Supporting Information

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SI Materials and Methods

Plasmid Construction. The *cas9* gene from *Neisseria meningitidis* strain 8013 was PCR-amplified and cloned into the pSimpleII plasmid (an OriP containing plasmid) under the control of the elongation factor-1α promoter. Nuclear localization signals and HA tag sequences were incorporated via the PCR primers. An *N. meningitidis* BsmBI-crRNA cassette and the *N. meningitidis* transacting clustered, regularly interspaced, short palindromic repeat (CRISPR) RNA (tracrRNA), both under the control of U6 RNA polymerase III promoters, were synthesized as gene blocks (Integrated DNA Technologies) and cloned into pSimpleII-Cas9 via blunt-end cloning, generating the pSimpleII-Cas9-Tracr-BsmBI plasmid that includes all elements needed for targeting. To insert specific spacer sequences into the crRNA cassette, synthetic oligonucleotides containing the desired spacer sequences were annealed to generate a duplex with overhangs compatible with those generated by BsmBI digestion of the pSimpleII-Cas9-Tracr-BsmBI plasmid. The insert was then ligated into the BsmBI-digested plasmid.

NmCas9 DNA Transfection and in Vitro Plasmid Digestion. All transfections with 293FT cells were done using Fugene HD (Promega) following the manufacturer's instructions. Roughly 2-μg plasmids and 6 μL of Fugene HD were used for one well of a six-well plate. Two days after transfection, 293FT cells expressing *N. meningitidis* Cas9 (NmCas9) were harvested by TrypLE (Life Technologies), washed once in PBS, and then lysed in PBS by sonication. Cellular debris was cleared by centrifugation and the supernatant was used in plasmid digestion assays. For the digestions, 1 μg tdTomato plasmid (Clontech) linearized by NdeI (New England Biolabs) was mixed with in vitro-transcribed tracrRNA, crRNA, and 293FT cell lysate and incubated at 37 °C for 1–4 h in digestion buffer (1× PBS with 10 mM $MgCl_2$). DNA from the reaction mix was then purified with a PCR clean-up kit (Qiagen) and resolved by agarose gel electrophoresis. To map the cleavage site of NmCas9, the digested plasmid DNA was excised from the agarose gel and purified using Gel Extraction Kit (Qiagen). The purified fragments were then sequenced to map the cleavage site.

In Vitro Transcription. Synthetic oligonucleotides (Integrated DNA Technologies) containing the T7 promoter sequence and *N. meningitidis* tracrRNA or crRNA sequences were annealed to generate dsDNA templates for run-off transcription. In vitro transcription was done using the MegaScript T7 In Vitro Transcription kit (Ambion) following the manufacturer's specifications.

Southern Blots. Genomic DNA of targeted clones is purified using PureGene core kit (Qiagen). Five micrograms of genomic DNA was digested with BamHI and then resolved on a 0.8% agarose gel. DIG-labeled DNA probe synthesis, DNA gel transfer, and blot hybridization and visualization were done according to Roche's DIG application manual.

Genome Editing Using Single-Guide RNA. A single-guide RNA (sgRNA) that targets tdTomato was put under the control of a U6 promoter and cloned into the EcoRV site of pstBlue-1 (Novagen). For electroporation, 7.5 μg of pstBlue-U6-sgRNA, 7.5 μg of pSimpleII-NLS-NmCas9-HA-NLS(s), and 5 μg of EBNA RNA was mixed with ~1×10^6 cells in a 4-mm cuvette (Bio-Rad) and immediately electroporated with a Bio-Rad Gene Pulser. Cells were then plated into appropriate Matrigel coated culture dishes in E8 supplemented with 10 μM ROCK inhibitor Y-27632.

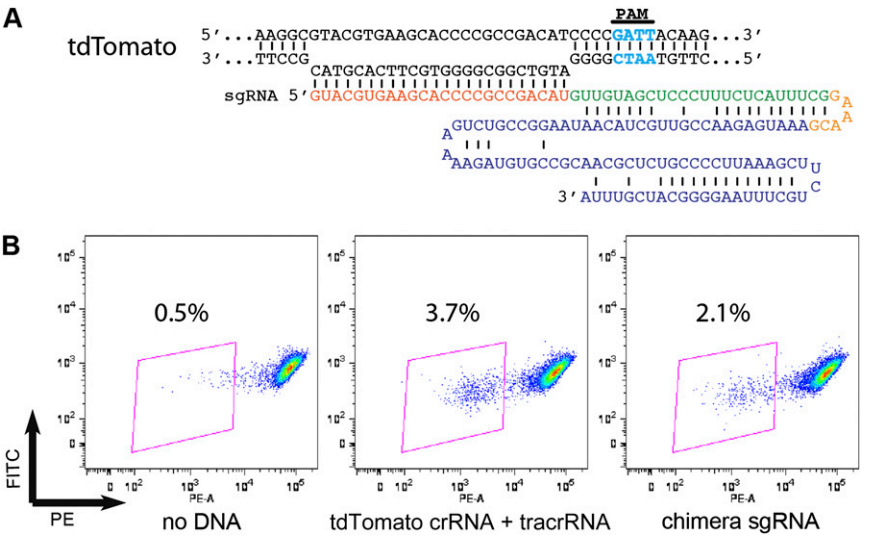

Fig. S1. sgRNA is able to direct NmCas9-catalyzed genome editing. (*A*) Design of an sgRNA that targets tdTomato. Red: spacer; green: crRNA repeat; orange: linker; Blue: tracrRNA. (*B*) FACS analysis of tdTomato reporter human ES cell lines after electroporation of the sgRNA construct and the NmCas9 expressing plasmid. The number in the plot indicates the percentage of tdTomato fluorescence-negative cells 5 d after electroporation.

Table S1. Plasmids used in this study

Plasmid no.	Plasmid name	Description
1	pSimpleII-NmCas9-FLAG	Flag tagged NmCas9 without NLS
2	pSimpleII-NLS-NmCas9-EGFP	NmCas9 EGFP fusion with N-terminal NLS
3	pSimpleII-NmCas9-HA-NLS	NmCas9 with C-terminal HA tag and NLS
4	pSimpleII-NLS-NmCas9-HA-NLS(s)	NmCas9 with dual NLS and HA tags
5	pSimpleII-U6-tracrRNA-U6-BsmBI-NLS-NmCas9-HA-NLS(s)	All-in-one plasmid containing NmCas9, tracrRNA expression cassette and U6-BsmBI cassette
6	pSimpleII-U6-tracrRNA-U6-crRNA(tdTomato)-NLS-NmCas9-HA-NLS(s)	All-in-one plasmid containing NmCas9, tracrRNA expression cassette and tdTomato-targeting crRNA expression cassette
7	pSimpleII-U6-tracrRNA-U6-crRNA(EGFP)-NLS-NmCas9-HA-NLS(s)	All-in-one plasmid containing NmCas9, tracrRNA expression cassette and EGFP-targeting crRNA expression cassette
8	pSimpleII-U6-tracrRNA-U6-crRNA(OCT4)-NLS-NmCas9-HA-NLS(s)	All-in-one plasmid containing NmCas9, tracrRNA expression cassette and OCT4-targeting crRNA expression cassette
9	pSTBlue-1-U6-sgRNA (tdTomato)	U6-driven sgRNA targeting tdTomato

Table S2. crRNA-encoding DNA sequences used in this study

crRNA	Encoding DNA sequence
EGFP targeting crRNA	gttcagcgtgtccggcgagggcgaGTTGTAGCTCCCTTTCTCATTTCG
OCT4 targeting crRNA	GacctggagtttgtgccagggtttGTTGTAGCTCCCTTTCTCATTTCG
tdTomato targeting crRNA (GATT PAM)	gtacgtgaagcaccccgccgacatGTTGTAGCTCCCTTTCTCATTTCG
tdTomato targeting crRNA (GATG PAM)	GccccgagggcttcaagtgggagcGTTGTAGCTCCCTTTCTCATTTCG
tdTomato targeting crRNA (GACT PAM)	ggacggcggtctggtgaccgtgacGTTGTAGCTCCCTTTCTCATTTCG
tdTomato targeting crRNA (GCTT PAM)	gattacaagaagctgtccttccccGTTGTAGCTCCCTTTCTCATTTCG
tdTomato targeting crRNA (CATT PAM)	GggcctcccagcccatggtcttctGTTGTAGCTCCCTTTCTCATTTCG
tdTomato targeting crRNA (CCAA PAM)	ggccgcccctacgagggcacccagGTTGTAGCTCCCTTTCTCATTTCG

All sequences are 5′ to 3′, left to right. Spacer regions are in lowercase and underlined, and CRISPR repeat regions are in uppercase. In some cases, the first nucleotide of the spacer is changed to a G to satisfy the requirement of the U6 promoter.

Table S3. Primers used in this study

Name	Forward	Reverse
For cleavage site mapping in tdTomato	ATGGTGAGCAAGGGCGAGGAG	CCGGTGCTGCCGGTGCCATGCCCCAG
For tdTomato indel mapping	AACACTGTCCCTCTCATGTCCCTGCTTC	CCGGTGCTGCCGGTGCCATGCCCCAG
For making a Southern blot probe for OCT4	GTGATGCCACCAAGAACCTT	ACAGCAGCGAGCAAATAGGT

All sequences are 5′ to 3′, left to right.

Table S4. Sequences of unprocessed and processed tracrRNA and crRNA in *N. meningitidis*

tracrRNA and crRNA	Sequence
Unprocessed tracrRNA	5′AUAUUGUCGCACUGCGAAAUGAGAACCGUUGCUACAAUAAGGCCGUCUGAAAAGAUGUGCC GCAACGCUCUGCCCCUUAAAGCUUCUGCUUUAAGGGGCAUCGUUUA 3′
Processed tracrRNA	5′AAAUGAGAACCGUUGCUACAAUAAGGCCGUCUGAAAAGAUGUGCCGCAACGCUCUGCCCCU UAAAGCUUCUGCUUUAAGGGGCAUCGUUUA 3′
Full-length spacer+repeat unit in CRISPR locus	5′NNNNNNNNNNNNNNNNNNNNNNNNNNNNNNGTTGTAGCTCCCTTTCTCATTTCGCAGTGCTA CAAT 3‘
Processed crRNA	5′NNNNNNNNNNNNNNNNNNNNNNNNNNGTTGTAGCTCCCTTTCTCATTTCG 3′

Sequences that are cleaved off during processing are underlined.

Other Supporting Information Files

Dataset S1 (DOCX)
Dataset S2 (DOCX)
Dataset S3 (DOCX)