Multiple sgRNAs facilitate base editing-mediated i-stop to induce complete and precise gene disruption

SUPPLEMENTARY INFORMATION

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Materials and methods



Supplementary figure 1. Efficient C-to-T substitution at *Tyr* and *PD1* loci in N2a by BE3-mediated base editing

(A) Schematic diagram of sgRNA-targeting sites at the *Tyr* locus. Red triangles indicate the predicted substitution sites for the sgRNAs. The PAM sequences and substitutions highted in blue and red, respectively; The expectedly edited codons are underlined. Wt, wild-type.
(B) Schematic diagram of sgRNA-targeting sites at the *Pdcd1* locus. Red triangles indicate the predicted substitution sites for the sgRNAs. The PAM sequences and substitutions highlighted in blue and red, respectively; The expectedly edited codons are underlined.

(C) Sanger sequencing chromatograms of BE3-mediated editing in N2a cells. Red star denotes the substituted nucleotides. The edited codons are underlined and substitutions highlighted in red.

(D) The sequencing results of the N2a using BE3-mediated base editing. The editing efficiencies were detected by sanger sequencing of TA clones for *Tyr* and *Pdcd1* in N2a. Each sample was analyzed at least ten TA clones. The PAM sequences and substitutions are highlighted in blue and red, respectively; The expectedly edited codons are underlined. The column on the right indicates frequencies of mutants and indels.

(E) The analysis of the base editing efficiency of BE3 in N2a co-transfected with different sgRNAs. The efficiencies were detected by Sanger sequencing of TA clones in N2a for *Tyr* and *Pdcd1*. Blue indicates percentage of i-stop mutations; Orange indicates percentage of indels. Each sample was analyzed at least ten TA clones.



Supplementary figure 2. sgRNA-targeting sites at the *Tyr* and *Pdcd1* loci.

(A) Schematic diagram of sgRNA-targeting sites at the *Tyr* locus. Red triangles indicate the predicted substitution sites for the sgRNAs. The PAM sequences and substitutions are highlighted in blue and red, respectively; The expectedly edited codons are underlined.
(B) Schematic diagram of sgRNA-targeting sites at the *Pdcd1* locus. Red triangles indicate the predicted substitution sites for the sgRNAs. The PAM sequences and substitutions are highlighted in blue and red, respectively; The expectedly edited codons are underlined.
(C) Sanger sequencing chromatogram of BE3-mediated editing in N2a cells. * denotes the substituted nucleotide.



• • Pdcd1-sg2 CGGTTT<u>CAA</u>GGCATGGTCAT(Q167stop)

* * * ~~~~~~ Pdcd1-sg3 CACAGC<u>CCA</u>AGTGAATGACC(W12stop)

Tyr-sg2

Pdcd1-sq2

Tyr-sg1

Pdcd1-sg1



	Tyr-	sg1	Frequency(%)
	ccct ccct	IGCGGC <u>TAG</u> CTTTCAGGCAGAGO IGCGGT <u>TAG</u> CTTTCAGGCAGAGO	6(10%,Q48stop) 6(40%,G47G,Q48stop)
	Tyr-	sg2	Frequency(%)
	CCT CCT CCT	FCTTCTCCTCC <u>TAA</u> CAAATAAGA FCTTCTCCTCC <u>TAA</u> CAGGTAAGA FCTTCTCCTCC <u>TGG</u> CAAGTAAGA	(22.2%,W272stop,Q273Q) (11.1%,W272stop) (11.1%,Q273Q)
	Tyr-	sg7	Frequency(%)
	CCAC CCAC	GGGTTTCTGCCT <u>TAA</u> CACAAACT GGGTTTCTGCCT <u>TAA</u> CACAGACT	2(11.1%,W210STOP,R212K) 2(22.2%,W210STOP)
	Pdc	d1-sg1	Frequency(%)
	TGA	AAAA <u>TAG</u> GCCGCCTTCTGTAATG	GG(30%,Q79stop)
	Pdc	d1-sg2	Frequency(%)
	GGC	CGGTTT <u>TAA</u> GGCATGGTCAT <mark>TG</mark> G	(50%,Q167stop)
	Pdc	d1-sg3	Frequency(%)
	CCTO CCTO CCTO CCTO	GGTCATTCACTTAAGCTATGCTC GGTCATTCACTTAAACTATGCTC GGTCATTCACTTAAACTGTGCTC GGTCATTCACTTAAGCTGTGCTC GGTCATTCACTTGGGCTATGCTC	<pre>(8.3%,W12stop,V14M) (8.3%,W12stop,A13T,V14M) (8.3%,W12stop,A13T) (8.3%,W12stop) (8.3%,W12stop) (8.3%,V14M)</pre>
i-stop fre	equen	cy	
inwanteo	d muta	ation only	
-	Ту	r-sg1	Frequency(%)
E	sa1		
	#1	CCCTGCGGC <u>TAG</u> CTTTCAGGCA CCCTGCGGC <u>TAA</u> CTTTCAGGCA CCCTGCGGC <u>CGG</u> CTTTCAGGCA	AGAGG(8.3%,Q48stop) AGAGG(8.3%,Q48stop) AGAGG(8.3%,Q48P)
	#4	CCCTGCGGT <u>TAG</u> CTTTCAGGCA CCCTGCGGT <u>CAG</u> CTTTCAGGCA	AGAGG(20%,G47G,Q48stop) AGAGG(10%,G47G)
	#6	CCCTGCGGT <u>CAG</u> CTTTCAGGCA CCCTGCGGT <u>CAA</u> CTTTCAGGCA	GAGG(42.8%,G47G) GAGG(14.3%,G47G,Q48Q)
	#9	CCCTGCGGC <u>TAG</u> CTTTCAGGCA	AGAGG(11.1%,Q48stop)
	Ту	r-sg1+sg2+sg7	Frequency(%)
	sg1 #7 #9 #10	CCCTGCGGC <u>TAG</u> CTTTCAGGCA CCCTGCGGT <u>TAG</u> CTTTCAGGCA CCCTGCGGT <u>TAG</u> CTTTCAGGCA	AGAGG (20%,G47G) AGAGG (50%,G47G,Q48stop) AGAGG (10%,G47G,Q48stop)
	sg2 #5	CCTTCTTCTCCCCCTAACAGG	PAAGA (10%-W272stop)
	#6	CCTTCTTCTCCTCA <u>TAG</u> CAAAT CCTTC:::::::::::AGGT	<pre>TAAAA (14.3%, S271S, W272stop, Q273Q) TAAGA (14.3%, -13)</pre>
)	#7	CCTTCTTCTCCTCC <u>TGA</u> CAGGT CCTTCTTCTCCTCC <u>TAG</u> CAGGZ	MAGA(33.3%,W272stop) MAAGA(11.1%,W272stop)
	#8	CCTTCTTCTCCTCC <u>TGA</u> CAGGI CCTTCTTCTCCTCCTAGCAGGI	PAGA (33.3%, W272stop) PAAGA (8.3%, W272stop)
	#9	CCTTCTTCTCCTCCTAGCAGGI	AAGA (33.3%, W272stop)
)	#10	CCTTCTTCTCCTCCTAGCAGGI	TAAGA (8.3%, W272stop)
V14M)	sg7	00110110100100 <u>100</u> 0A001	
V14M)	#3	CCAGGGTTTCTGCCT <u>TAG</u> CACA	GACT (91.7%, W210STOP)
1 43.43	#4	CCAGGGTTTCTGCCT <u>TAG</u> CACA	AACT (100%, W210STOP, R212K)
14M)	#5 #6	CCAGGGTTTCTGCCT <u>TAG</u> CACA	AGACT(10%,W210STOP)
⊥4M)	₩υ	CCAGGGTTTCTGCCT <u>TAA</u> CACA	MGACI(14.35,WZIUSTUP)

Frequency(%) TGAAAAATAGGCCGCCTTCTGTAATGG (30%,079stop) TGAAAAATAGGCCGCCTTCTGTAATGG (10%, Q79stop) Frequency(%)

Tyr-sg7

Pdcd1-sq3

Pdcd1-sg1+sg2+sg3

sg1 #2 TGAAAAA<u>TAG</u>GCCGCCTTCTGTAATGG(33.3%,Q79stop #4 TGAAAAATAGGCCGCCTTCTGTAATGG(70%,Q79stop)

- sa2
- #4 #7 GGCCGGTTT<u>TAA</u>GGCATGGTCATTGG(20%,Q167stop) GGCCGGTTT<u>TAA</u>GGCATGGTCATTGG(20%,Q167stop)
- #8 GGCCGGTTT<u>TAA</u>GGCATGGTCATTGG(8.3%,Q167stop)
- #9 GGCCGGTTTTAAGGCATGGTCATTGG(14.3%,0167stop
- sg3 CCTGGTCATTCACTTAAGCTATGCTG(11.1%, W12stop, #3
- CCTGGTCATTCACTTGAGCTATGCTG(11.1%,W12stop, CCTGGTCATTCACT<u>TGG</u>GCTATGCTG(11.1%,V14M) CCTGGTCATTCACT<u>TGA</u>GCTATGCTG(8.3%,W12stop,V #5 CCTGGTCATTCACT<u>TAG</u>GCTATGCTG (8.3%, W12stop, V CCTGGTCATTCACT<u>TGT</u>GCTGTGCTG (8.3%, W12C)
- CCTGGTCATTCACTTGGGCTATGCTG (50%, V14M) CCTGGTCATTCACT<u>TAGG</u>CTGTGCTG(14.3%,W12stop) CCTGGTCATTCACT<u>TGT</u>GCTGTGCTG(14.3%,W12C) #8
- CCTGGTCATTCACTTAGGCTGTGCTG(14.3%,W12stop,A13T) CCTGGTCATTCACTTGGCTATGCTG(14.3%,V14M) #9

#7 CCAGGGTTTCTGCCTTGGCACAAACT (50%, R212K) CCAGGGTTTCTGCCT<u>TAA</u>CACAAACT (50%, W210STOP, R212K) #9 CCAGGGTTTCTGCCTTGGCACAAACT (33.3%, R212K) CCAGGGTTTCTGCCT<u>TGG</u>CACACACT(33.3%,R212T) #10 CCTTCTTCTCCCCCC<u>TAA</u>CAGGTAAGA(8.3%,W272stop)

Supplementary figure 3. Efficient C-to-T substitution at Tyr and Pdcd1 loci in N2a and mouse embryos by BE4max-mediated base editing

(A) Sanger sequencing chromatograms of BE4max-mediated editing in N2a cells. Red star

denotes the substituted nucleotides. The edited codons are underlined and substitutions

highlighted in red.

(B) The sequencing results of the N2a using BE4-mediated base editing. The editing

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С

D

sg1

#8

ž

Pdcd1

Pdcd1-sg1

efficiencies were detected by sanger sequencing of TA clones for *Tyr* and *Pdcd1* in N2a. Each sample was analyzed at least ten TA clones. The PAM sequences and substitutions are highlighted in blue and red, respectively; The expectedly edited codons are underlined. The column on the right indicates frequencies of mutants and indels.

(C) The analysis of the base editing efficiency of BE4max in N2a co-transfected with different sgRNAs. The efficiencies were detected by Sanger sequencing of TA clones in N2a for *Tyr* and *Pdcd1*. Blue indicates percentage of i-stop mutations; Orange indicates percentage of unwanted mutation. Each sample was analyzed at least ten TA clones.

(D) The editing efficiencies were detected by sanger sequencing of TA clones for *Pdcd1* of mouse embryos, each sample was analyzed at least ten TA clones. The PAM sequences and substitutions are highlighted in blue and red, respectively; The expectedly edited codons are underlined. The column on the right indicates frequencies of mutants.

(E) The editing efficiencies were detected by sanger sequencing of TA clones for *Tyr* of mouse embryos, each sample was analyzed at least ten TA clones. The PAM sequences and substitutions are highlighted in blue and red, respectively; The expectedly edited codons are underlined. The column on the right indicates frequencies of mutants.

А				Pdo	:d1-sg1	Frequency(%)	
	Tyr-	sg1	Frequency(%)	sg1 #5 #6	TGA AAAA<u>TAG</u>GCCGCCTT TGAAAAA <mark>TAG</mark> GCCGCCTT	CTGTAATGG(20%,Q79stop) CTGTAATGG(18%,Q79stop)	
	sg1 #5 C #9 C	CCTGCGGT <u>CAG</u> CTTTCA	GGCAGAGG(11%,G47G) GGCAGAGG(8%,G47G)	Pdo sg1	:d1-sg1+sg2	Frequency(%)	
				#1 sg2	TGAAAAA <u>TAG</u> GCCGCCTT	CTGTAATGG(33%,Q79stop)	
	Tyr-	sg1+sg2	Frequency(%)	#1 #7	GGC CGGTTT<u>TAA</u>GGCATG GGC CGGTTT<u>TAA</u>GGCATG	GTCATTGG(10%,Q167stop) GTCATTGG(12%,Q167stop)	
	#2 C #3 C #6 C	CCCTGCGGT <u>CAG</u> CTTTCA CCCTGCGGT <u>CAG</u> CTTTCA CCCTGCGGT <u>CAG</u> CTTTCA	GGCAGAGG (20%,G47G) GGCAGAGG (13%,G47G) GGCAGAGG (10%,G47G,Q48stop)	Pdo sg1	:d1-sg1+sg2+sg3	Frequency(%)	
В	C C C C C C C C C C C C C C C C C C C	CCTGCGGT <u>CAG</u> CTTTCAC CTTCTTCTCCTCCTGGCi CTTCTTCTCCTCCTGGCi CTTCTTCTCCTCCTGGCi CTTCTTCTCCTCCTGGCi CTTCTTCTCCTCCTGACi CTTCTTCTCCTCCTAACi CTTCTTCTCCTCCTAACi CCTCCTCCTCCTCCTAACi CCTCCTCCTCCTCCTCAACi CCTCCTCCTCCTCCTCAACi	GGCAGAGG (10%, G47G) AGGTAAGA (22%, W272stop) AAGTAAAA (28%, Q273Q) AGGTAAAA (28%, Q273top) AGGTAAAA (10%, W272stop) AGGTAAAA (10%, W272stop, Q273Q) AGGTAAAA (6%, W272stop) AGGTAAAA (6%, W272stop) GGGTAAAA (6%, W272stop) CAGGTAAGA (33%, W272stop) CAGGTAAGA (33%, W272stop)	#1 #3 sg2 #1 #2 sg3 #5 #7 #8 \$ g2 #14 sg3 #8	IGAAAAATAGGCCGCCTT IGAAAAATAGGCCGCCTT IGAAAAATAGGCCGCCTT IGACGCCGGTTTTAAGGCATG IGCCGGTTTTAAGGCATG ICCTGGTCATTCACTIGA ICCTGGTCATTCACTIGA ICCTGGTCATTCACTIGA ICCTGGTCATTCACTIGA ICCTGGTCATTCACTIGA ICCTGGTCATTCACTIAAA ICCTGGTCATTCACTIAAA ICCTGGTCATTCACTIAAA ICCTGGTCATTCACTIAAA ICCTGGTCATTCACTIAAA ICCTGGTCATTCACTIAAA	CTGTAATGG (14%,Q79stop) CTGTAATGG (13%,Q79stop) GTCATTGG (13%,Q167stop) GTCATTGG (7%,Q167stop) CTGTGCTG (11%,W12stop,A13T) CTATGCTG (20%,V14M) CTATGCTG (30%,W12c,A13T,V14M) CTGTGCTG (4%,W12c,A13T) CTGTGCTG (5%,M12stop,A13T) CTGTGCTG (5%,M12stop,A13T) CTGTGCTG (10%,W12stop,A13T) CTGTGCTG (10%,W12stop,A13T) CTGTGCTG (10%,W12stop,A13T) CTGTGCTG (10%,W12stop,A13T) CTGTGCTG (30%,Q167stop) AGGCTGTGCTG (41.2%,W12stop,A13 GGCTGTGCTG (8.3%,V12stop,A13 GGCTATGCTG (8.3%,V12stop,A13 GGCTATGCTG (8.3%,V14M)) 3T,V14M)
С				#9 #11 #13	CCTGGTCATTCACT <u>I</u> CCTGGTCATTCACT <u>I</u> CCTGGTCATTCACT <u>I</u> CCTGGTCATTCACT <u>I</u>	GACTGTGCTG(/./«,W12.,-2) GACTGTGCTG(28.6%,A13T) GACTGTGCTG(50%,W12stop) GGCTATGCTG(50%,V14M)	
	Ту	r-sg1+sg2+sg7	Frequency(%)	0.91	Pdcd1-sg1+sg2+sg3	Frequency(%)	
	sg1 #3 #4 #5 #16 #17 #18 sg2	CCCTGCGGTCAGCTTT CCCTGCGGTAGCTTT CCCTGCGGTCAGCTTT CCCTGCGGTCAGCTTT CCCTGCGGTCAGCTTT CCCTGCGGTAGCTTT CCCTGCGGTAGCTTT	MAGCAAGAGG (30%,G47G,S50L) MAGCAAGAGG (30%,G47G,Q48stop) SAGCAAGAGG (30%,G47G,Q48stop) SAGCAAGAGG (40%,G47G) SAGCAAGAGG (57.1%,G47G) SAGCAAGAGG (14.3%,G47G,Q48stop) SAGCAAGAGG (53%,G47G,Q48stop) SAGCAAGAGG (50%,Q48stop)	sg1 #15 0L)#16 #18 sg2 #16 #17 #18 #19	TGAAAAA <u>TAG</u> GCCGCC TGAAAAA <u>TAG</u> GCCGCC TGAAAAA <u>TAG</u> GCCGCC GGCCGGTTT <u>TAA</u> GGC GGCCCGGTTT <u>TAA</u> GGC GGCCCGGTTT <u>TAA</u> GGC	CTTCTGTAATGG (100%, Q79stop) TTCTGTAATGG (100%, Q79stop) CTTCTGTAATGG (100%, Q79stop) ATGGTCATTGG (100%, Q167stop) ATGGTCATTGG (100%, Q167stop) ATGGTCATTGG (25%, Q167stop)	
	#1 #3 #4 #5 #15 #16 #17 #18 #20	CCTTCTTCTCCCCCG CCTTCTTCTCCCCCG CCTTCTTCTCCCCCG CCTTCTTCTCCCCCG CCTTCTTCTCCCCCG CCTTCTTCTCCCCCG CCTTCTTCTCCCCCG CCTTCTTCTCCCCCG CCTTCTTCTCCCCCG CCTTCTTCTCCCCCG CCTTCTTCTCCCCCG CCTTCTTCTCCCCCG	CAAGTAAGA (100%, W272stop, Q273 CAAGTAAGA (16.6%, W272stop, Q273 (CAGGTAAAA (33.3%, W272stop) CCAGGTAAAA (71.4%, W272stop) CCAGGTAAGA (28.6%, W272stop) CCAGGTAAGA (45%, Q273Q) CCAGGTAAGA (40%, W272stop) CAGGTAAGA (40%, W272stop) CAGGTAAGA (75%, W272stop) CCAGGTAAAA (75%, W272stop) CCAGGTAAAA (55%, W272stop) CCAGGTAAAA (50%, W272stop)	#19 (2) sg3 #4 #5 #16 #17 #18	CCTGGTCATTCACT <u>H</u> CCTGGTCATTCACT <u>H</u> CCTGGTCATTCACT <u>H</u> CCTGGTCATTCACT <u>H</u> CCTGGTCATTCACT <u>H</u> CCTGGTCATTCACT <u>H</u> CCTGGTCATTCACT <u>H</u> CCTGGTCATTCACT <u>H</u>	EGGCTATGCTG (100%, V14M) 3A:::::GCTG (22%, W12stop, -5) AGCTGTGTGTG (39%, W12stop) AACTTATGCTG (91.7%, W12stop, A1 GACTGTGCTG (8.3%, A13T) 3ACTTGCTG (90%, W12stop, A137) 3GCTATGCTG (66.7%, V14M) AGCTATGCTG (33.3%, W12stop, V1	13T) ?) 14M)
	#16 #17 #18	CCAGGGTTTCTGCCT <u>T</u> CCAGGGTTTCTGCCT <u>T</u> CCAGGGTTTCTGCCT <u>T</u> CCAGGGTTTCTGCCT <u>T</u>	AACACAGACT (100%, W210STOP) AGCACAGACT (90%, W210STOP) AACACAGACT (50%, W210STOP) AGCACAAACT (50%, W210STOP, R212K)				

Supplementary figure 4. The sequencing results of the embryos and mutant mice using BE3-mediated base editing.

(A) The editing efficiencies were detected by sanger sequencing of TA clones for *Tyr* and *Pdcd1* of mouse embryos, each sample was analyzed at least ten TA clones. The PAM sequences and substitutions are highlighted in blue and red, respectively; The expectedly

edited codons are underlined. The column on the right indicates frequencies of mutants and indels.

(B) Sanger sequencing chromatograms of DNA from mice co-injecting with sgRNAs (Tyr-sg1+2, Pdcd1-sg1+2+3) and BE3, each sample was analyzed at least ten TA clones. The PAM sequences and substitutions are highlighted in blue and red, respectively; The expectedly edited codons are underlined. The column on the right indicates frequencies of mutants and indels.

(C) Sanger sequencing chromatograms of DNA from mice co-injecting with sgRNAs (Tyr-sg1+2+7, Pdcd1-sg1+2+3) and BE3, each sample was analyzed at least ten TA clones. The PAM sequences and substitutions are highlighted in blue and red, respectively; The expectedly edited codons are underlined. The column on the right indicates frequencies of mutants and indels.



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sgRNA	potential off-target sequences	Wt	#16	#18	#16 (exclude Wt)	#18 (exclude Wt)
Pdcd1-sg1	175	0	0	0	0	0
Pdcd1-sg2	961	0	0	0	0	0
Pdcd1-sg3	690	0	0	0	0	0
Tyr-sg1	1069	0	0	0	0	0
Tyr-sg2	2414	0	0	0	0	0
Tyr-sg7	1919	0	0	0	0	0

Supplementary figure 5. The off-target sites.

(A) Off-target sites for Tyr-sg1, the lowercase in white cell represents the mismatch nucleotide.

(B) Off-target sites for Tyr-sg2, the lowercase in white cell represents the mismatch nucleotide.

(C) Off-target sites for Tyr-sg7, the lowercase in white cell represents the mismatch nucleotide.

(D) Off-target sites for Pdcd1-sg1, the lowercase in white cell represents the mismatch nucleotide.

(E) Off-target sites for Pdcd1-sg2, the lowercase in white cell represents the mismatch nucleotide.

(F) Off-target sites for Pdcd1-sg3, the lowercase in white cell represents the mismatch nucleotide.

(G) Summary of potential off-target sequences. Up to 2-bp mismatch in seed region and 3-bp mismatch in non-seed region with NRG PAM (using CasOT).

sgRNA	Upstream oligos (5' to 3')	Downstream oligos (5' to 3')
Tyr-sg1	ACCGTGCGGCCAGCTTTCAGGCAG	AAACCTGCCTGAAAGCTGGCCGCA
Tyr-sg2	ACCGTACCTGCCAGGAGGAGAAGA	AAACTCTTCTCCTCCTGGCAGGTA
Tyr-sg3	ACCGGGACCTCAGTTCCCCTTCAA	AAACTTGAAGGGGAACTGAGGTCC
Tyr-sg4	ACCGCTATGGCCAAATGAACAA	AAACTTGTTCATTTGGCCATAG
Tyr-sg5	ACCGCATCTCTCCAATCCCAGTA	AAACTACTGGGATTGGAGAGATG
Tyr-sg6	ACCGTGGAACAAGCCAGTCGTATC	AAACGATACGACTGGCTTGTTCCA
Tyr-sg7	ACCGCTGTGCCAAGGCAGAAACCC	AAACGGGTTTCTGCCTTGGCACAG
Pdcd1-sg1	ACCGAAAACAGGCCGCCTTCTGTAA	AAACTTACAGAAGGCGGCCTGTTTT
Pdcd1-sg2	ACCGCGGTTTCAAGGCATGGTCAT	AAACATGACCATGCCTTGAAACCG
Pdcd1-sg3	ACCGCACAGCCCAAGTGAATGACC	AAACGGTCATTCACTTGGGCTGTG
Pdcd1-sg4	ACCGGCTGCAGTTGAGCTGGCAATC	AAACGATTGCCAGCTCAACTGCAGC
Pdcd1-sg5	ACCGCTGGCAATCAGGGTGGCTTCT	AAACAGAAGCCACCCTGATTGCCAG
Pdcd1-sg6	ACCGAGCAACCAGACTGAAAAAC	AAACGTTTTTCAGTCTGGTTGCT
Pdcd1-sg7	ACCGGATCATACAGCTGCCCAAC	AAACGTTGGGCAGCTGTATGATC

Supplementary Table 1: The sequence of sgRNA oligos

Supplementary Table 2: Genotyping primers

sgRNA	Forward sequence (5' to 3')	Reverse sequence (5' to 3')
Tyr-sg1	CCAGGGGTTGCTGGAAAAGA	GCAGAAACCCTGGTGCTTCA
Tyr-sg2	GTTCTTTTCTTACCTCACTTTAGCA	AGGAGGTGCTAACAATACAGTGG
Tyr-sg3	CCAGGGGTTGCTGGAAAAGA	GCAGAAACCCTGGTGCTTCA
Tyr-sg4	CCAGGGGTTGCTGGAAAAGA	GCAGAAACCCTGGTGCTTCA
Tyr-sg5	GTTCTTTTCTTACCTCACTTTAGCA	AGGAGGTGCTAACAATACAGTGG
Tyr-sg6	TCAAAAGATCCCAAAAGCAGAGAA	GGAAAACAAAAATGTGCCTCAAGT
Tyr-sg7	GTTCTTTTCTTACCTCACTTTAGCA	AGGAGGTGCTAACAATACAGTGG
Pdcd1-sg1	CACATTCCTCTCCAGGGGGT	ATGGCAGAGAGCCTAAGAGGT
Pdcd1-sg2	GTCCCCAATGGGCCCTG	CATACTTGTTGAGCAGAAGACAGC
Pdcd1-sg3	ACTGCTACTGAAGGCGACAC	GCCAGGGATATGAACTCGGG
Pdcd1-sg4	ACTGCTACTGAAGGCGACAC	GCCAGGGATATGAACTCGGG
Pdcd1-sg5	ACTGCTACTGAAGGCGACAC	GCCAGGGATATGAACTCGGG
Pdcd1-sg6	GTCCCCAATGGGCCCTG	CATACTTGTTGAGCAGAAGACAGC
Pdcd1-sg7	GTCCCCAATGGGCCCTG	CATACTTGTTGAGCAGAAGACAGC

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Nunnlemenfary	Table	- 1 *	Deen	seamencing	nrimers
Suppremental j	Iunic	•••	Deep	sequencing	Primers

On-/Off-target site	Forward sequence (5' to 3')	Reverse sequence (5' to 3')
Tyr-sg1-ontarget	AGCCTGTGCCTCCTCTAAGAA	ACGGTCATCCACCCCTTTGA
Tyr-sg1-OST1	TTCCTGTGGAGTTGGCTGGA	CTGCATTTCAAGGGGACACG
Tyr-sg1-OST2	TCACTCTGCAGGAGACTTGGG	AGCTTTCAGGACCAAGGACAC
Tyr-sg1-OST3	GATGCCTGCTCCAGCATTTG	AGGTACAAGAGGAATGGTGCTG
Tyr-sg1-OST4	CCCTTAGAGAGAGCATGGGGC	AGCCAAGCTCAGCTGCTACA
Tyr-sg1-OST5	GACCAATGCCCCATAATTGGC	GCTACAGTGCAGACGTGGTA
Tyr-sg2-ontarget	GTACTTGGGAGGTCGTCACC	AGAAGAAGGATGCTGGGCTGA
Tyr-sg2-OST1	AATGAAGGCCACACCACTCTC	ATTGACCAGGGCAGAGGAGC
Tyr-sg2-OST2	CTGCTGCCCTAATTGCACCT	CAGCCGTCCTCCAAAAGCAC
Tyr-sg2-OST3	AGTATACATGAGGCCAGGGAG	GACCATGCTGCTGATCTTGTC
Tyr-sg2-OST4	TCCTGGGAAGTTAGATAGCTCCA	AGCCAGCCAAGTAAGAGCAG
Tyr-sg7-ontarget	TCAAGGGACACACTGCTTGG	CTGCATCTCTCCAATCCCAGT
Tyr-sg7-OST1	TGCTCTGGGCCTTTGGATTT	AAACCTCTGGGGGCTTGCATT
Tyr-sg7-OST2	GAGGCCGGCGGTTTATCTA	CCCACAGCTCTTCGTGTTGT
Tyr-sg7-OST3	GATGAGTCAGCAGAGGGCTG	AAGCAGGAGGAGGGAAAACC
Tyr-sg7-OST4	AAAGGAGTATTCCCTCCCAGC	ACACATCAGCACCACAAATGTT
Tyr-sg7-OST5	GACACACCACTGACTTTGCC	GAATCCCACAGCTGACAAATGG
Pdcd1-sg1-ontarget	TCAGAGGGAGCAAATGCCAC	AGCTGTATGATCTGGAAGCGG
Pdcd1-sg1-OST1	AACTTGCTTGCAGGTTCACAT	AAACACTGTTGCGCCTCATC
Pdcd1-sg1-OST2	GAAAGTGATGCTTCTCGGCG	TGTCTGTATCAAGTTCAGGGACC
Pdcd1-sg1-OST3	TGTATTGTAGACCTGGCTGCTG	CACCCAAGTTGCTTCTAGCCT
Pdcd1-sg1-OST4	GGCACTGTCTCGGTGATGAG	GTCGTGAGGCAATACAGGCT
Pdcd1-sg1-OST5	GAAGGAAGGTGACCCACAGG	GCAGTTCCCTTCCCAACAGA
Pdcd1-sg2-ontarget	ACAAGATATCCCAGCCCCTC	AGCAGGGTATGATGAGCCTT
Pdcd1-sg2-OST1	TGTGTGAAAGGAAGGTGGGTA	ACAGTCAGATGTTCGGTGTG
Pdcd1-sg2-OST2	AAGATGATGCTGCCCCTCAA	CCACAGCTGGCTTGATGTTTA
Pdcd1-sg2-OST3	CTATGTGCCGCTAGCCTCTG	TAGAGCTGAGACCCAACGGA
Pdcd1-sg2-OST4	TGGCAACTGCATTAGTGGCT	GAGTTGCCATTGTGTGACCA
Pdcd1-sg2-OST5	GAGCCTGCCTAACCAGACAT	GTGCCAAGAAGTAAACGCAGA
Pdcd1-sg3-ontarget	GGAGACTGCTACTGAAGGCG	AACCGAGGGTGAACGTTTCT
Pdcd1-sg3-OST1	TTGCAGGTTGGGTTTTGGTTT	AGGAAACGAAGGCGATGACA
Pdcd1-sg3-OST2	TGGGGGAGATGCTCTTGGATA	GCCTCAGCTGAAAACCGTTC
Pdcd1-sg3-OST3	GATCCCACATCCCTAGGAGC	TACAGACAACTCTGCCTGAGC
Pdcd1-sg3-OST4	CTGATGCTGTGAGTCAGGGT	ATTGCAACAGTCATGGGCAC
Pdcd1-sg3-OST5	AGCTACTGTACTAGGAGGCAAGA	TGGCCTAGGAAGAGACCTGA

Materials and methods

Animal ethics statement

The use and care of animals complied with the guideline of the Institutional Animal Care and Use Committee (IACUC) of CAM-SU Genomic Resource Center (CAM-SU GRC), Suzhou, China. Mice were maintained in a SPF (specific pathogen–free) facility under a 12 h dark-light cycle. C57BL/6J and ICR mouse strains were used as embryo donors and foster mothers, respectively.

Vector construct

SgRNA oligos were annealed into pGL3-sgRNA expression vector with U6 promoter and EGFP from Addgene (#107721). The sequence of sgRNA oligos were listed in Table S1.

Cell culture and transfection

Mouse-derived Neuro-2a (N2a) cells were obtained from ATCC (ATCC, CCL-131) and maintained at 37 °C with 5% CO2 inside the Modified Eagle Medium (DMEM) (Hyclone, SH30243.01) containing 10% fetal bovine serum (FBS) and 1% penicillin–streptomycin. The transfection procedure was carried out using Lipofectamine 2000 Reagent (ThermoFisher Scientific, 11668019) according to the manufacturer's instructions. In brief, N2a cells were seeded on Poly-D-lysine (Sigma, P4707) coated 12-well plates (JETBIOFIL, TCP010012), and transfection were performed at approximately 70% density about 12 hours after seeding, 0.5µg sgRNA plasmids and 1µg BE3 plasmids were transfected with 3µl Lipofectamine 2000. The medium was replaced with fresh medium at 6h after transfection, cells were harvested using flow cytometry sorting technology at 3 days after transection. Cells were incubated at 37 °C with 5% CO2.

In vitro transcription

The BE3 and BE4max plasmid was linearized by AgeI enzyme (NEB, R3552L), and transcribed *in vitro* using T7 ULTRA (Ambion, AM1345). BE3 mRNA and BE4 mRNA was purified using RNeasy Mini Kit (QIAGEN, 74104). SgRNA oligos were annealed into

pUC57-sgRNA expression vector with T7 promoter (Addgene, 51132). Then the targeted sgRNA was amplified and transcribed using MEGAshorttranscript T7 KIT (Ambion, AM1345). The sgRNAs were purified using MEGAclear Kit (Ambion, AM1908) and recovered by alcohol precipitation.

Microinjection of one-cell embryos and embryo transfer.

Super ovulated female C57BL/6J mice were mated to C57BL/6J males, and zygotes were collected from oviducts at E0.5. BE3 mRNA (50 ng/µl) or BE4max mRNA and sgRNAs (25 ng/µl for total sgRNAs in all experiments) were mixed and injected into the cytoplasm of zygotes with well recognized pronuclei. Injected zygotes were cultured to blastocysts for genomic analysis or transferred at 2-cell stage into the oviduct of pseudopregnant ICR female mice to generate offsprings.

Genomic DNA extraction and genotyping

Genomic DNA was extracted from blastocyst stage embryos and tails of newborn mice, and then subjected to targeted deep sequencing and Sanger sequencing. Embryos genomic DNA was extracted using QuickExtractTM DNA Extraction Solution (Lucigen, QE09050), 10 μ l QuickExtractTM DNA Extraction Solution were added to blastocysts that developed normally, following digest was performed according to the manufacture's protocol. Genomic DNA of N2a cells and mouse tails was extracted using phenol-chloroform method. 500 μ l Lysis buffer (10 μ M Tris-HCl, 0.4 M NaCl, 2 μ M EDTA, 1% SDS) supplemented with 5 μ l proteinase K (10 mg/mL) (Yeasen, 10401ES60) was added to harvested cells or mouse tails and digest at 55 °C overnight. The primers used for genotyping were listed in Table S2.

Western blot

Total proteins were extracted from thymus of mice using RIPA lysate containing 1% Phenylmethanesulfonyl fluoride (PMSF) and 1% Protease Inhibitor Cocktail (PIC). Protein was then quantified using a Bradford assay. Equal amounts of soluble protein were separated by SDS/PAGE and transferred onto a polyvinylidene difluoride membrane

(Roche). Immunoblotting was conducted using antibodies specific for PD-1(1:1,000, R&D Systems) and GAPDH (1: 3,000, Sigma-Aldrich). Variations in sample loading were estimated by densitometry and corrected based on the GAPDH band intensities.

Targeted deep sequencing

Possible off-target sites were predicted from Cas-OFFinder

(http://www.rgenome.net/cas-offinder) and were amplified from mouse genomic DNA using Phanta® Max Super-Fidelity DNA Polymerase (Vazyme, P505-d3). The off-target sites were listed in Table S3 and the primers used for targeted deep sequencing were listed in Table S4. Non-specific sequences in the PCR products were eliminated by gel electrophoresis. PCR products with different barcodes were pooled together for deep sequencing using the Illumina HiseqXten (2×150) platform at Novogene, Nanjing, China.

Whole-genome sequencing

Genomic DNA was extracted by phenol-chloroform method. Libraries were sequenced using the Novaseq platform (Illumina) at Novogene, Nanjing, China.

Data availability

High-throughput sequencing data has been deposited in the NCBI Sequence Read Archive database under accession code (). Plasmids pGL3-U6-sgRNA-EGFP (#107721) and pCMV-BE3 (#73021) are available from Addgene.