Supplementary Materials

Ethics declarations

All animal experiments were performed following the Guide for the Care and Use of Laboratory Animals (Ministry of Science and Technology of the People's Republic of China, Policy No. 2006398) and approved by the Animal Care and Use Center of Northwest A&F University, China.

Vector construction

The backbone vector to express Cas9 endonuclease was pSpCas9(BB)-2A-PURO (PX459) v2.0 (Ran et al., 2013). Single guide RNA (sgRNA) expression cassettes were constructed based on pSpCas9(BB)-2A-PURO using T4 DNA ligases. We used CRISPOR (http://crispor.tefor.net/) to design porcine target sgRNAs and predict potential off-target sites (Concordet & Haeussler, 2018). The polycistronic transfer RNA (tRNA)-sgRNA cassettes (PTG) were synthesized by Tsingke Biotechnology Co., Ltd, Beijing, China. All vectors used are provided in Supplementary Table S1. Sequence information about PTG cassettes is presented in Supplementary Table S2.

Cell culture and transfection

Porcine fetal fibroblasts (PFFs) were obtained from fetal pigs at 35 days of gestation and cultured at 37 °C with 5 % CO_2 in Dulbecco's Modified Eagle Medium (DMEM, Hyclone, USA) with 15% fetal bovine serum (FBS, VISTECH, New Zealand) (Ma et al., 2018; Niu et al., 2017).

Electrotransfection was carried out as described previously (Zhang et al., 2019). Approximately 3×10^6 PFFs were resuspended in 250 μL of electroporation buffer containing 25 μg of plasmids and treated for 1 ms at 300 V with BTX-ECM 830 (BTX, USA) in a 2 mm gap. After 12 h, the cells were cultured in culture medium with 2.5 μg/mL puromycin at 37 °C with 5 % CO_2 for 48 h to screen PFFs successfully transfected with the target plasmids. Limiting dilution was performed and cells were incubated at 37 °C with 5 % CO_2 for 12 days to obtain single-cell clones (Fufa et al., 2019).

Polymerase chain reaction (PCR) and Sanger sequencing

Single-cell clones selected by puromycin were collected for further genotyping assay. The cell genome was extracted using a TIANamp Genomic DNA Kit (Tiangen, China). Target fragments were amplificated by PCR and further tested by Sanger sequencing. To quantify the editing efficiency of the different sgRNA expression strategies, the sequencing peak maps were uploaded for TIDE analysis (https://tide.nki.nl/) (Brinkman et al., 2014). To test whether the sgRNAs led to significant off-targets, we performed DNA sequencing for the

top three potential off-target sites predicted by CRISPOR. All primers used are listed in Supplementary Table S3.

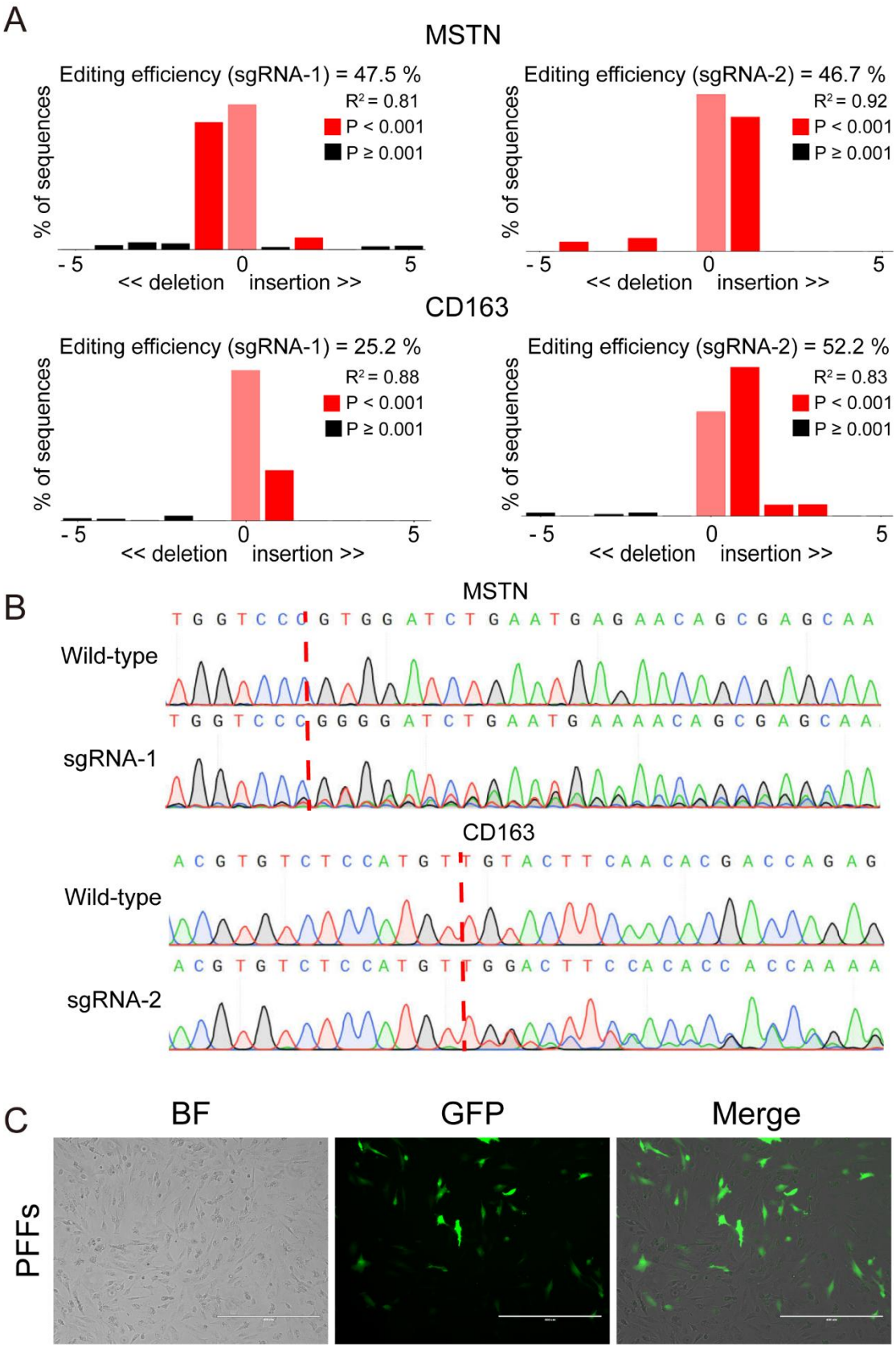
Somatic cell nuclear transfer (SCNT) and embryo transfer

Both SCNT and embryo transfer were performed following previously published protocols (Li et al., 2021; Wei et al., 2013). We collected porcine oocytes and cultured them at 38.5 °C with 5 % CO_2 *in vitro* for 40-44 h . We then produced enucleated oocytes with micromanipulators. Donor cells with positive gene-editing were injected into the pre-prepared enucleated oocytes and treated with an Electro Cell Fusion Generator (LF201, Nepa Gene, Chiba, Japan) at a voltage of 200 V/mm for 20 μs. The fused embryos were then activated with a voltage of 150 V/mm for 100 μs in activation medium and cultured at 38.5 °C with 5 % CO_2 in *vitro* for 7 days.

For embryo transfer, we selected crossbred prepubertal gilts as recipients and surgically transferred the reconstructed embryos into their oviducts. Pregnancy diagnosis was carried out using an ultrasound scanner (HS-101 V, Honda Electronics Co. Ltd., Toyohashi, Japan) after 30 days. The genetically modified piglets were obtained after about 114 days.

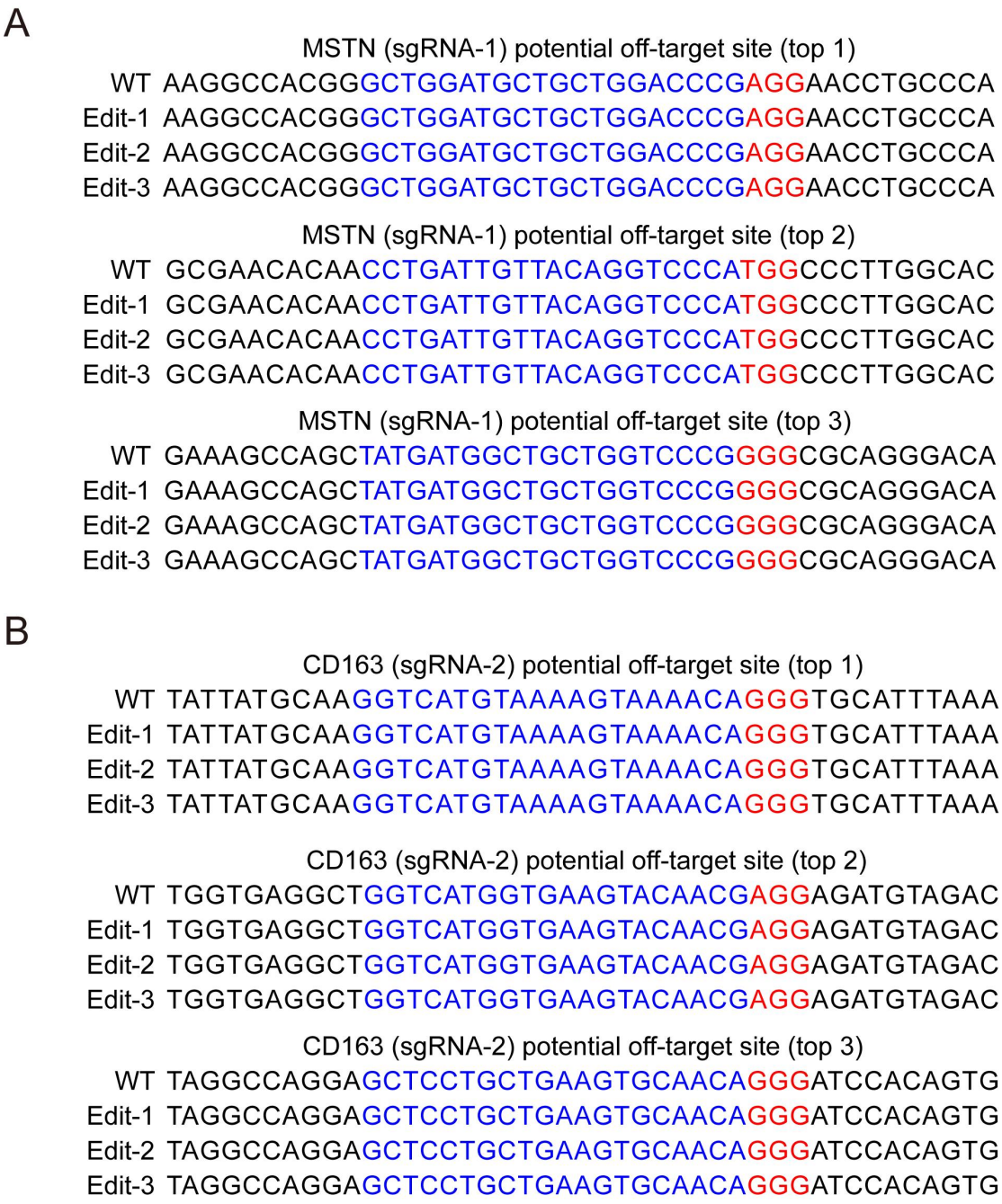
Whole-genome sequencing and data analysis

We performed whole-genome sequencing to further investigate whether our operation could lead to unexpected mutations caused by off-targets, following pre-published standard protocols (Li et al., 2019). The whole-genome sequencing data were uploaded to the National Center for Biotechnology Information Sequence Read Archive (SRA) database under accession number PRJNA866354. Genomic DNA from the gene-edited pigs and primary cells used for gene editing was extracted and sequenced (BGI, China). The reference genome for sequencing data was Sscrofa11.1. Single-nucleotide variants (SNVs) and small indels of the two pigs and porcine primary cells were identified by GATK (v4.1.4.1) (McKenna et al., 2010). Shared SNVs and indels of the genome-edited pigs and porcine primary cells were eliminated. The distribution of SNVs and indels was measured using ChIPseeker (version 1.5.1) (Yu et al., 2015).


A
MSTN
Editing efficiency (sgRNA-1) = 47.5 %
R² = 0.81
P < 0.001
P ≥ 0.001
% of sequences
- 5
0
5
<< deletion
insertion >>
Editing efficiency (sgRNA-2) = 46.7 %
R² = 0.92
P < 0.001
P ≥ 0.001
% of sequences
- 5
0
5
<< deletion
insertion >>
CD163
Editing efficiency (sgRNA-1) = 25.2 %
R² = 0.88
P < 0.001
P ≥ 0.001
% of sequences
- 5
0
5
<< deletion
insertion >>
Editing efficiency (sgRNA-2) = 52.2 %
R² = 0.83
P < 0.001
P ≥ 0.001
% of sequences
- 5
0
5
<< deletion
insertion >>
B
MSTN
Wild-type
sgRNA-1
CD163
Wild-type
sgRNA-2
C
BF
GFP
Merge
PFFs

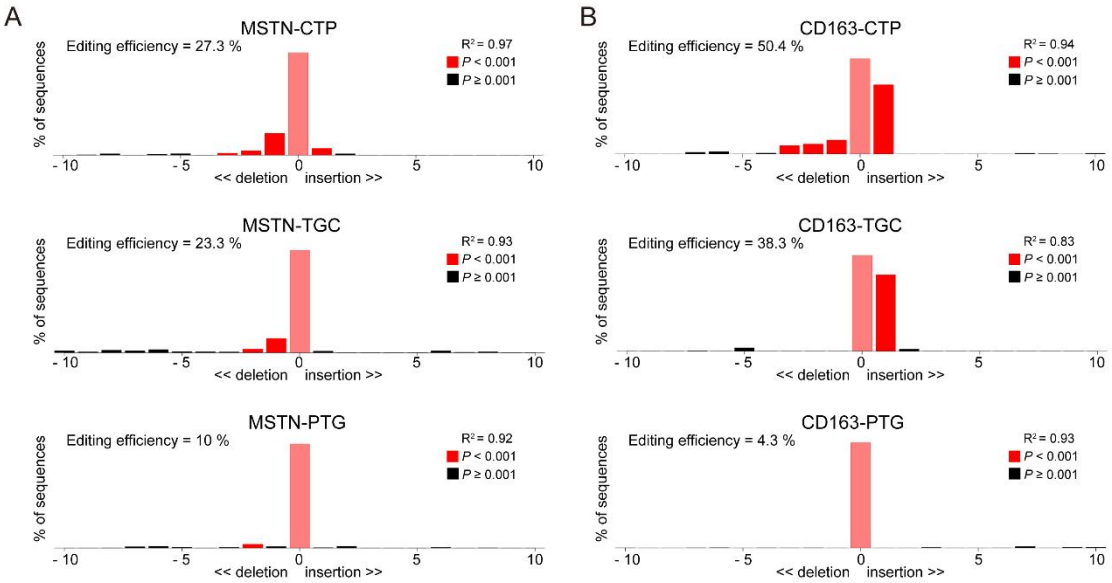
Supplementary Figure S1 Selection of sgRNAs targeting MSTN and CD163 with high efficiency

(A) Histogram of indels caused by sgRNAs. Editing efficiency was quantified by TIDE analysis. (B) Representative sequencing chromatograms of sgRNA targets. Editing start sites are marked by red dotted lines. (C) Vector transfection efficiency in PFFs. Cells carrying transfected vectors displayed GFP-positive. Scale bar, 4 00 μm.



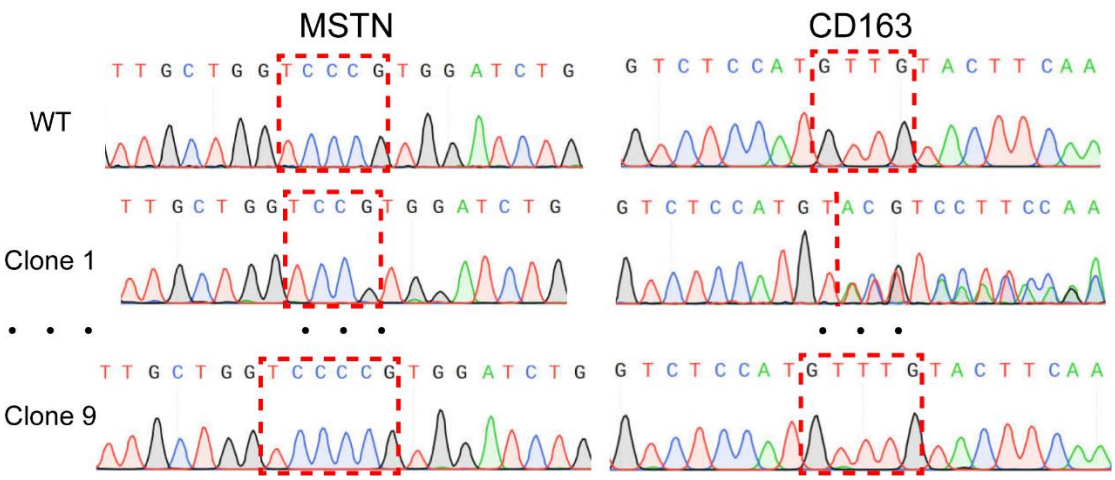
Supplementary Figure S2 Selected sgRNAs targeting MSTN and CD163 display low off-target rate

(A, B) Sanger sequencing of top-ranked potential off-target sites of MSTN and CD163 between wild-type (WT) and gene-edited cells. Protospacer adjacent motif (PAM) is in red and sgRNAs are in blue.



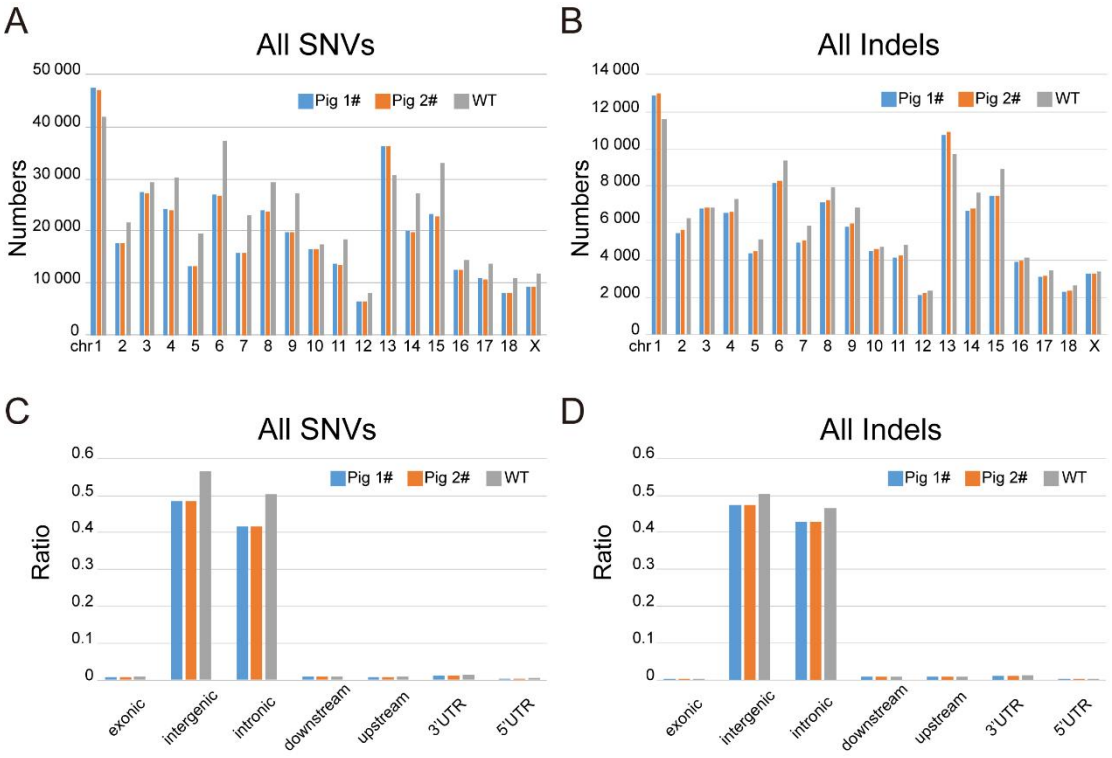
Supplementary Figure S3 Tandem sgRNA expression cassettes display high multiplex gene-editing efficiency

(A) Histograms of MSTN indels by different sgRNA expression strategies. (B) Histograms of CD163 indels by different sgRNA expression strategies. Editing efficiency is presented in upper left. CTP, strategy of co-transfection of two plasmids. TGC, strategy of tandem sgRNA expression cassettes. PTG, strategy of polycistronic tRNA-sgRNA cassettes.



Supplementary Figure S4 Generation of multiplex gene-edited single-cell-derived clones

Representative sequencing chromatograms at editing regions of gene-edited clones. Edited loci are marked by red dotted lines and squares.



Supplementary Figure S5 Analysis of off-targeting mutations in gene-edited pigs

(A and B) Distribution of all SNVs and indels in porcine chromosomes. WT represents genotype of wild-type porcine primary cells. (C and D) Genome distribution analysis of SNVs and indels.

Supplementary Table S1 List of all vectors used in this study

Vector name	Source
pSpCas9(BB)-2A-Puro (PX459)	addgene #48139
PX459-sgRNA-MSTN-1	this work
PX459-sgRNA-CD163-1	this work
PX459-sgRNA-MSTN-2	this work
PX459-sgRNA-CD163-2	this work
PX459-U6-sgMSTN-U6-sgCD163	this work
PX459-U6-PTG-sgMSTN-PTG-sgCD163	this work

Supplementary Table S2 Sequence information of PTG strategy used in this study

PTG-sgMSTN-PTG-sgCD163

AACAAAGCACCAGTGGTCTAGTGGTAGAATAGTACCCTGCCACGGTACAG
ACCCGGGTTCGATTCCCGGCTGGTGCAGCTGATTGTTGCTGGTCCCGGT
TTTAGAGCTAGAAATAGCAAGTTAAAATAAGGCTAGTCCGTTATCAACTTGA
AAAAGTGGCACCGAGTCGGTGCAACAAAGCACCAGTGGTCTAGTGGTAG
AATAGTACCCTGCCACGGTACAGACCCGGGTTCGATTCCCGGCTGGTGC
AGGTCGTGTTGAAGTACAACAGTTTTAGAGCTAGAAATAGCAAGTTAAAAT
AAGGCTAGTCCGTTATCAACTTGAAAAAGTGGCACCGAGTCGGTGC

Supplementary Table S3 Information on all primers used in this study

Application	Primer name	Forward Primer (5' -> 3')	Reverse Primer (5' -> 3')
sgRNA	sgRNA-MSTN-1	CACCGGCTGATTGTTGCTGGTCCCG	AAACCGGGACCAGCAACAATCAGCC
sgRNA	sgRNA-CD163-1	CACCGGGTCGTGTTGAAGTACAACA	AAACTGTTGTACTTCAACACGACCC
sgRNA	sgRNA-MSTN-2	CACCGGGCTGTGTAATGCATGTATG	AAACCATACATGCATTACACAGCCC
sgRNA	sgRNA-CD163-2	CACCGGGAAACCCAGGCTGGTTGGA	AAACTCCAACCAGCCTGGGTTTCCC
Clone	Clone-2sgRNA	CAAATGGCTCTAGAGGTACCGAG GGCCTATTTCCCATGATTCCTT	TAAGTTATGTAACGGGTACCAA AAAAGCACCGACTCGGTGCCA
PCR	Indel-MSTN-Test	AGATTCATTGTGGAGCAAGAGCC	CAAGGAGCCATCACTGCTGTCAT
PCR	Indel-CD163-Test	TATGGGTTCCAGAAGGCAAAGTC	TCACTTGAGCAGACTACGCCGAC
PCR	Off-target-MSTN-1	AAAGCGGTGAAAAGTCAGTCTGAG	TCTGATCCCCACACGAGGTTG
PCR	Off-target-MSTN-2	TGAAGAAAAGCACACCAACCAAG	GCTGAGCCTGGTTGGGATAGA
PCR	Off-target-MSTN-3	GCTCTGTGCTAGATGCGGAGG	CGGTTAGGATCAAACGCCAA
PCR	Off-target-CD163-1	CTTGCCCTAAGCAACATTCTCAAG	GAGGGCAACTGCTGGTCTCAATA
PCR	Off-target-CD163-2	CATTGGCACCCCTTACCAGTTTA	GCCCCGCCACTGTCATACTTAC
PCR	Off-target-CD163-3	GGAACTGCCTGATGGGAATGT	GCTGTGAAGAGGACTGTGGGGT

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