown under the cleaved lanes. **d** *In vitro* cleavage activity of AaCas12b at various temperatures. The cleavage rate is shown under the cleaved lanes. **e** *In vitro* cleavage activity of AaCas12b under various pH conditions. The cleavage rate is shown under the cleaved lanes. **f** *In vitro* cleavage assay showing the Mg^{2+}-dependent endonuclease activity of AaCas12b. The cleavage rate is shown under the cleaved lanes. **g** *In vitro* cleavage assay of AaCas12b in the presence of indicated metals, Ca^{2+}, Mn^{2+}, Sr^{2+}, Ni^{2+}, Fe^{2+}, Co^{2+}, Zn^{2+}, Cu^{2+}. The cleavage rate is shown under the cleaved lanes. **h** *In vitro* validation of the PAM requirements of AkCas12b showing that PAMs matching the 5'-TTTN sequence can be efficiently cleaved. The cleavage rate is shown under the cleaved lanes. **i** Cleavage site determination of AaCas12b by sequencing the cleavage products. The cleavage sites are indicated by red triangles in the left panel. TS, target strand; NTS, non-target strand.

Figure S2

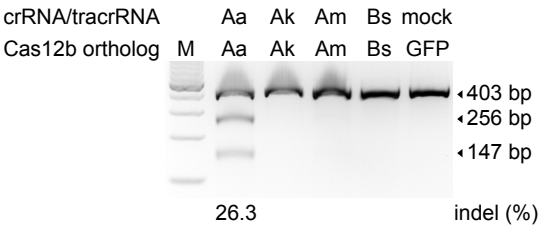
a



b

Human *RNF2* target 2

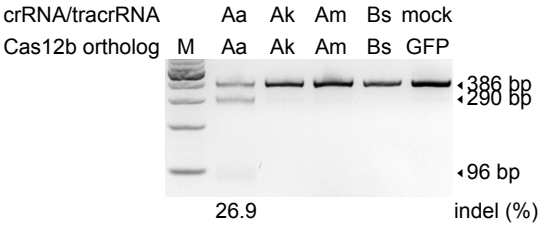




c

Mouse *Nrl* target 1





d

Human *RNF2* target 2

```
TGAATTAATGTGCCCAATTTGTTTGGATATGTTGAAGAACACCATGACTACAAGGAGTGTTTACATCGTT  Wild-type
TGAATTAATGTGCCCAATTTGTTTG----TGTTGAAGAACACCATGACTACAAAGGAGTGTTTACATCGTT  Δ4
TGAATTAATGTGCCCAATTTGTTTGGATA-----AAGAACACCATGACTACAAAGGAGTGTTTACATCGTT  Δ5
TGAATTAATGTGCCCAATTTGTTTGGATATGT-----AACACCATGACTACAAAGGAGTGTTTACATCGTT  Δ5
TGAATTAATGTGCCCAATTTGTTT--------TGAAGAACACCATGACTACAAAGGAGTGTTTACATCGTT  Δ8
TGAATTAATGTGCCCAATTTGTTTGGATA--------AACACCATGACTACAAAGGAGTGTTTACATCGTT  Δ8
TGAATTAATGTGCCCAATTTGTTTGGATA----------------------------TGTTTACATCGTT  Δ29
```

e

Mouse *Nrl* target 1

```
CTCAGTCCCAGAATGGCTTTCCTCCCAGTCCCTTG-GCTATGGAATATGTTAATGACTTTGATTTGATGA  Wild-type
CTCAGTCCCAGAATGGCTTTCCCTCCCAGTCCCTTGcGCTATGGAATATGTTAATGACTTTGATTTGATGA  +1
CTCAGTCCCAGAATGGCTTTCCCTCCCAGTCCCTTG-GC--TGGAATATGTTAATGACTTTGATTTGATGA  Δ2
CTCAGTCCCAGAATGGCTTTCCCTCCCAGTCCCTTG-------GAATATGTTAATGACTTTGATTTGATGA  Δ6
CTCAGTCCCAGAATGGCTTTCCCTCCCAGTCCCTT----------TGTTAATGACTTTGATTTGATGA  Δ12
CTCAGTCCCAGAATGGCTTTCCCTCCCAGTCCCTT----------GTTAATGACTTTGATTTGATGA  Δ13
CTCAGTCCCAGAATGGCTTTCCCTCCCAGTCCCTT----------------------------TGATGA  Δ29
```

Fig. S2 Cas12b nucleases mediate robust genome editing in mammalian cells. a Immunofluorescence staining showing two nuclear localization signals (NLSs) ensured nuclear compartmentalization of AaCas12b in HeLa cells. Scale bar, 20 μm. **b** (*Left*) Schematic illustration of the human *RNF2* target site 2 of AaCas12b and crRNA/tracrRNA duplex. Red letters indicate the PAM sequence. (*Right*) T7EI analysis of indels produced by Cas12b orthologues (AaCas12b, AkCas12b, AmCas12b and BsCas12b) at the human RNF2 gene target site 2. The indel rate is shown under the lane with mutation. mock, an U6 empty vector without crRNA/tracrRNA expression. GFP, an empty backbone vector without Cas12b protein expression. **c** (*Left*) Schematic illustration of the mouse *Nrl* target site 1 of AaCas12b and crRNA/tracrRNA duplex. Red letters indicate the PAM sequences. (*Right*) T7EI analysis of indels produced by Cas12b orthologues (AaCas12b, AkCas12b, AmCas12b and BsCas12b) at the mouse *Nrl* gene target site 1. The indel rate is shown under the lane with mutation. mock, an U6 empty vector without crRNA/tracrRNA expression. GFP, an empty backbone vector without Cas12b protein expression. **d** Sanger sequencing results showing the indels in human *RNF2* target site 2 produced by AaCas12b. Blue dashes, deleted bases; red uppercases, PAM. **e** Sanger sequencing results showing the indels in mouse *Nrl* target site 1 produced by AaCas12b. Blue dashes, deleted bases; purple lowercases, insertions or mutations; red uppercases, PAM.

Figure S3

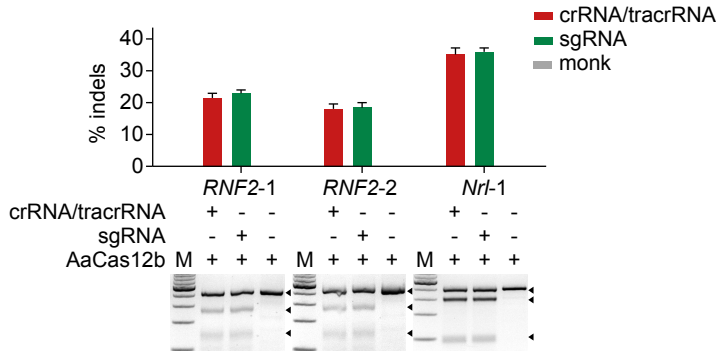
Gene	Sequence (5' - 3')	% indel frequency		
		AaCas12b	AsCas12a	SpCas9
CCR5	GAATTCTTTGGCCTGAATAATTGCAGTAGCTCTAACAGGTTGGACCAA	19.4 ± 0.5	21.1 ± 1.0	18.8 ± 0.8
CCR5	TTCTCCTGAACACCTTCCAGGAATTCTTTGGCCTGAATAATTGCAGTA	10.4 ± 0.9	ND	35.1 ± 1.3
DNMT1	GACTTTTCCTTTTATTTCCCTTCAGCTAAAATAAAGGAGGAGGAAGCT	28.1 ± 2.8	20.1 ± 2.0	16.7 ± 1.1
DNMT1	CGTTAATGTTTCCTGATGGTCCATGTCTGTTACTCGCCTGTCAAGTGG	13.0 ± 0.9	15.6 ± 0.8	21.5 ± 1.2
DYRK1A	CATGAGGTGACCCATTTCCATTCAAGGGTTTTAGAAGCACATCAAGGA	22.0 ± 1.8	ND	24.3 ± 1.5
EMX1	CGATGTCACCTCCAATGACTAGGGTGGGCAACCACAAACCCACGAGGG	5.2 ± 0.8	11.9 ± 1.1	20.1 ± 1.3
RNF2	CCCAATTTGTTTGGATATGTTGAAGAACACCATGACTACAAAGGAGTG	26.7 ± 0.4	12.7 ± 2.1	29.5 ± 3.0
Nrl	ATGGCTTTCCCTCCCAGTCCCTTGGCTATGGAATATGTTAATGACTTT	35.9 ± 0.7	20.9 ± 0.6	30.8 ± 1.2

ND, not detectable

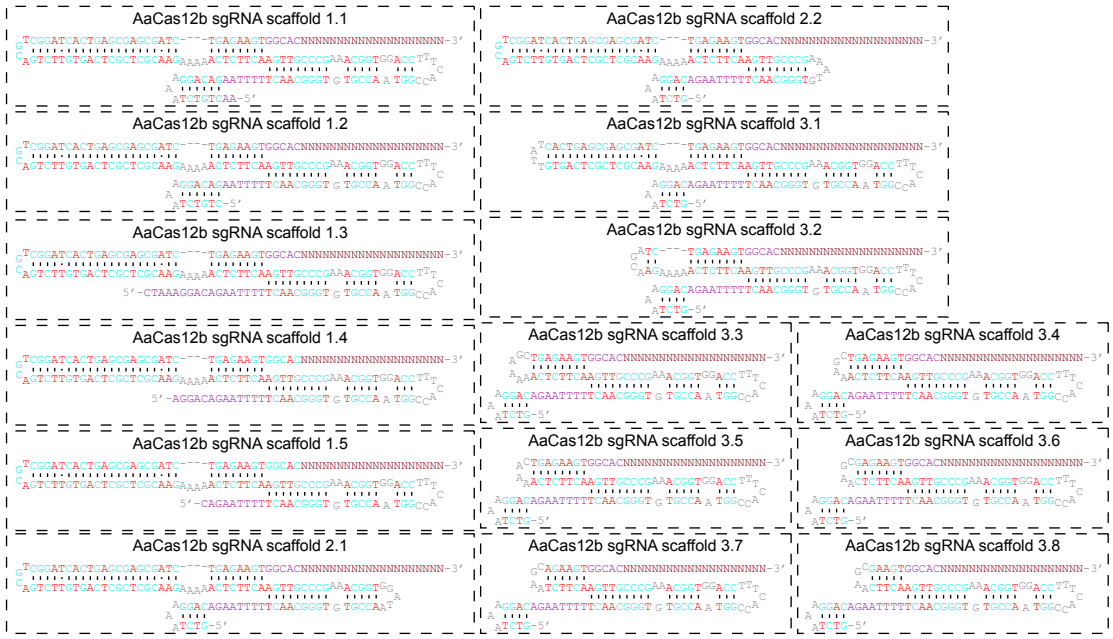
Fig. S3 Frequency of AaCas12b-, AsCas12a- and SpCas9-mediated targeted indel mutations at on-target sites in mammalian cells. Target genomic DNA sequences and the resulted indel frequencies of AaCas12b, AsCas12a and SpCas9 are shown. Target sites for AaCas12b, AsCas12a and SpCas9 are shown and underlined in red, green and blue, respectively. Mutation frequencies were assessed by T7EI assay. Error bars indicate standard errors of the mean (s.e.m.), n = 2.

Figure S4

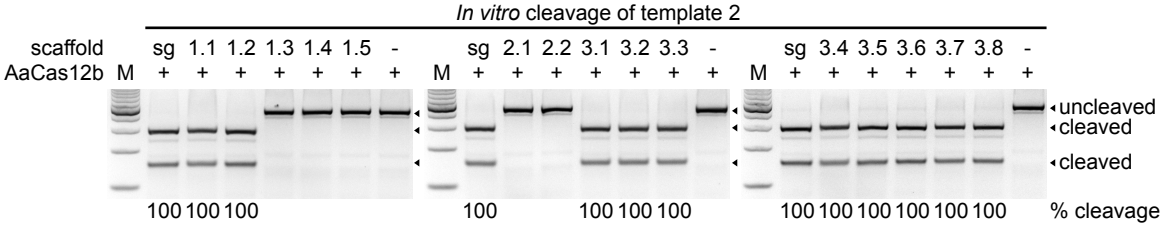
a



b



c



d

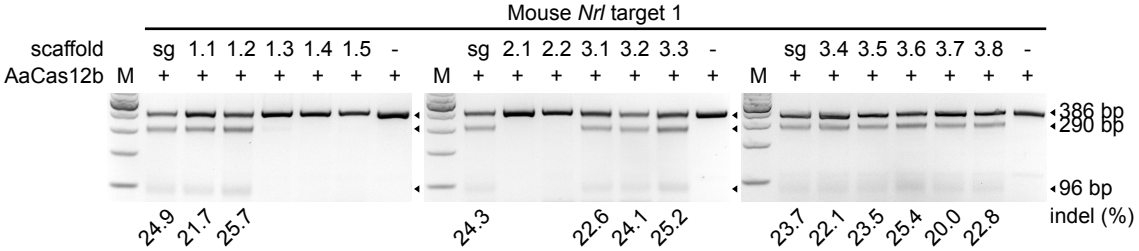
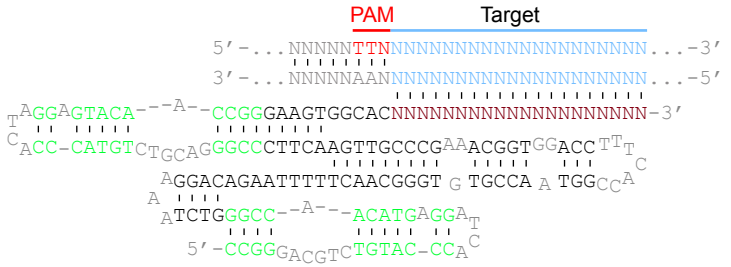


Fig. S4 Engineered AaCas12b chimeric sgRNAs for genome editing. a Indel frequencies induced by AaCas12b directed by crRNA/tracrRNA duplexes and sgRNAs. Indel frequencies are calculated by T7EI assay. Error bars indicate standard errors of the mean (s.e.m.), n = 3. **b** Schematic illustration of 5' truncated sgRNAs on stem loop 1, 2 and 3. **c** *In vitro* DNA cleavage assay of AaCas12b complexed with the optimized sgRNAs. The cleavage rate is shown under the cleaved lanes. **d** Targeting of mouse *Nrl* gene by AaCas12b complexed with the optimized sgRNAs. The indel rate is shown under the lanes with mutation.

Figure S5

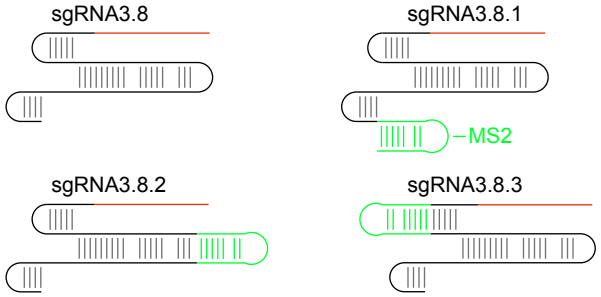
a



b



c



d

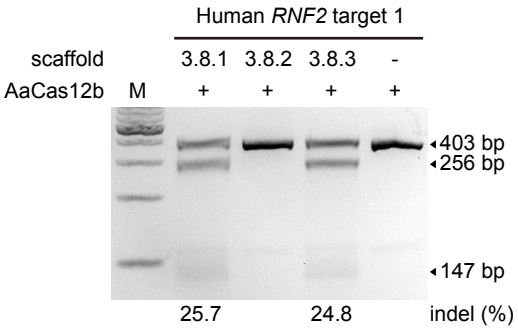
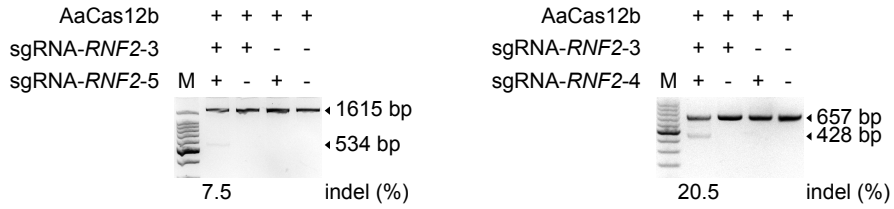


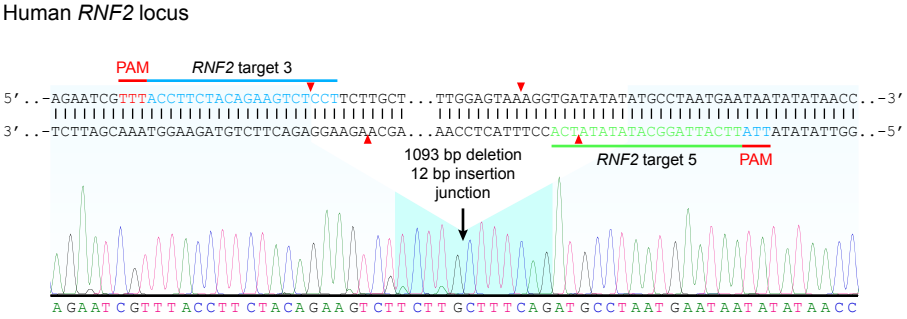
Fig. S5 Engineer MS2 hairpin into sgRNA scaffold. a Schematic illustration of the sgRNA3.8 scaffold constructed with the MS2 RNA hairpin. **b** Alignment of the sequences of sgRNA scaffolds engineered with MS2 RNA hairpin. **c** Schematic illustration of AaCas12b sgRNA scaffolds inserted with the MS2 RNA hairpin. **d** Insertion of the MS2 RNA hairpin into AaCas12b sgRNA stem loop 1 and 3, but not the stem loop 2, maintains the cleavage activity of AaCas12b in human cells. The indel rate is shown under the lanes with mutation.

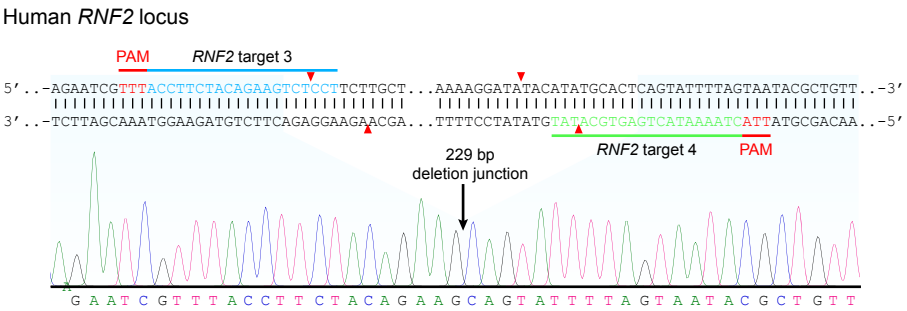
Figure S6

a



b





c

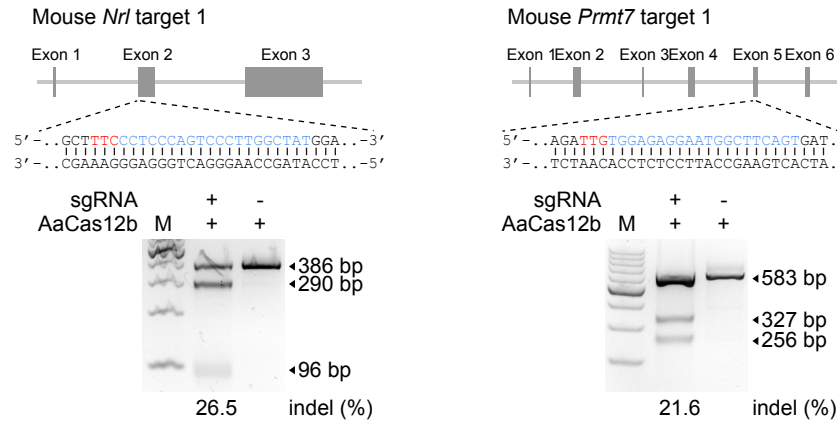


Fig. S6 Multiplex genome editing using AaCas12b combined with sgRNA. **a** AaCas12b-mediated large genomic deletions by simultaneously targeting human *RNF2* target sites 3 and 5 (~1093 bp deletion) (*left*), and human *RNF2* target sites 3 and 4 (~229 bp deletion) (*right*) in 293FT cells. The indel rate is shown under the lanes with mutation. **b** Sanger sequencing results showing the AaCas12b-mediated large genomic deletion by simultaneously targeting human *RNF2* target sites 3 and 5 (~1093 bp deletion) (*left*), and human *RNF2* target sites 3 and 4 (~229 bp deletion) (*right*) in 293FT cells in Fig. S6a. **c** AaCas12b facilitated multiplex genome editing by simultaneously targeting the mouse *Nrl* and *Prmt7* genes using two sgRNAs in the mouse genome. The indel rate is shown under the lanes with mutation.

Figure S7

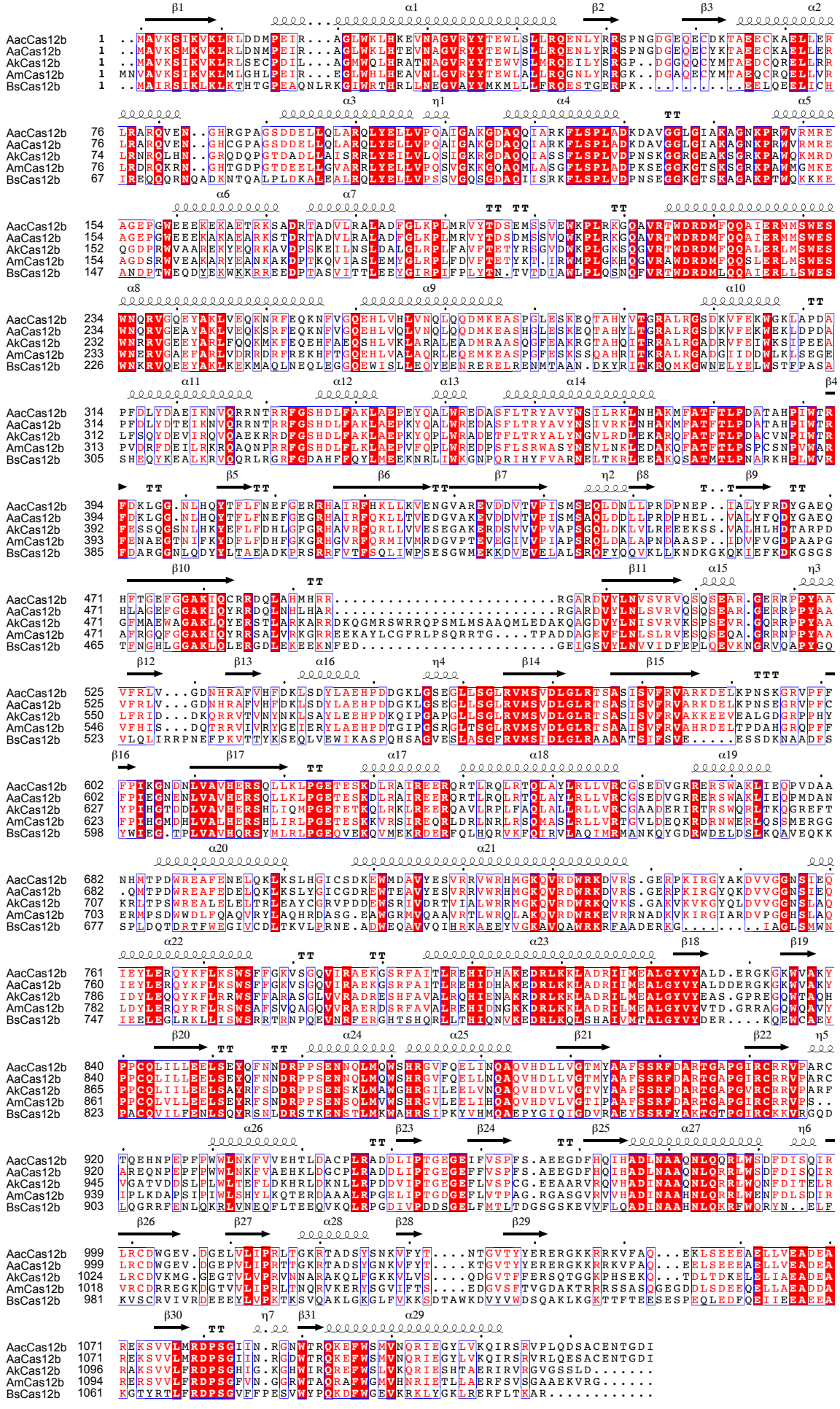


Fig. S7 Protein alignment of Cas12b orthologs. Multiple sequence alignment of amino acid sequences of AaCas12b, AkCas12b, AmCas12b and BsCas12b shows highly conserved residues. Strict identical residues are highlighted with the red background and conserved mutations are highlighted with an outline and red font.

Figure S8

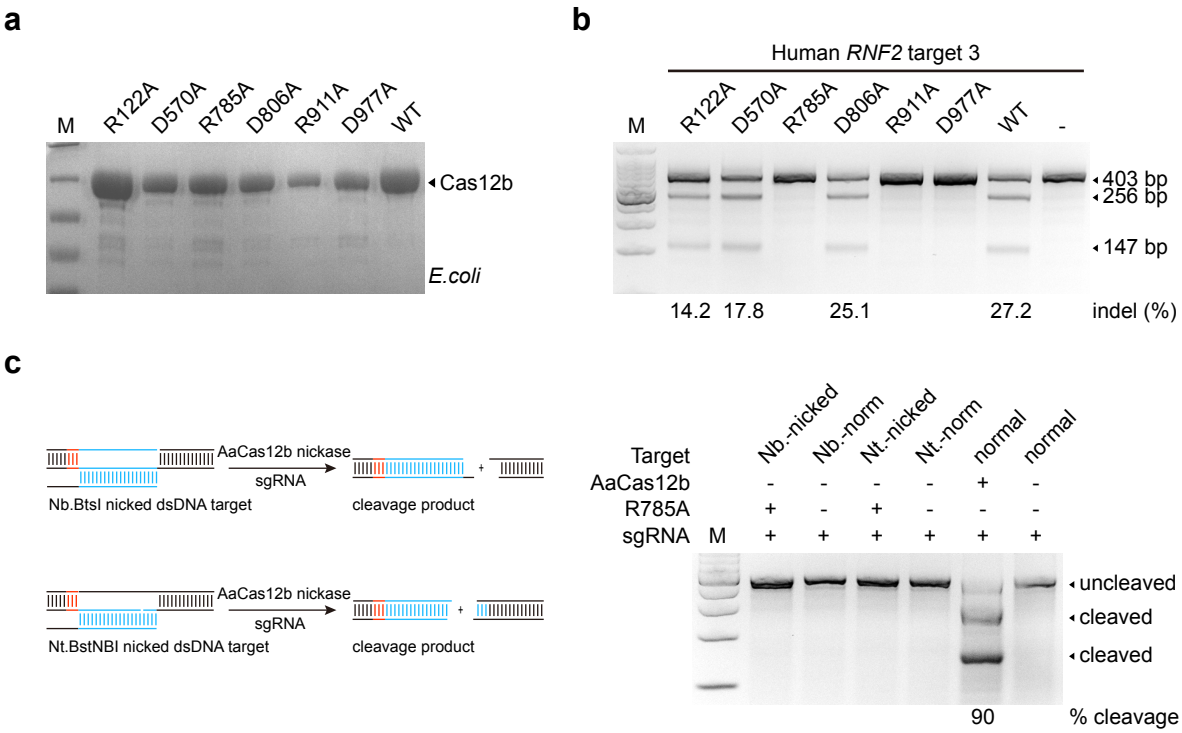

a
M
R122A
D570A
R785A
D806A
R911A
D977A
WT
Cas12b
E.coli
b
Human RNF2 target 3
M
R122A
D570A
R785A
D806A
R911A
D977A
WT
-
403 bp
256 bp
147 bp
14.2
17.8
25.1
27.2
indel (%)
c
AaCas12b nickase
sgRNA
Nb.BtsI nicked dsDNA target
cleavage product
AaCas12b nickase
sgRNA
Nt.BstNBI nicked dsDNA target
cleavage product
Target
AaCas12b
R785A
sgRNA
M
Nb.-nicked
Nb.-norm
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Nt.-norm
normal
normal
uncleaved
cleaved
cleaved
90
% cleavage

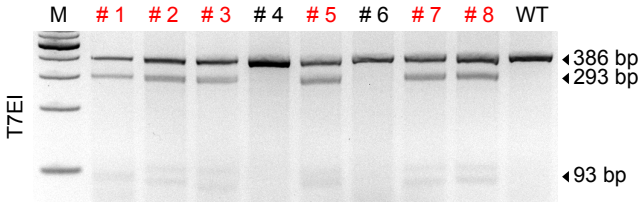
Fig. S8 Catalytic residues required for AaCas12b-mediated DNA cleavage. a Coomassie blue staining of AaCas12b variants (R122A, D570A, R785A, D806A, R911A and D977A) and WT purified from *E. coli*. **b** Effect of mutation of catalytic residues of AaCas12b on DNA targeting in 293FT cells. The indel rate is shown under the lanes with mutation. GFP, an empty backbone vector without Cas12b protein expression. **c** (*Left*) Schematic of *in vitro* cleavage of Nb.BtsI- and Nt.BstNBI-nicked dsDNA fragments using site-directed mutated AaCas12b. (*Right*) *In vitro* nicked dsDNA cleavage analysis of the catalytic residue R785A of AaCas12b. The cleavage rate is shown under the cleaved lanes.

Figure S9

a

Target gene	No. of injected embryos (%)	No. of 2-cell stage embryos (%)	No. of blastocysts (%)	Mutation ratio (%) (no. of mutated/total blastocysts)
Nrl	16	12 (75)	8 (50)	75 (6/8)

b



c

```
Embryo # 1                                                                                    Indel (bp)
CAGTCCCAGAATGGCTTTCCCTCCCAGTCCCTTGGCTATGGAATATGTTAATGACTTTGATTTGATGAAGTTCGAAAT  WT
CAGTCCCAGAATGGCTTTCCCTCCCAGTCCCTTGGCTATG-------------ACTTTGATTTGATGAAGTTCGAAAT  Δ13
CAGTCCCAGAATGGCTTTCCCTCCCAGTC--------------------attaACTTTGATTTGATGAAGTTCGAAAT  Δ24, +4
CAGTCCCAGAATGGCTTT-----------------------------------------GATTTGATGAAGTTCGAAAT  Δ40
CAGTCCCAGAATG-------------------------(Δ 233 bp)------------------------------  Δ233

Embryo # 2
CAGTCCCAGAATGGCTTTCCCTCCCAGTCCCTTGGCTATGGAATATGTTAATGACTTTGATTTGATGAAGTTCGAAAT  WT
CAGTCCCAGAATGGCTTTCCCTCCCAGTCCCTTGGg---GGAATATGTTAATGACTTTGATTTGATGAAGTTCGAAAT  Δ4, +1
CAGTCCCAGAATGGCTTTCCCTCCCAGTCCC-----TATGGAATATGTTAATGACTTTGATTTGATGAAGTTCGAAAT  Δ5
CAGTCCCAGAATGGCTTTCCCTCCCAGTCCCTTGG-----------GTTAATGACTTTGATTTGATGAAGTTCGAAAT  Δ11

Embryo # 3
CAGTCCCAGAATGGCTTTCCCTCCCAGTCCCTTGGCTATGGAATATGTTAATGACTTTGATTTGATGAAGTTCGAAAT  WT
CAGTCCCAGAATGGCTTTCCCTCCCAGTCCCTTG--TATGGAATATGTTAATGACTTTGATTTGATGAAGTTCGAAAT  Δ2
CAGTCCCAGAATGGCTTTCCCTCCCAGTCCCTTGGCTA---AATATGTTAATGACTTTGATTTGATGAAGTTCGAAAT  Δ3
CAGTCCCAGAATGGCTTTCCCTCCCAGTCCCTTGtt-----AATATGTTAATGACTTTGATTTGATGAAGTTCGAAAT  Δ7, +2
CAGTCCCAGAATGGCTTTCCCTCCCAGTCCCT--------agATATGTTAATGACTTTGATTTGATGAAGTTCGAAAT  Δ10, +2

Embryo # 5
CAGTCCCAGAATGGCTTTCCCTCCCAGTCCCTTGGCTATGGAATATGTTAATGACTTTGATTTGATGAAGTTCGAAAT  WT
CAGTCCCAGAATGGCTTTCCCTCCCAGTCCCTT--------AATATGTTAATGACTTTGATTTGATGAAGTTCGAAAT  Δ8
CAGTCCCAGAATGGCTTTCCCTCCCAGTCCCT----------ATATGTTAATGACTTTGATTTGATGAAGTTCGAAAT  Δ10
CAGTCtctg-----------------------------(Δ 95 bp)--------------------------------  Δ95
CAGTCCCAGAATGGCTTTCCCTCCCAGT----------------(Δ 132 bp)-----------------------  Δ132

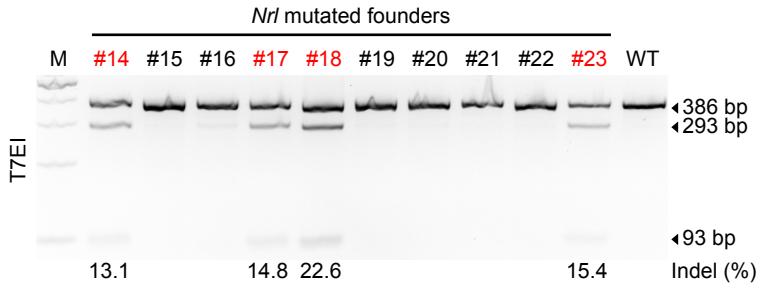
Embryo # 7
CAGTCCCAGAATGGCTTTCCCTCCCAGTCCCTTGGCTATGGAATATGTTAATGACTTTGATTTGATGAAGTTCGAAAT  WT
CAGTCCCAGAATGGCTTTCCCTCCCAGTCCCTT-----TGGAATATGTTAATGACTTTGATTTGATGAAGTTCGAAAT  Δ5
CAGTCCCAGAATGGCTTTCCCTCCCAGTCCCTT------GGAATATGTTAATGACTTTGATTTGATGAAGTTCGAAAT  Δ6
CAGTCCCAGAATGGCTTTCCCTCCCAGTCCCTTG--------cTATGTTAATGACTTTGATTTGATGAAGTTCGAAAT  Δ9, +1
CAGTCCCAGAATGGCTTTCCCTCCCAGTCCCT---------AATATGTTAATGACTTTGATTTGATGAAGTTCGAAAT  Δ9
CAGTCCCAGAATGGCTTTCCCTCCCAGTCCCTT-----------ATGTTAATGACTTTGATTTGATGAAGTTCGAAAT  Δ11

Embryo # 8
CAGTCCCAGAATGGCTTTCCCTCCCAGTCCCTTGGCTATGGAATATGTTAATGACTTTGATTTGATGAAGTTCGAAAT  WT
CAGTCCCAGAATGGCTTTCCCTCCCAGTCCCTTG-CTATGGAATATGTTAATGACTTTGATTTGATGAAGTTCGAAAT  Δ1
CAGTCCCAGAATGGCTTTCCCTCCCAGTCCC------ATGGAATATGTTAATGACTTTGATTTGATGAAGTTCGAAAT  Δ6
CAGTCCCAGAATGGCTTTCCCTCCCAGTCCCT-----------TATGTTAATGACTTTGATTTGATGAAGTTCGAAAT  Δ11
CAGTCCCAGAATGGCT---------------------ATGGAATATGTTAATGACTTTGATTTGATGAAGTTCGAAAT  Δ21
```

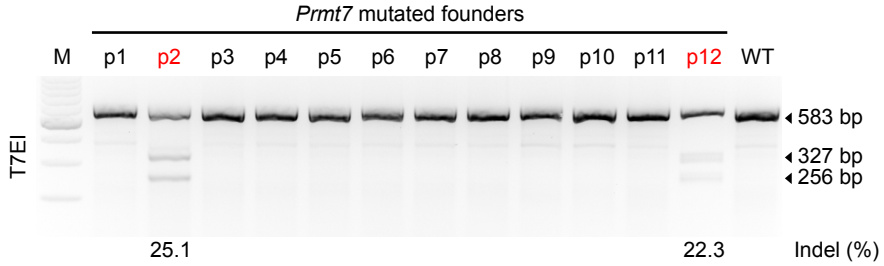
Figure S9. Mutated embryos generated by AaCas12b RNP microinjection. a Summary of mutated embryos generated by preassembled AaCas12b RNP microinjection. **b** T7EI-based genotyping analysis of mouse embryos with AaCas12b RNP injected. The red font highlighted numbers denote induced mutants. **c** Representative indels in mutated embryos in Fig. S9b. Blue dashes, deleted bases; purple lowercases, insertions or mutations; red uppercases, PAM.

Figure S10

a



b



c

	Indel (bp)	Frequency (%)
Mouse #1		
CAGTCCCAGAATGGCTTTCCCTCCCAGTCCCTTGGCTATGGAATATGTTAATGACTTTGATTTGATGAAGTTCGAAAT	WT	
CAGTCCCAGAATGGCTTTCCCTCCCAGTC----------TAATGACTTTGATTTGATGAAGTTCGAAAT	Δ19	4.8 (1/21)
CAGTCCCAGAATGGCTTTCCC----------aaAATGACTTTGATTTGATGAAGTTCGAAAT	Δ28, +2	19.0 (4/23)
Mouse #4		
CAGTCCCAGAATGGCTTTCCCTCCCAGTCCCTTGGCTATGGAATATGTTAATGACTTTGATTTGATGAAGTTCGAAAT	WT	
CAGTCCCAGAATGGCTTTCCCTCCCAGTCCCTTGG-----GAATATGTTAATGACTTTGATTTGATGAAGTTCGAAAT	Δ5	28.6 (6/21)
Mouse #5		
CAGTCCCAGAATGGCTTTCCCTCCCAGTCCCTTGGCTATGGAATATGTTAATGACTTTGATTTGATGAAGTTCGAAAT		
CAGTCCCAGAATGGCTTTCCCTCCCAGTCC----------ATGTTAATGACTTTGATTTGATGAAGTTCGAAAT	Δ14	17.2 (5/19)
CAGTCCCAGAATGGCTTTCCCTCCCAGTCCC----------GTTAATGACTTTGATTTGATGAAGTTCGAAAT	Δ15	10.3 (3/19)
Mouse #8		
CAGTCCCAGAATGGCTTTCCCTCCCAGTCCCTTGGCTATGGAATATGTTAATGACTTTGATTTGATGAAGTTCGAAAT	WT	
CAGTCCCAGAATGGCTTTCCCTCCCAGTC----------ATGTTAATGACTTTGATTTGATGAAGTTCGAAAT	Δ15	26.0 (7/26)
Mouse #9		
CAGTCCCAGAATGGCTTTCCCTCCCAGTCCCTTGGCTATGGAATATGTTAATGACTTTGATTTGATGAAGTTCGAAAT	WT	
CAGTCCCAGAATGGCTTTCCCTCCCAGTCCCT----------TGTTAATGACTTTGATTTGATGAAGTTCGAAAT	Δ13	28 (7/25)
Mouse #10		
CAGTCCCAGAATGGCTTTCCCTCCCAGTCCCTTGGCTATGGAATATGTTAATGACTTTGATTTGATGAAGTTCGAAAT	WT	
CAGTCCCAGAATGGCTTTCCCTCCCAGTCCCTT-------GAATATGTTAATGACTTTGATTTGATGAAGTTCGAAAT	Δ7	10.3 (3/29)
CAGTCCCAGAATGGCTTTCCCTCCCAGTCC----------ATATGTTAATGACTTTGATTTGATGAAGTTCGAAAT	Δ12	6.7 (2/29)
CAGTCCCAGAATGGCTTTCCCTCCCAGTCCC----------TATGTTAATGACTTTGATTTGATGAAGTTCGAAAT	Δ12	17.3 (5/29)
Mouse #14	Indel (bp)	Frequency (%)
CAGTCCCAGAATGGCTTTCCCTCCCAGTCCCTTGGCTATGGAATATGTTAATGACTTTGATTTGATGAAGTTCGAAAT	WT	
CAGTCCCAGAATGGCTTTCCCTCCCAGTCCCTTGGCTATt---TATGTTAATGACTTTGATTTGATGAAGTTCGAAAT	Δ4, +1	15 (3/20)
Mouse #17		
CAGTCCCAGAATGGCTTTCCCTCCCAGTCCCTTGGCTATGGAATATGTTAATGACTTTGATTTGATGAAGTTCGAAAT	WT	
CAGTCCCAGAATGGCTTTCCCTCCCAGTCCCTTGtt-----AATATGTTAATGACTTTGATTTGATGAAGTTCGAAAT	Δ7, +2	21.7 (5/23)
Mouse #18		
CAGTCCCAGAATGGCTTTCCCTCCCAGTCCCTTGGCTATGGAATATGTTAATGACTTTGATTTGATGAAGTTCGAAAT	WT	
CAGTCCCAGAATGGCTTTCCCTCCCAGTCCCTTG--------ATATGTTAATGACTTTGATTTGATGAAGTTCGAAAT	Δ8	54.5 (12/22)
Mouse #23		
CAGTCCCAGAATGGCTTTCCCTCCCAGTCCCTTGGCTATGGAATATGTTAATGACTTTGATTTGATGAAGTTCGAAAT	WT	
CAGTCCCAGAATGGCTTTCCCTCCCAGTCCC----------ATGTTAATGACTTTGATTTGATGAAGTTCGAAAT	Δ13	50 (12/24)

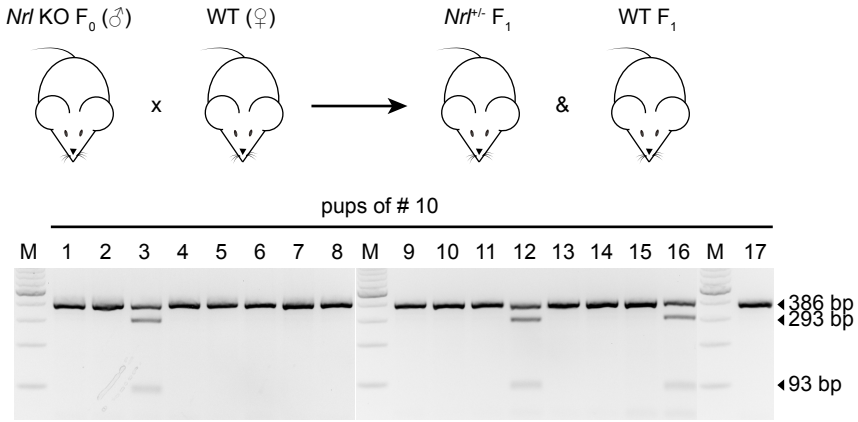
d

	Indel (bp)	Frequency (%)
Mouse p4		
GAGGCTGCTGTGAAGATTGTGGAGAGGAATGGCTTCAGTGATAAGATTAAAGTCATTAACAAGCACTCCACTGAGGTG	WT	
GAGGCTGCTGTGAAGATTGTGGAGAGGA----------gggaaaatgagcCATTAACAAGCACTCCACTGAGGTG	Δ25, +12	37.8 (14/37)
Mouse p12		
GAGGCTGCTGTGAAGATTGTGGAGAGGAATGGCTTCAGTGATAAGATTAAAGTCATTAACAAGCACTCCACTGAGGTG	WT	
GAGGCTGCTGTGAAGATTGTGGAGAGGAATGGCT-----GATAAGATTAAAGTCATTAACAAGCACTCCACTGAGGTG	Δ5	66.7 (16/24)

Fig. S10 Mutated mice generated by AaCas12b RNP microinjection. a *Nrl* mutated founder mice generated by microinjection of AaCas12b RNPs. The numbers in red denote newborn mice with induced indel mutations. The indel rate is shown under the lanes with mutation. **b** *Prmt7* mutated founder mice generated by microinjection of AaCas12b RNPs. The numbers in red denote newborn mice with induced indel mutations. The indel rate is shown under the lanes with mutation. **c** Sanger sequencing of targeted *Nrl* alleles in mutated mice induced by injection of AaCas12b RNPs in Fig. 5d and S10a. Blue dashes, deleted bases; purple lowercases, insertions or mutations; red uppercases, PAM. Indel frequencies are indicated. **d** Sanger sequencing of targeted *Prmt7* alleles in mutated mice induced by injection of AaCas12b RNPs in Fig. S10b. Blue dashes, deleted bases; purple lowercases, insertions or mutations; red uppercases, PAM. Indel frequencies are indicated.

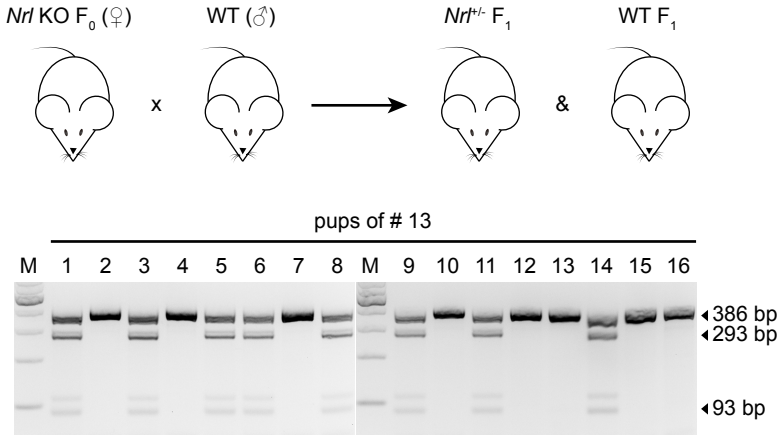
Figure S11

a




```
WT  CCAGAATGGCTTTCCCTCCCAGTCCCTTGGCTATGGAATATGTTAATGACTTTGATTTGATGAAGTTCG
3   CCAGAATGGCTTTCCCTCCCAGTCCCTT-------GAATATGTTAATGACTTTGATTTGATGAAGTTCG  Δ7
12  CCAGAATGGCTTTCCCTCCCAGTCCCTT-------GAATATGTTAATGACTTTGATTTGATGAAGTTCG  Δ7
16  CCAGAATGGCTTTCCCTCCCAGTCCCTT-------GAATATGTTAATGACTTTGATTTGATGAAGTTCG  Δ7
```

b




```
WT  CCAGAATGGCTTTCCCTCCCAGTCCCTTGGCTATGGAATATGTTAATGACTTTGATTTGATGAAGTTCG
1   CCAGAATGGCTTTCCCTCCCAGTCCCTT------------------------TGATTTGATGAAGTTCG  Δ24
3   CCAGAATGGCTTTCCCTCCCAGTCCCTT------------------------TGATTTGATGAAGTTCG  Δ24
5   CCAGAATGGCTTTCCCTCCCAGTCCCTT------------------------TGATTTGATGAAGTTCG  Δ24
6   CCAGAATGGCTTTCCCTCCCAGTCCCTT------------------------TGATTTGATGAAGTTCG  Δ24
8   CCAGAATGGCTTTCCCTCCCAGTCCCTT------------------------TGATTTGATGAAGTTCG  Δ24
9   CCAGAATGGCTTTCCCTCCCAGTCCCTT------------------------TGATTTGATGAAGTTCG  Δ24
11  CCAGAATGGCTTTCCCTCCCAGTCCCTT------------------------TGATTTGATGAAGTTCG  Δ24
14  CCAGAATGGCTTTCCCTCCCAGTCCCTT------------------------TGATTTGATGAAGTTCG  Δ24
```

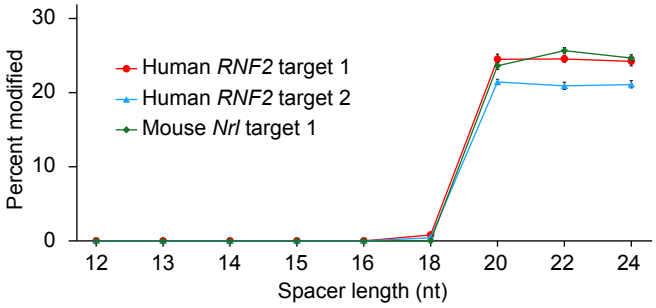
Fig. S11 Successful germline transmission of mutated founders. a *Nrl*-mutant male founder #10 crossed with WT female mouse (*top*) and the genotypes of the pups were determined by T7EI assay (*middle*). Sanger sequencing reads represent genotypes of mutants (*bottom*). **b** *Nrl*-mutant female founder #13 crossed with WT male mouse (*top*) and the genotypes of the pups were determined by T7EI assay (*middle*). Sanger sequencing reads represent genotypes of mutants (*bottom*).

Figure S12

a

Human *RNF2* target 2

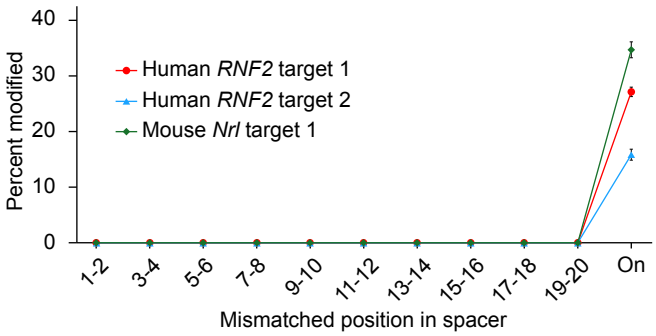
sgRNA	5′-...GGCACNNNNNNNNNNNNNNNNNNNN-3′
24 nt	TAGTCATGGTGTTCTTCAACATAT
22 nt	TAGTCATGGTGTTCTTCAACAT
20 nt (WT)	TAGTCATGGTGTTCTTCAAC
18 nt	TAGTCATGGTGTTCTTCA
16 nt	TAGTCATGGTGTTCTT
15 nt	TAGTCATGGTGTTCT
14 nt	TAGTCATGGTGTTC
13 nt	TAGTCATGGTGTT
12 nt	TAGTCATGGTGT



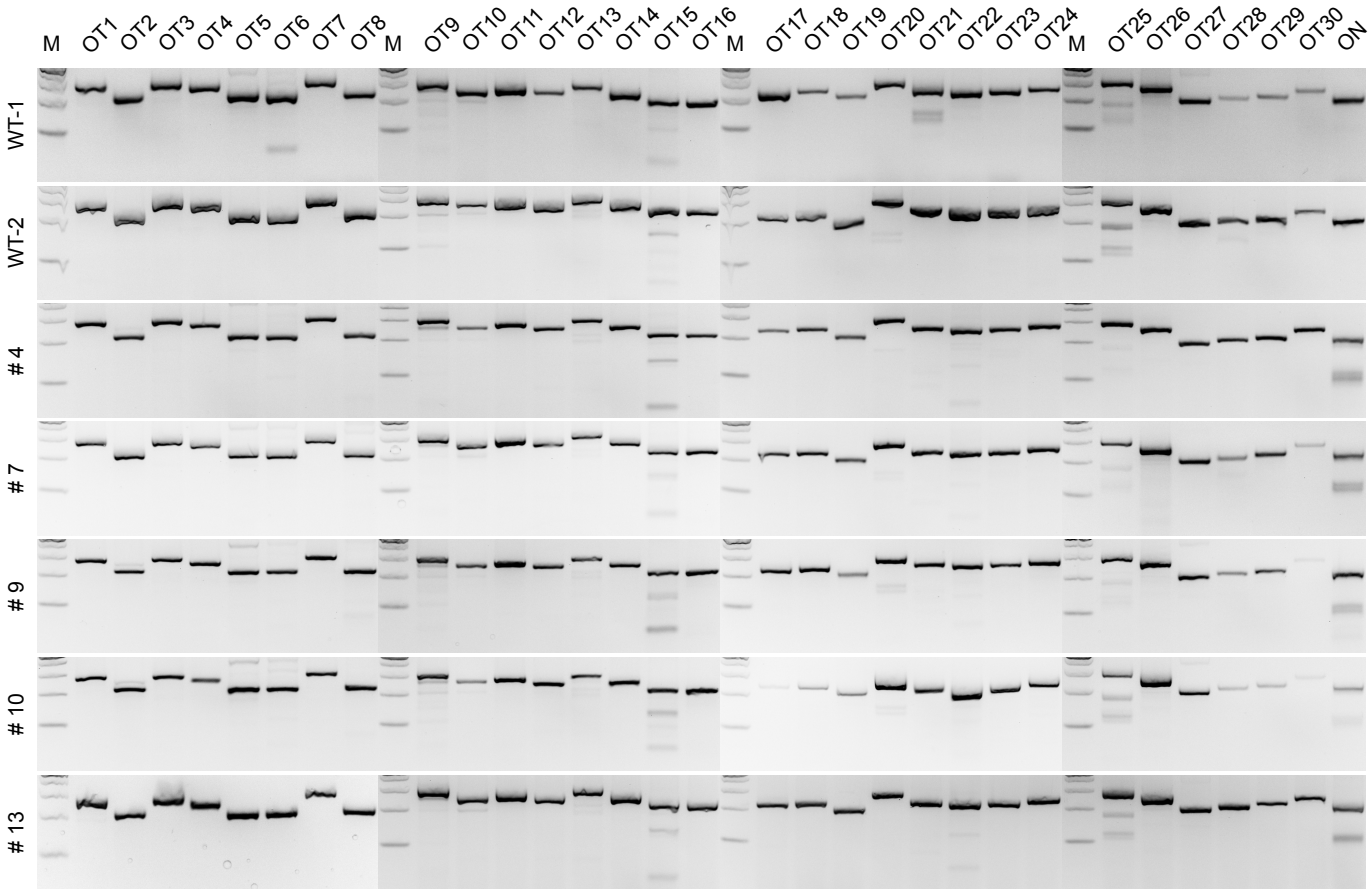
b

Human *RNF2* target 2

sgRNA	5′-...GGCACNNNNNNNNNNNNNNNNNNNN-3′
WT (On)	TAGTCATGGTGTTCTTCAAC
mut_1-2	gcGTCATGGTGTTCTTCAAC
mut_3-4	TAtgCATGGTGTTCTTCAAC
mut_5-6	TAGTacTGGTGTTCTTCAAC
mut_7-8	TAGTCAgtGTGTTCTTCAAC
mut_9-10	TAGTCATGtgGTTCTTCAAC
mut_11-12	TAGTCATGGTtgTCTTCAAC
mut_13-14	TAGTCATGGTGTgaTTCAAC
mut_15-16	TAGTCATGGTGTTCggCAAC
mut_17-18	TAGTCATGGTGTTCTTacAC
mut_19-20	TAGTCATGGTGTTCTTCAca



c



d

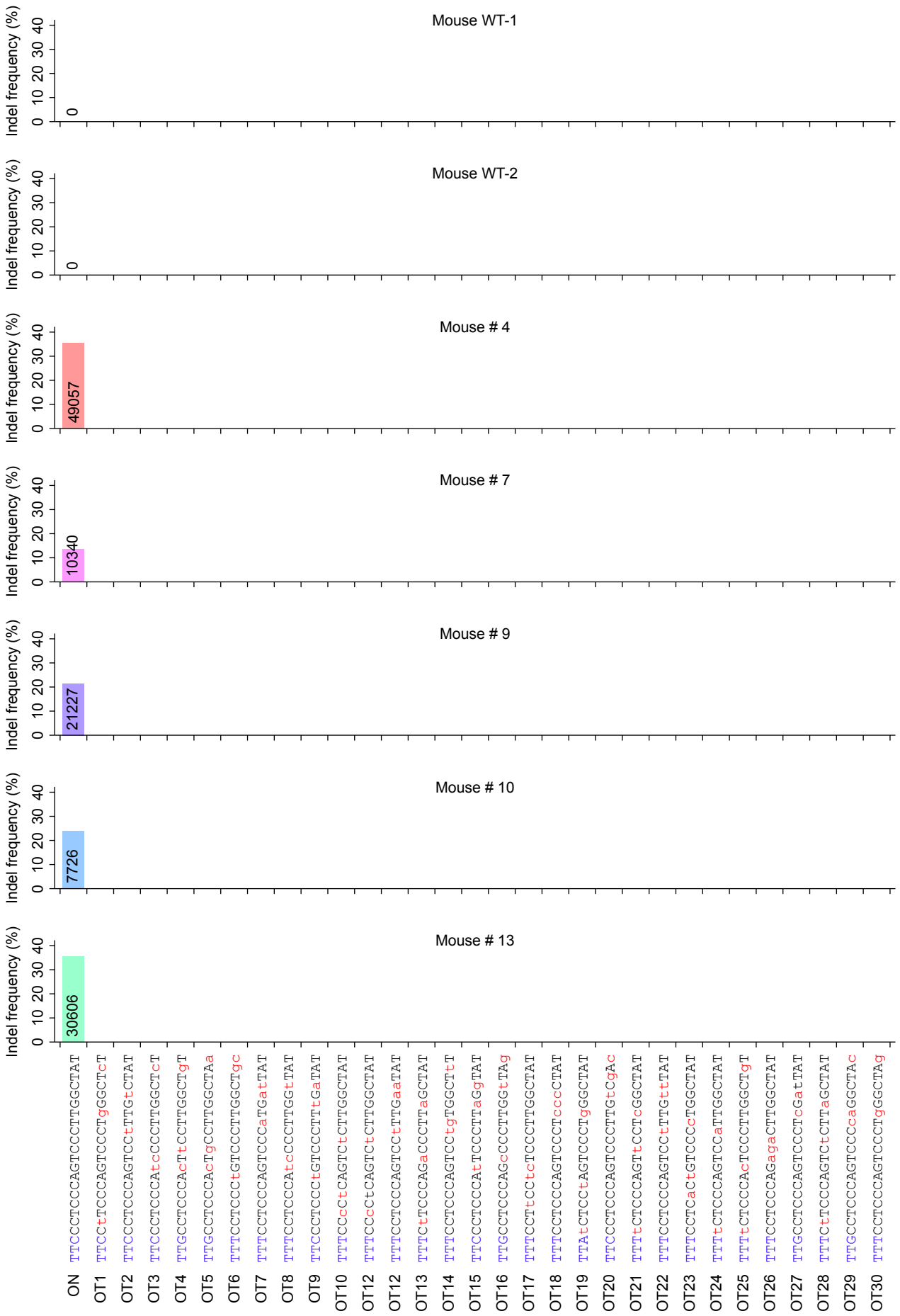

Mouse WT-1
Mouse WT-2
Mouse # 4
Mouse # 7
Mouse # 9
Mouse # 10
Mouse # 13
Indel frequency (%)
0 10 20 30 40
0
49057
10340
21227
7726
30606
ON TTCCCTCCCAGTCCCTTGGCTAT
OT1 TTCCtTCCCAGTCCCTgGGCTcT
OT2 TTCCCTCCCAGTCCtTTGtCTAT
OT3 TTCCCTCCCAtccCCCTTGGCTcT
OT4 TTGCCTCCCAcTtCCTTGGCTgT
OT5 TTGCCTCCCAcTgCCTTGGCTAa
OT6 TTTCCTCCCtGTCCCTTGGCTgc
OT7 TTTCCTCCCAGTCCCaTGatTAT
OT8 TTTCCTCCCAtcCCCTTGGtTAT
OT9 TTCCCTCCCtGTCCCTTtGaTAT
OT10 TTTCCCCtCAGTCtCTTGGCTAT
OT12 TTTCCCCtCAGTCtCTTGGCTAT
OT12 TTTCCTCCCAGTCCtTTGaaTAT
OT13 TTTCtTCCCAGaCCCTTaGCTAT
OT14 TTTCCTCCCAGTCCtgTGGCTtT
OT15 TTCCCTCCCAtTCCCTTaGgTAT
OT16 TTGCCTCCCAGcCCCTTGGtTAg
OT17 TTTCCTtCCtcTCCCTTGGCTAT
OT18 TTTCCTCCCAGTCCCTcccCTAT
OT19 TTAtCTCCtAGTCCCTgGGCTAT
OT20 TTCCCTCCCAGTCCCTTGtCgAc
OT21 TTTtCTCCCAGTtCCTcGGCTAT
OT22 TTTCCTCCCAGTCCtTTGttTAT
OT23 TTTCCTCaCtGTCCCcTGGCTAT
OT24 TTTtCTCCCAGTCCaTTGGCTAT
OT25 TTTtCTCCCAcTCCCTTGGCTgT
OT26 TTTCCTCCCAGagaCTTGGCTAT
OT27 TTGCCTCCCAGTCCCTcGatTAT
OT28 TTTCtTCCCAGTCtCTTaGCTAT
OT29 TTGCCTCCCAGTCCCcaGGCTAc
OT30 TTTCCTCCCAGTCCCTgGGCTAg

Fig. S12 Analysis of off-target effects in mammalian genomes. a Effects of spacer length on AaCas12b/sgRNA cleavage activity in human and mouse cells. Error bars indicate standard errors of the mean (s.e.m.), n = 3. **b** Analysis of cleavage specificity of AaCas12b/sgRNA in human and mouse cells using sgRNAs carrying double base-pair mismatches in the guide sequence. Error bars indicate s.e.m., n = 3. **c** T7EI analysis of the 30 potential off-targets related to *Nrl* target in the mouse genome. Two WT mice and five indicated founder mice were applied for detection and no detectable off-target effects existed by T7EI assay. **d** Pooled PCR products containing the 30 off-target sites per mice were subjected to deep sequencing. Sequences containing insertions and deletions around the cleavage site were considered to be AaCas12b-induced mutations.

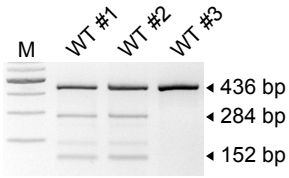
Figure S13

a

Sample-specific variant observed in WGS data

	Chromosome	2
	Position	151978463
	Reference	ATTCTCACCCAGACCCTTTGCT-AGTCATC
	Alternate	ATTCTCACCCAGACCCTTTGCTcAGTCATC
Frequency	#4 (♀)	41.0% (16 / 39)
	# 7 (♀)	43.2% (16 / 37)
	# 9 (♂)	40% (14 / 35)
	# 10 (♂)	100% (19 / 19)

b

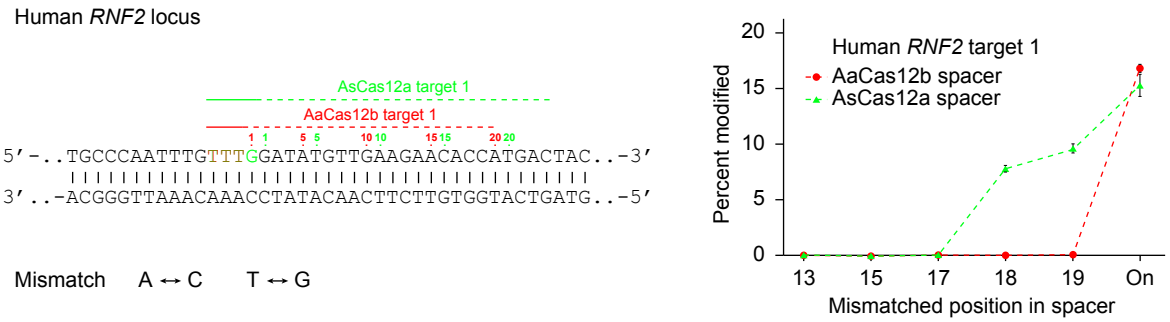



```
Reference  TGTGTAATTCTCACCCAGACCCTTTGCT-AGTCATCTATGTT
WT #1      TGTGTAATTCTCACCCAGACCCTTTGCTcAGTCATCTATGTT
WT #2      TGTGTAATTCTCACCCAGACCCTTTGCTcAGTCATCTATGTT
WT #3      TGTGTAATTCTCACCCAGACCCTTTGCT-AGTCATCTATGTT
```

Fig. S13 Sample-specific variant is not induced by off-target effects. a Sample-specific variant observed in WGS data. All the four *Nrl*-mutated founders contain the same variant and the indel frequencies are shown. **b** T7EI assay (*top*) and Sanger sequencing (*bottom*) showing the sample-specific variant in Fig. 6e also observed in wild-type siblings (WT #1 and #2).

Figure S14

a



b

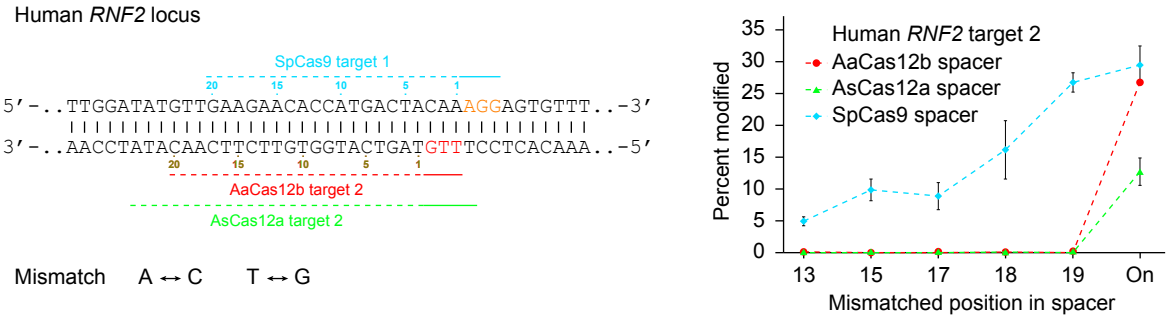
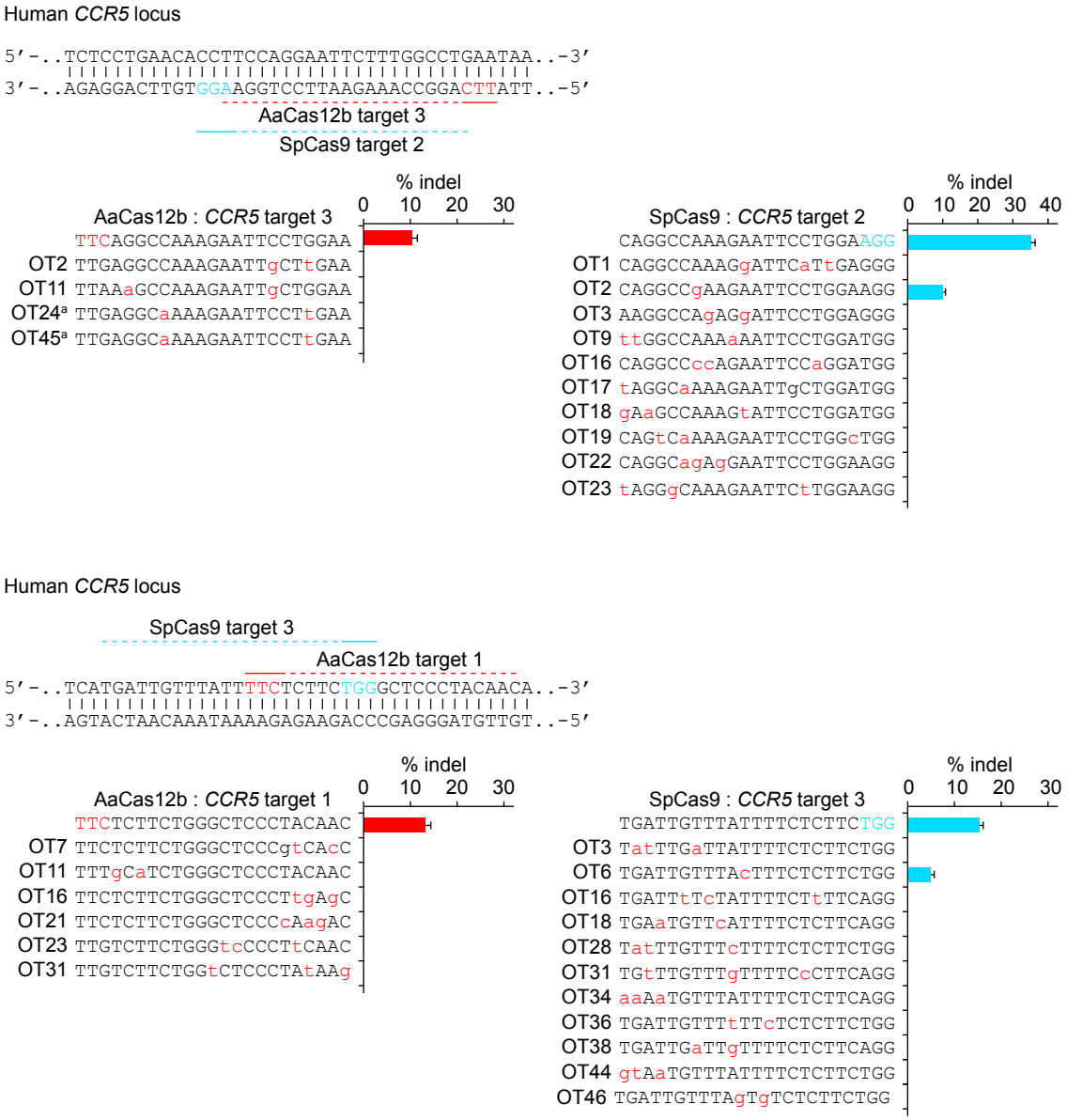


Fig. S14 Comparison of off-target effects of AaCas12b, AsCas12a and SpCas9 in mammalian cells. a-b (*Left*) Schematic showing the targeting sequences of AaCas12b, AsCas12a and SpCas9 in human *RNF2* locus. (*Right*) Activities of AaCas12b, AsCas12a and SpCas9 targeted to the human *RNF2* locus using respective guide RNAs with single mismatches in human 293FT cells. Mutation frequencies were assessed by T7EI assay. Error bars indicate standard errors of the mean (s.e.m.), n = 3.

Figure S15

a



b

Fig. S15 AaCas12b generates minimal genome-wide off-target effects compared to SpCas9. a-b (*Upper*) Schematic showing the targeting sequences of AaCas12b and SpCas9 in the human *CCR5* locus. (*Lower*) Frequencies of induced indels at on- and off-target sites by AaCas12b and SpCas9 in human 293FT cells. Mutation frequencies were assessed by T7EI assay. Error bars indicate standard errors of the mean (s.e.m.), n = 2.

Table S1. Oligonucleotides (oligos) used for Cas12b coding gene and guide RNA synthesis.

See separate Excel file

Table S2. Target sequences bearing various 5' PAM sequences used for *in vitro* DNA cleavage assay. Target sequences used for in vitro DNA cleavage analysis of PAM sequences were commercially synthesized with EcoRI 5' and SphI 3' overhangs highlighted with yellow and green backgrounds, respectively. Annealed oligos were constructed into EcoRI and SphI double-digested p11-LacY-wtx1 vector.

See separate Excel file

Table S3. Protospacer sequences of mammalian genomic targets. Protospacer targets designed based on CRISPR-Cas12b loci with their requisite PAMs against different genes in human and mouse genomes. The PAMs are highlighted in blue uppercase and mismatches in red lowercase.

See separate Excel file

Table S4. Frequency of AaCas12b-induced indel mutations at potential off-target sites in mammalian genomes. Sites in the human and mouse genomes bearing 1 to 3 mismatches from the *CCR5*, *RNF2* and *Nrl* on-target sites for AaCas12b were detected by targeted deep sequencing. PAMs were highlighted in red, and mismatches were within lowercase.

See separate Excel file

Table S5. Frequency of AaCas12b- and SpCas9-induced indel mutations at potential off-target sites in human 293FT cells. Sites in the human genome bearing 1 to 3 mismatches from the *CCR5* and *RNF2* on-target sites for AaCas12b and SpCas9 were detected by T7EI assay. PAMs were highlighted in red, and mismatches were within lowercase.

See separate Excel file

Table S6. List of primer sequences used in this study.

See separate Excel file

Supplementary Sequences

Accession information for the four Cas12b orthologs and vectors used in this study.

Cas12b coding sequence from *Alicyclobacillus acidiphilus* NBRC 100859 (GeneBank ID: NZ_BCQI01000053.1)

ATGGCCGTTAAATCCATGAAAGTGAAACTTCGCCTCGATAATATGCCGGAGATTCGGGCTGG
TTTATGGAAACTCCATACGGAGGTCAACGCGGGGGTTCGATATTACACGGAATGGCTGAGTC
TTCTGCGTCAAGAGAATTTGTATCGAAGAAGTCCGAATGGGGACGGAGAGCAAGAATGTTAT
AAGACTGCAGAAGAATGCAAAGCCGAATTGTTGGAGCGGCTGCGCGCGCGTCAAGTGGAGAA
TGGACACTGTGGTCCGGCGGGATCGGACGATGAATTGCTGCAGTTGGCTCGTCAACTTTATG
AACTGTTGGTTCCGCAGGCGATAGGTGCGAAAGGCGATGCGCAGCAAATTGCGCGCAAGTTT
TTGAGCCCCTTAGCCGACAAGGATGCAGTGGGTGGGCTTGGAATCGCGAAGGCGGGGAACAA
ACCGCGGTGGGTTCGCATGCGCGAAGCGGGAGAACCTGGCTGGGAAGAGGAGAAGGCGAAGG
CTGAGGCGAGGAAATCTACGGATCGAACTGCGGATGTTTTGCGCGCGCTCGCGGATTTTGGG
TTAAAGCCACTGATGCGCGTGTACACCGATTCTGACATGTCATCTGTTCAGTGGAAACCGCT
TCGGAAGGGCCAAGCGGTTCGGACGTGGGACAGGGATATGTTCCAACAGGCCATCGAGCGGA
TGATGTCGTGGGAGTCGTGGAATCAGCGCGTTGGCGAAGCGTACGCGAAACTGGTAGAGCAA
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GTTGCAACAAGATATGAAAGAAGCATCGCACGGGCTCGAATCGAAAGAGCAAACCGCACATT
ATCTGACGGGACGGGCATTGCGCGGATCGGACAAAGTGTTTGAGAAGTGGGAGAAACTCGAC
CCTGATGCGCCATTCGATTTGTACGACACCGAAATCAAGAACGTGCAGAGACGTAACACGAG
GCGATTCGGCTCACACGACTTGTTCGCGAAATTGGCGGAACCGAAGTATCAGGCCCTGTGGC
GCGAAGATGCTTCGTTTCTCACGCGTTACGCGGTGTACAACAGCATCGTTCGCAAACTGAAT
CACGCCAAAATGTTCGCGACGTTTACTTTACCGGATGCAACTGCGCATCCGATTTGGACTCG
CTTTGATAAATTGGGCGGGAATTTGCACCAGTACACCTTTTTGTTCAACGAATTCGGAGAAG
GCAGGCACGCGATTCGTTTTCAAAAGCTGTTGACCGTCGAAGATGGTGTCGCAAAAGAAGTT
GATGATGTAACGGTGCCCATTTCCATGTCAGCGCAATTGGATGATCTGCTGCCAAGAGATCC
CCATGAACTGGTTGCACTATATTTTCAAGATTATGGAGCCGAACAGCATTTGGCGGGTGAAT
TCGGTGGCGCGAAGATTCAGTACCGTCGGGATCAACTAAATCATTTGCACGCACGCAGAGGG
GCGAGGGATGTTTATCTCAATCTCAGCGTACGTGTGCAGAGCCAGTCTGAGGCACGGGGAGA
ACGCCGCCCGCCGTATGCCGCAGTATTCCGCCTGGTCGGGGACAACCATCGTGCGTTTGTCC
ATTTTGATAAATTATCGGATTATCTTGCGGAACATCCGGATGATGGGAAGCTTGGATCGGAG
GGGCTGCTTTCCGGGCTACGGGTGATGAGTGTCGATCTCGGCCTTCGCACATCGGCATCGAT
TTCCGTTTTTCGCGTTGCCCGGAAGGACGAGTTGAAGCCGAACTCGGAAGGGCGTGTCCCAT
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AAGCTGCCTGGCGAAACAGAGTCAAAGGACCTGCGGGCTATCCGAGAAGAGCGCCAACGGAC
CCTGCGGCAGCTGCGGACGCAACTGGCGTATTTGCGGCTGCTCGTGCGGTGTGGGTCGGAAG
ATGTGGGACGGCGTGAACGGAGTTGGGCAAAGCTTATTGAGCAGCCCATGGATGCCAATCAG
ATGACACCGGATTGGCGCGAAGCCTTTGAAGACGAACTTCAGAAGCTTAAGTCACTCTATGG
TATCTGTGGCGACAGGGAATGGACGGAGGCTGTCTACGAGAGCGTTCGCCGCGTGTGGCGCC
ATATGGGCAAACAGGTTCGCGATTGGCGAAAGGACGTACGGAGTGGAGAGCGGCCGAAGATT
CGCGGCTATCAAAAAGATGTGGTCGGCGGAAATTCGATTGAGCAAATTGAGTATCTTGAACG

GCAGTACAAGTTTCTCAAGAGTTGGAGCTTTTTTGGCAAGGTATCGGGACAAGTGATTCGTG
CGGAGAAGGGATCCCGATTTGCGATCACGCTGCGTGAACACATTGATCACGCGAAGGAAGAC
CGGCTGAAGAAATTGGCGGATCGCATCATTATGGAGGCGCTCGGTTATGTGTACGCGTTGGA
TGATGAGCGCGGCAAAGGAAAGTGGGTTGCGAAGTATCCGCCGTGCCAGCTCATCCTGCTGG
AGGAATTGAGCGAGTACCAGTTCAATAACGACAGGCCTCCGAGTGAAAACAATCAGTTGATG
CAATGGAGCCATCGCGGCGTGTTCCAGGAGTTGTTGAATCAGGCCCAAGTCCACGATTTACT
CGTTGGGACGATGTATGCAGCGTTCTCGTCGCGATTCGACGCGCGAACCGGGGCACCGGGTA
TCCGCTGTCGCAGGGTACCGGCGCGTTGCGCTCGGGAGCAGAATCCAGAACCATTTCCTTGG
TGGCTGAACAAGTTTGTGGCGGAACACAAGTTGGATGGTTGTCCCTTACGGGCAGACGACCT
CATCCCCACGGGTGAAGGAGAGTTTTTTGTCTCGCCGTTCAGTGCGGAGGAAGGGGACTTTC
ATCAGATTCATGCCGACCTGAATGCGGCGCAAAACCTGCAGCGGCGACTCTGGTCTGATTTT
GATATCAGTCAAATTCGGTTGCGGTGTGATTGGGGTGAAGTGGACGGTGAACCCGTTCTGAT
CCCAAGGACCACAGGAAAGCGAACGGCGGATTCATATGGCAACAAGGTGTTTTATACCAAAA
CAGGTGTCACCTATTATGAGCGAGAGCGGGGGAAGAAGCGGAGAAAGGTTTTCGCGCAAGAG
GAATTGTCGGAGGAAGAGGCGGAGTTGCTTGTGGAAGCAGACGAGGCAAGGGAGAAATCGGT
CGTTTTGATGCGTGATCCGTCCGGCATTATCAATCGTGGCGACTGGACCAGGCAAAAGGAGT
TTTGGTCGATGGTGAACCAGCGGATTGAAGGATACTTGGTCAAGCAGATTCGCTCGCGCGTT
CGCTTACAAGAAAGTGCGTGTGAAAACACGGGGGATATT

Humanized AaCas12b coding sequence

ATGGCCGTGAAGAGCATGAAGGTGAAGCTGCGCCTGGACAACATGCCCGAGATCCGCGCCGG
CCTGTGGAAGCTGCACACCGAGGTGAACGCCGGCGTGCGCTACTACACCGAGTGGCTGAGCC
TGCTGCGCCAGGAGAACCTGTACCGCCGCAGCCCCAACGGCGACGGCGAGCAGGAGTGCTAC
AAGACCGCCGAGGAGTGCAAGGCCGAGCTGCTGGAGCGCCTGCGCGCCCGCCAGGTGGAGAA
CGGCCACTGCGGCCCCGCCGGCAGCGACGACGAGCTGCTGCAGCTGGCCCGCCAGCTGTACG
AGCTGCTGGTGCCCCAGGCCATCGGCGCCAAGGGCGACGCCCAGCAGATCGCCCGCAAGTTC
CTGAGCCCCCTGGCCGACAAGGACGCCGTGGGCGGCCTGGGCATCGCCAAGGCCGGCAACAA
GCCCCGCTGGGTGCGCATGCGCGAGGCCGGCGAGCCCGGCTGGGAGGAGGAGAAGGCCAAGG
CCGAGGCCCGCAAGAGCACCGACCGCACCGCCGACGTGCTGCGCGCCCTGGCCGACTTCGGC
CTGAAGCCCCTGATGCGCGTGTACACCGACAGCGACATGAGCAGCGTGCAGTGGAAGCCCCT
GCGCAAGGGCCAGGCCGTGCGCACCTGGGACCGCGACATGTTCCAGCAGGCCATCGAGCGCA
TGATGAGCTGGGAGAGCTGGAACCAGCGCGTGGGCGAGGCCTACGCCAAGCTGGTGGAGCAG
AAGAGCCGCTTCGAGCAGAAGAACTTCGTGGGCCAGGAGCACCTGGTGCAGCTGGTGAACCA
GCTGCAGCAGGACATGAAGGAGGCCAGCCACGGCCTGGAGAGCAAGGAGCAGACCGCCCACT
ACCTGACCGGCCGCGCCCTGCGCGGCAGCGACAAGGTGTTCGAGAAGTGGGAGAAGCTGGAC
CCCGACGCCCCCTTCGACCTGTACGACACCGAGATCAAGAACGTGCAGCGCCGCAACACCCG
CCGCTTCGGCAGCCACGACCTGTTCGCCAAGCTGGCCGAGCCCAAGTACCAGGCCCTGTGGC
GCGAGGACGCCAGCTTCCTGACCCGCTACGCCGTGTACAACAGCATCGTGCGCAAGCTGAAC
CACGCCAAGATGTTCGCCACCTTCACCCTGCCCGACGCCACCGCCCACCCCATCTGGACCCG
CTTCGACAAGCTGGGCGGCAACCTGCACCAGTACACCTTCCTGTTCAACGAGTTCGGCGAGG
GCCGCCACGCCATCCGCTTCCAGAAGCTGCTGACCGTGGAGGACGGCGTGGCCAAGGAGGTG
GACGACGTGACCGTGCCCATCAGCATGAGCGCCCAGCTGGACGACCTGCTGCCCCGCGACCC
CCACGAGCTGGTGGCCCTGTACTTCCAGGACTACGGCGCCGAGCAGCACCTGGCCGGCGAGT
TCGGCGGCGCCAAGATCCAGTACCGCCGCGACCAGCTGAACCACCTGCACGCCCGCCGCGGC

GCCCGCGACGTGTACCTGAACCTGAGCGTGCGCGTGCAGAGCCAGAGCGAGGCCCGCGGCGA
GCGCCGCCCCCCCTACGCCGCCGTGTTCCGCCTGGTGGGCGACAACCACCGCGCCTTCGTGC
ACTTCGACAAGCTGAGCGACTACCTGGCCGAGCACCCCGACGACGGCAAGCTGGGCAGCGAG
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AaCas12b protein sequence

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Cas12b coding sequence from *Alicyclobacillus kakegawensis* NBRC 103104 (GeneBank ID: NZ_BCRP01000027.1)

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Humanized AkCas12b coding sequence

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AkCas12b protein sequence

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Cas12b coding sequence from *Alicyclobacillus macrosporangiidus* strain DSM 17980 (GeneBank ID: FPBV01000001.1)

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Humanized AmCas12b coding sequence

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GAGCGGCGCCGCCGAGAAGGTGCGCGGC

AmCas12b protein sequence

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Cas12b coding sequence from *Bacillus sp*. NSP2.1 (GeneBank ID: NZ_KI301973.1)

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Humanized BsCas12b coding sequence

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BsCas12b protein sequence

MAIRSIKLKLKTHTGPEAQNLRKGIWRTHRLLNEGVAYYMKMLLLFRQESTGERPKEELQEE
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pCAG-2AeGFP partial sequence

(CAG-NLS-*XmaI*-*NheI*-NLS-T2A-eGFP-SV40**)**

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BPK2104-*ccdB* partial sequence

(*lac*I-T7-*lac*O-NLS-*XmaI*-*SpeI*-His_{10}-terminator)

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pUC19-U6 partial sequence

(U6-*BasI*-*HindIII***)**

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TTTAAAATGGACTATCATATGCTTACCGTAACTTGAAAGTATTTCGATTTCTTGGCTTTATA
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pUC19-U6-Aa_tracrRNA-tRNA-crRNA partial sequence

(U6-Aa_tracrRNA-tRNA-crRNA_scaffold-*BasI*-*BasI*-terminator**)**

TGTAAAACGACGGCCAGTGAATTCGAGGGCCTATTTCCCATGATTCCTTCATATTTGCATAT
ACGATACAAGGCTGTTAGAGAGATAATTGGAATTAATTTGACTGTAAACACAAAGATATTAG
TACAAAATACGTGACGTAGAAAGTAATAATTTCTTGGGTAGTTTGCAGTTTTAAAATTATGT
TTTAAAATGGACTATCATATGCTTACCGTAACTTGAAAGTATTTCGATTTCTTGGCTTTATA
TATCTTGTGGAAAGGACGAAACACCGGGTCTAAAGGACAGAATTTTTCAACGGGTGTGCCAA
TGGCCACTTTCCAGGTGGCAAAGCCCGTTGAACTTCTCAAAAAGAACGCTCGCTCAGTGTTC
TGACAACAAAGCACCAGTGGTCTAGTGGTGGAATAGTACCCTGCCACGGTACAGACCCGGGT
TCGATTCCCGGCTGGTGCAGTCGGATCACTGAGCGAGCGATCTGAGAAGTGGCACA*GAGACC*
GAGAGAG*GGTCTC*Attttttttt AAGCTTGGCGTAATCATGGTCATAGCTGTTTCCTG

pUC19-U6-Ak_tracrRNA-tRNA-crRNA partial sequence

(U6-Ak_tracrRNA-tRNA-crRNA_scaffold-BasI-BasI-terminator)

TGTAAAACGACGGCCAGTGAATTCGAGGGCCTATTTCCCATGATTCCTTCATATTTGCATAT
ACGATACAAGGCTGTTAGAGAGATAATTGGAATTAATTTGACTGTAAACACAAAGATATTAG
TACAAAATACGTGACGTAGAAAGTAATAATTTCTTGGGTAGTTTGCAGTTTTAAAATTATGT
TTTAAAATGGACTATCATATGCTTACCGTAACTTGAAAGTATTTCGATTTCTTGGCTTTATA

TATCTTGTGGAAAGGACGAAACACCGGGTCGTCTATAGGACGGCGAGGACAACGGGAAGTGC
CAATGTGCTCTTTCCAAGAGCAAACACCCCGTTGGCTTCAAGATGACCGCTCGCTCAGCGAT
CTGACAACAAAGCACCAGTGGTCTAGTGGTGGAATAGTACCCTGCCACGGTACAGACCCGGG
TTCGATTCCCGGCTGGTGCAAACGGATCGCTGAGCGAGCGGTCTGAGAAGTGGCACAGAGAC
CGAGAGAGGGTCTCAttttttttAAGCTTGGCGTAATCATGGTCATAGCTGTTTCCTG

pUC19-U6-Am_tracrRNA-tRNA-crRNA partial sequence

(U6-Am_tracrRNA-tRNA-crRNA_scaffold-BasI-BasI-terminator)

TGTAAAACGACGGCCAGTGAATTCGAGGGCCTATTTCCCATGATTCCTTCATATTTGCATAT
ACGATACAAGGCTGTTAGAGAGATAATTGGAATTAATTTGACTGTAAACACAAAGATATTAG
TACAAAATACGTGACGTAGAAAGTAATAATTTCTTGGGTAGTTTGCAGTTTTAAAATTATGT
TTTAAAATGGACTATCATATGCTTACCGTAACTTGAAAGTATTTCGATTTCTTGGCTTTATA
TATCTTGTGGAAAGGACGAAACACCGGGGAATTGCCGATCTATAGGACGGCAGATTCAACGG
GATGTGCCAATGCACTCTTTCCAGGAGTGAACACCCCGTTGGCTTCAACATGATCGCCCGCT
CAACGGTCCGATAACAAAGCACCAGTGGTCTAGTGGTGGAATAGTACCCTGCCACGGTACAG
ACCCGGGTTCGATTCCCGGCTGGTGCAGTCGGATCACTGAGCGAGCGATCTGAGAAGTGGCA
CAGAGACCGAGAGAGGGTCTCAttttttttAAGCTTGGCGTAATCATGGTCATAGCTGTTTC
CTG

pUC19-U6-Bs_tracrRNA-tRNA-crRNA partial sequence

(U6-Bs_tracrRNA-tRNA-crRNA_scaffold-BasI-BasI-terminator)

TGTAAAACGACGGCCAGTGAATTCGAGGGCCTATTTCCCATGATTCCTTCATATTTGCATAT
ACGATACAAGGCTGTTAGAGAGATAATTGGAATTAATTTGACTGTAAACACAAAGATATTAG
TACAAAATACGTGACGTAGAAAGTAATAATTTCTTGGGTAGTTTGCAGTTTTAAAATTATGT
TTTAAAATGGACTATCATATGCTTACCGTAACTTGAAAGTATTTCGATTTCTTGGCTTTATA
TATCTTGTGGAAAGGACGAAACACCGGCCATAAGTCGACTTACATATCCGTGCGTGTGCATT
ATGGGCCCATCCACAGGTCTATTCCCACGGATAATCACGACTTTCCACTAAGCTTTCGAATA
ACAAAGCACCAGTGGTCTAGTGGTGGAATAGTACCCTGCCACGGTACAGACCCGGGTTCGAT
TCCCGGCTGGTGCAGTTCGAAAGCTTAGTGGAAAGCTTCGTGGTTAGCACAGAGACCGAGAG
AGGGTCTCAttttttttAAGCTTGGCGTAATCATGGTCATAGCTGTTTCCTG

pUC19-U6-AasgRNA partial sequence

(U6-AasgRNA_scaffold-*BasI*-*BasI*-terminator**)**

TGTAAAACGACGGCCAGTGAATTCGAGGGCCTATTTCCCATGATTCCTTCATATTTGCATAT
ACGATACAAGGCTGTTAGAGAGATAATTGGAATTAATTTGACTGTAAACACAAAGATATTAG
TACAAAATACGTGACGTAGAAAGTAATAATTTCTTGGGTAGTTTGCAGTTTTAAAATTATGT
TTTAAAATGGACTATCATATGCTTACCGTAACTTGAAAGTATTTCGATTTCTTGGCTTTATA
TATCTTGTGGAAAGGACGAAACACCGGGTCTAAAGGACAGAATTTTTCAACGGGTGTGCCAA
TGGCCACTTTCCAGGTGGCAAAGCCCGTTGAACTTCTCAAAAAGAACGCTCGCTCAGTGTTC
TGACGTCGGATCACTGAGCGAGCGATCTGAGAAGTGGCACA*GAGACC*GAGAGAG*GGTCTC*At
ttttttAAGCTTGGCGTAATCATGGTCATAGCTGTTTCCTG

pUC19-U6-AksgRNA partial sequence

(U6-AksgRNA_scaffold-*BasI*-*BasI*-terminator**)**

TGTAAAACGACGGCCAGTGAATTCGAGGGCCTATTTCCCATGATTCCTTCATATTTGCATAT
ACGATACAAGGCTGTTAGAGAGATAATTGGAATTAATTTGACTGTAAACACAAAGATATTAG
TACAAAATACGTGACGTAGAAAGTAATAATTTCTTGGGTAGTTTGCAGTTTTAAAATTATGT
TTTAAAATGGACTATCATATGCTTACCGTAACTTGAAAGTATTTCGATTTCTTGGCTTTATA
TATCTTGTGGAAAGGACGAAACACCGGtcgtctataGGACGGCGAGGACAACGGGAAGTGCC
AATGTGCTCTTTCCAAGAGCAAACACCCCGTTGGCTTCAAGATGACCGCTCGCTCAGCGATC
TGACAACGGATCGCTGAGCGAGCGGTCTGAGAAGTGGCACA*GAGACC*GAGAGAG*GGTCTC*At
ttttttttAAGCTTGGCGTAATCATGGTCATAGCTGTTTCCTG

pUC19-U6-AmsgRNA partial sequence

(U6-AmsgRNA_scaffold-*BasI*-*BasI*-terminator**)**

TGTAAAACGACGGCCAGTGAATTCGAGGGCCTATTTCCCATGATTCCTTCATATTTGCATAT
ACGATACAAGGCTGTTAGAGAGATAATTGGAATTAATTTGACTGTAAACACAAAGATATTAG
TACAAAATACGTGACGTAGAAAGTAATAATTTCTTGGGTAGTTTGCAGTTTTAAAATTATGT
TTTAAAATGGACTATCATATGCTTACCGTAACTTGAAAGTATTTCGATTTCTTGGCTTTATA
TATCTTGTGGAAAGGACGAAACACCGGggaattgccgatctaTAGGACGGCAGATTCAACGG
GATGTGCCAATGCACTCTTTCCAGGAGTGAACACCCCGTTGGCTTCAACATGATCGCCCGCT
CAACGGTCCGATGTCGGATCGTTGAGCGGGCGATCTGAGAAGTGGCACA*GAGACC*GAGAGAG
*GGTCTC*Atttttttt AAGCTTGGCGTAATCATGGTCATAGCTGTTTCCTG

pUC19-U6-BssgRNA partial sequence

(U6-BssgRNA_scaffold-*BasI*-*BasI*-terminator**)**

TGTAAAACGACGGCCAGTGAATTCGAGGGCCTATTTCCCATGATTCCTTCATATTTGCATAT
ACGATACAAGGCTGTTAGAGAGATAATTGGAATTAATTTGACTGTAAACACAAAGATATTAG
TACAAAATACGTGACGTAGAAAGTAATAATTTCTTGGGTAGTTTGCAGTTTTAAAATTATGT
TTTAAAATGGACTATCATATGCTTACCGTAACTTGAAAGTATTTCGATTTCTTGGCTTTATA
TATCTTGTGGAAAGGACGAAACACCGGCCATAAGTCGACTTACATATCCGTGCGTGTGCATT
ATGGGCCCATCCACAGGTCTATTCCCACGGATAATCACGACTTTCCACTAAGCTTTCGAATG
TTCGAAAGCTTAGTGGAAAGCTTCGTGGTTAGCACA*GAGACC*GAGAGAG*GGTCTC*Atttttt
ttAAGCTTGGCGTAATCATGGTCATAGCTGTTTCCTG