SUPPLEMENTAL DATA

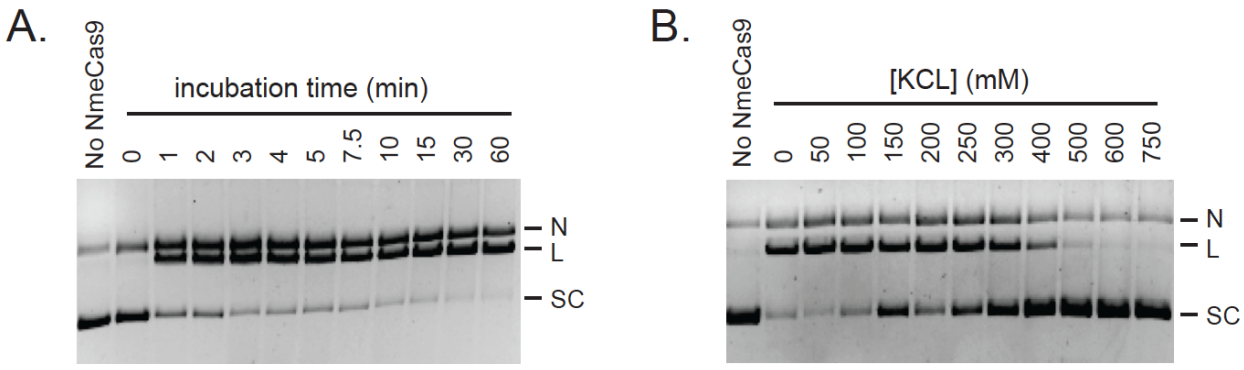


Figure S1. Biochemical Characterization of NmeCas9-catalyzed Plasmid DNA Cleavage, Related to Figure 1.

A) Time course analysis for NmeCas9 cleavage. Reactions were done as in Fig. 1C. Cleavage by NmeCas9 is rapid under these conditions, nearing completion within five minutes. N, nicked; L, linearized; SC: supercoiled.

B) Effect of salt (KCl) concentration on NmeCas9 cleavage. The optimum KCl concentration for NmeCas9 cleavage is 0-300 mM, while KCl concentrations ≥ 400 mM are inhibitory. N, nicked; L, linearized; SC: supercoiled.

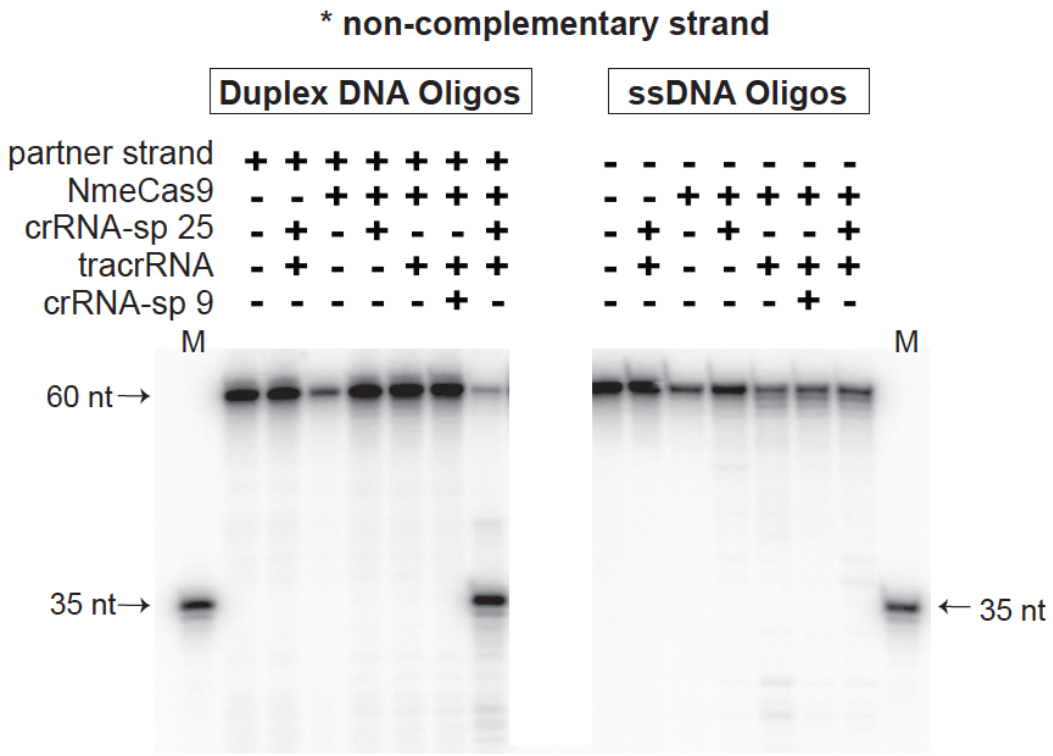


Figure S2. NmeCas9 Cleavage of the Non-complementary Strand Requires the TracrRNA, Related to Figure 3.

NmeCas9/RNA complexes were assayed for cleavage of double- (left) or single- (right) stranded DNA substrates bearing a previously verified functional target for sp 25. The non-complementary strand was 5' ^{32}P-labeled. Reactions were performed as in Fig. 2C. M, size markers. The sizes of the substrates, cleavage products and markers are indicated.

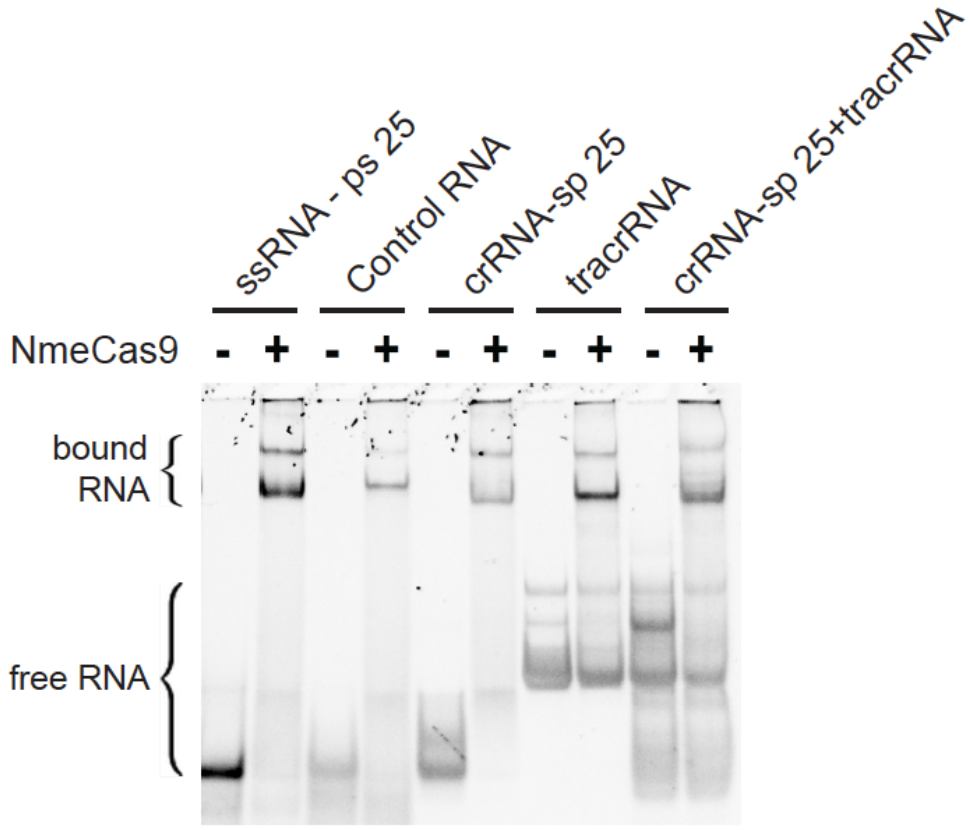


Figure S3. Promiscuous RNA Binding by NmeCas9, Related to Figure 3.
Electrophoretic mobility shift assays (EMSAs) were performed using NmeCas9 (500 nM) and various small RNAs (500 nM) as indicated. Binding was performed in cleavage reaction buffer (but with Mg^{2+} omitted) at room temperature for 10 min, and then resolved by 6% native PAGE. RNAs were visualized by SYBR Gold staining. NmeCas9 binds promiscuously to all RNAs tested. These RNAs include annealed crRNA and tracrRNA, crRNA alone, tracrRNA alone, a ps 25-containing ssRNA "target" (see Fig. 4D, RNA substrate 3), and even a 40-nt control ssRNA (see Table S3) that is unrelated to CRISPR.

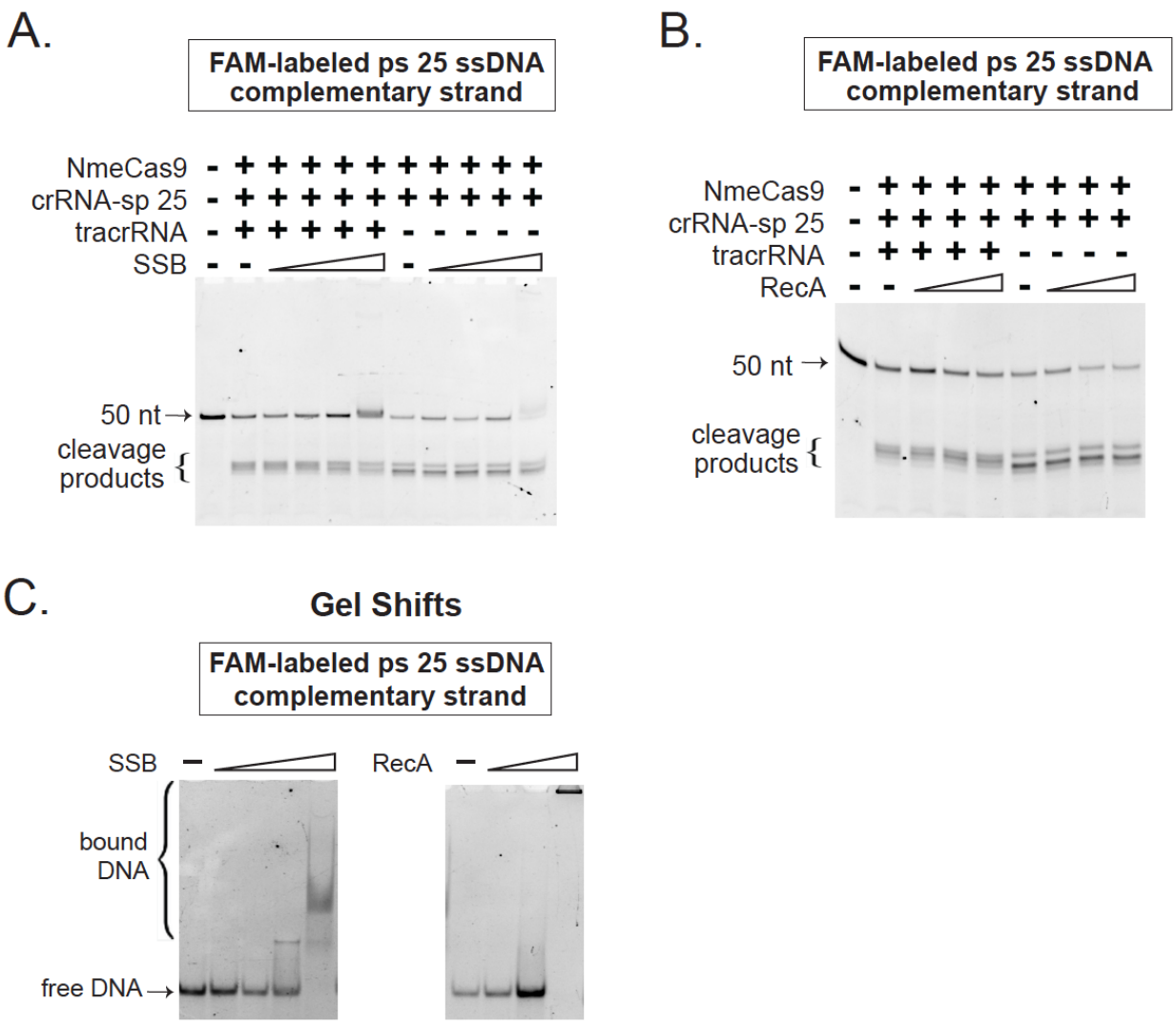
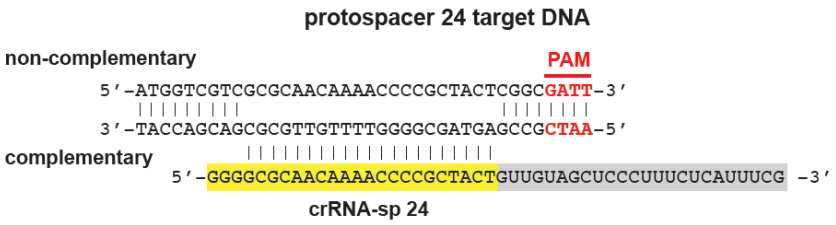


Figure S4. NmeCas9 Cleaves SsDNA Pre-bound with SSB or RecA Proteins, Related to Figure 3.

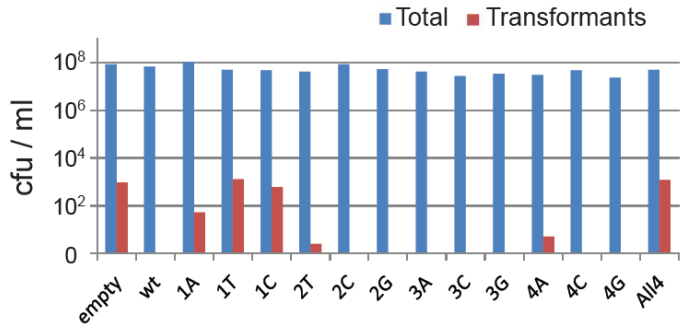
(A, B) NmeCas9 cleaves a FAM-labeled ssDNA target pre-incubated with SSB protein (A) or RecA protein (B), regardless of the presence or absence of tracrRNA. The FAM-labeled ssDNA oligonucleotide was the same as used in Fig. 4, bearing a target for sp 25. Cleavage reactions were performed as for Fig. 4, except that the ssDNA target was pre-incubated with increasing amounts of SSB or RecA in standard cleavage buffer before the addition of crRNA, tracrRNA and NmeCas9. The sizes of the substrates and cleavage products are indicated.

(C) FAM-labeled ssDNA targets are bound by higher concentrations of SSB or RecA after the pre-incubation, as revealed by EMSA.

A.



B.



C.

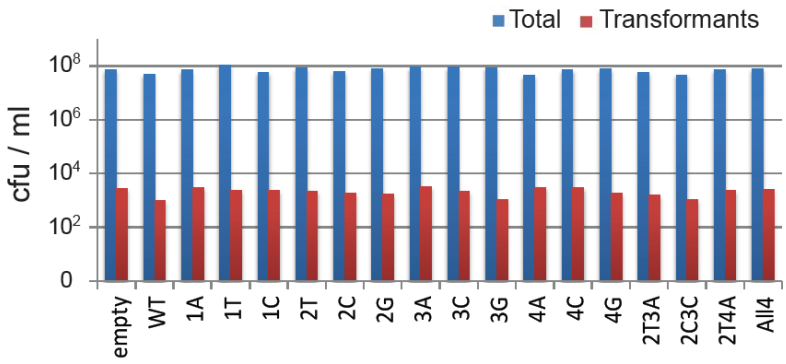


Figure S5. NmeCas9's Strong Preference for the 1st Guanine within PAM is Not a Spacer-specific Effect, Related to Figure 5.

(A) A schematic of the ps 24-containing DNA targets and sp 24 sgRNA. In red, PAM; highlighted in yellow, crRNA spacer; highlighted in gray, crRNA repeat.

(B) pYZEJS040 derivatives with WT and mismatched targets for sp 24 were tested by natural transformation assays as in Fig. 5B. Experiments were done twice, and the data shown here are from one representative experiment.

(C) Interference Defects for PAM variants are not due to deficiencies in natural transformation. PYZEJS040 derivatives tested in Fig. 5B (all single-nt PAM variants and selected two-nt PAM variants) were analyzed by natural transformation assay using an interference-defective strain of MC8013 (*cas9*::Tn) as the recipient (Zhang et al., 2013). In the absence of CRISPR interference, all pYZEJS040 derivatives tested were as efficiently transformed as the empty vector. Experiments were done twice, and the data shown here are from one representative experiment.

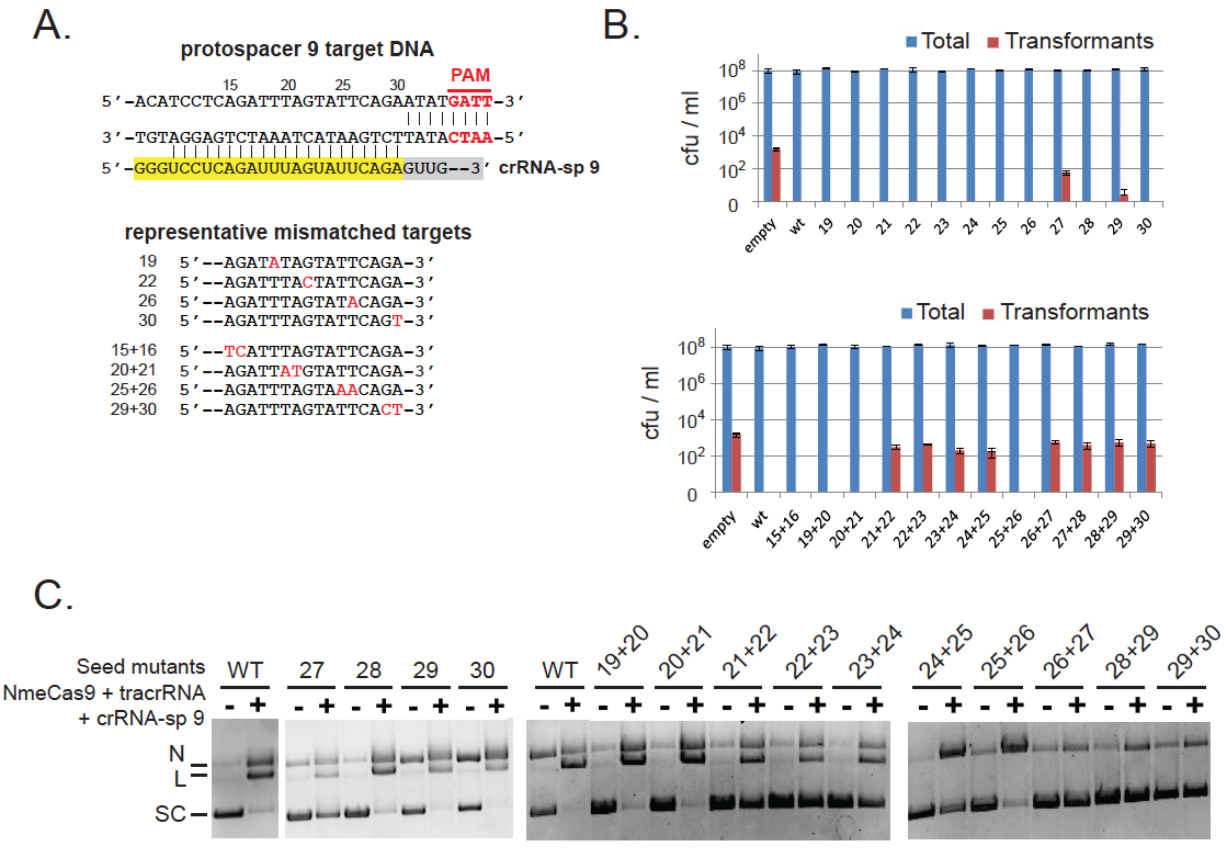


Figure S6. NmeCas9's Seed Sequence Requirements in Bacterial Cells and *in vitro*, Related to Figure 6.

(A) A schematic of ps 9-containing DNA targets and sp 9 crRNA. In red, PAM; highlighted in yellow, spacer 9 of the crRNA; highlighted in gray, crRNA repeat. Representative mismatched targets are shown below, with mutations marked in red. Positions of the mutated nts within ps 25 (counting from the PAM-distal end) are indicated on the left.

(B) NmeCas9 tolerates most of the 1-nt but not 2-nt mismatches in the seed region during interference in bacteria. pGCC2 derivatives containing ps 9 with various 1-nt and 2-nt seed mutations were tested by natural transformation assays as in Fig. 5B.

(C) NmeCas9 tolerates many 1-nt but not 2-nt mismatches in the seed region *in vitro.* Representatives of the plasmids tested in (B) were analyzed *in vitro* by a plasmid cleavage assay using NmeCas9, sp 9 crRNA, and tracrRNA. Reactions were performed as in Fig. 1C. N, nicked; L, linearized; SC: supercoiled.

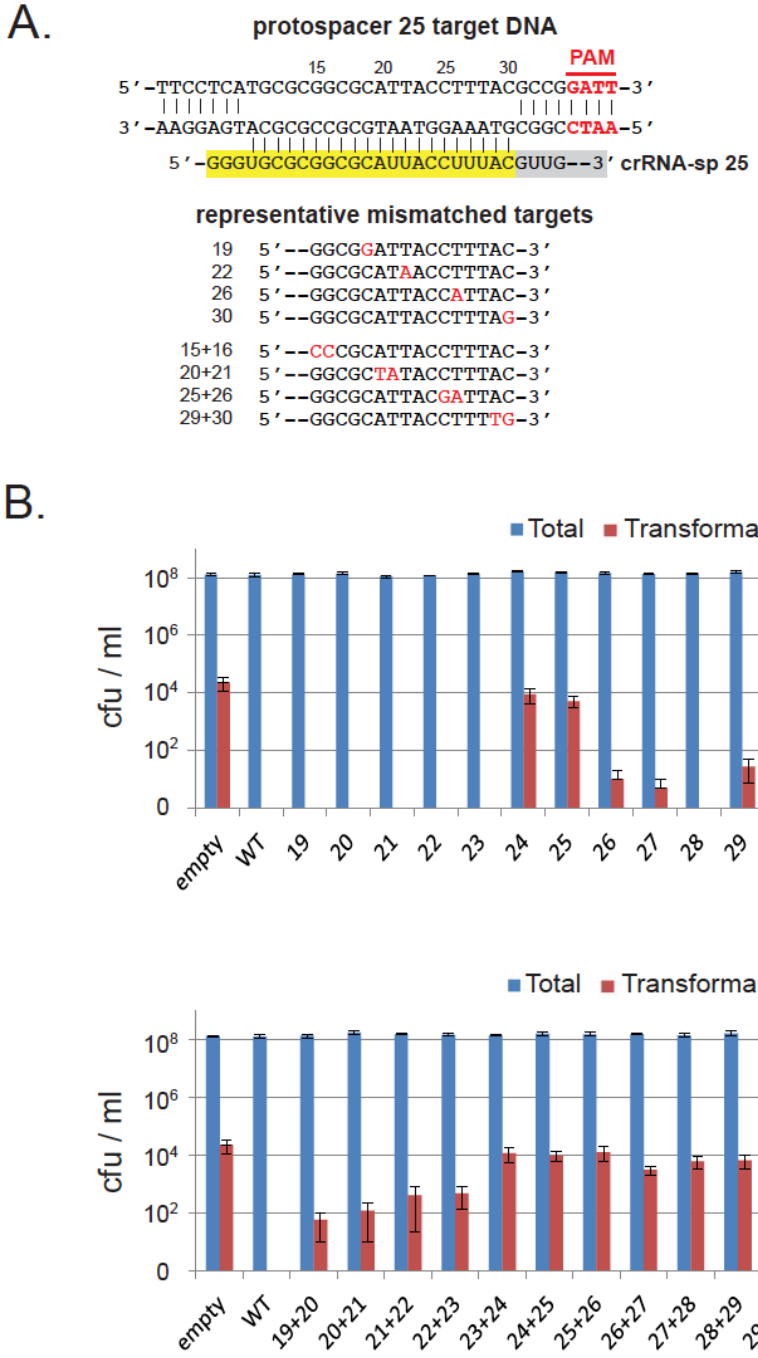


Figure S7. NmeCas9's Seed Sequence Requirements in Bacterial Cells are not Spacer Context-dependent, Related to Figure 6.

(A) A Schematic of ps 25-containing DNA targets and sp 25 crRNA. In red, PAM; highlighted in yellow, crRNA spacer; highlighted in gray, crRNA repeat. Representative mismatched targets are shown below, with mismatches marked in red and positions of the mutated nts indicated on the left.

(B) NmeCas9 tolerates many 1-nt but not 2-nt mismatches in the seed region of protospacer 25 during interference in bacteria. pYZEJS040 derivatives containing ps 25 with various 1-nt and 2-nt seed mutations were assayed for interference using natural transformation as in Fig. 5B.

Table S1. Bacterial strains used in this study, related to Figures 5-7, and S5-S7.

Strain names	Relevant genotypes	Source
N. meningitidis 8013	Wild type	Dr. Hank Seifert lab collection
	Δ*crispr*Δ*tracr*	This study
	Δ*crispr*Δ*tracr*/pGCC2	This study
	Δ*crispr*Δ*tracr*/pGCC2-Sp25 sgRNA FL	This study
	Δ*crispr*Δ*tracr*/pGCC2-Sp25 sgRNA Δ7nt	This study
	Δ*crispr*Δ*tracr*/pGCC2-Sp25 sgRNA Δ14nt	This study
	Δ*crispr*Δ*tracr*/pGCC2-Sp25 sgRNA Δ22nt	This study
	Δ*crispr*Δ*tracr*/pGCC2-Sp25 sgRNA Δ45nt	This study
	Δ*crispr*Δ*tracr*/pGCC2-Sp25 sgRNA Δ57nt	This study
	Δ*crispr*Δ*tracr*/pGCC2-Sp25 sgRNA Δ69nt	This study
	Δ*crispr*Δ*tracr*/pGCC2-Sp25 sgRNA int1	This study
	Δ*crispr*Δ*tracr*/pGCC2-Sp25 sgRNA int2	This study
	Δ*crispr*Δ*tracr*/pGCC2-Sp25 sgRNA int3	This study
	cas9::Tn	Zhang *et al*., 2013

Table S2. Plasmids used in this study for strain creation and in vitro assays, related to Figures 1, 2, 5A, 7, S1, and S6.

Plasmids	Relevant characteristics	Source
Plasmids for creating the Δ*crispr*Δ*tracr* strain used in Fig. 7		
pYZEJS067	pSmartHCAmp/Δ*crispr*/ SalI+SpeI	This study
pYZEJS068	pSmartHCAmp/Δ*crispr*/ *CAT+rpsL*	This study
Plasmids for creating the *sgRNA* complementation strains in Δ*crispr*Δ*tracr* background used in Fig. 7		
pYZEJS001	pGCC2 empty	Dr. Hank Seifert lab collection
pYZEJS079	pGCC2/promoter+Sp25 sgRNA FL	This study
pYZEJS162	pGCC2/promoter+Sp25 sgRNA Δ22nt	This study
pYZEJS163	pGCC2/promoter+Sp25 sgRNA Δ45nt	This study
pYZEJS164	pGCC2/promoter+Sp25 sgRNA Δ57nt	This study
pYZEJS165	pGCC2/promoter+Sp25 sgRNA Δ69nt	This study
pYZEJS175	pGCC2/promoter+Sp25 sgRNA Δ7nt	This study
pYZEJS176	pGCC2/promoter+Sp25 sgRNA Δ14nt	This study
pYZEJS177	pGCC2/promoter+Sp25 sgRNA int1	This study
pYZEJS178	pGCC2/promoter+Sp25 sgRNA int2	This study
pYZEJS179	pGCC2/promoter+Sp25 sgRNA int3	This study
Plasmids for purification of NmeCas9 proteins		
pYZEJS265	pMCSG7/NmeCas9 wt	This study
pYZEJS266	pMCSG7/NmeCas9 D16A	This study
pYZEJS267	pMCSG7/NmeCas9 H588	This study
pYZEJS268	pMCSG7/NmeCas9 D16A+H588A (dm)	This study
Plasmids for *in vitro* cleavage in Figs. 1, 2, 5A, S1, S6 (also used in interference assays in Fig. S6)		
pYZEJS012	pGCC2/protospacer 9 wt	Zhang *et al*., 2013
pYZEJS095	pGCC2/protospacer 9 1C	This study
pYZEJS096	pGCC2/protospacer 9 2T	This study
pYZEJS097	pGCC2/protospacer 9 3A	This study
pYZEJS098	pGCC2/protospacer 9 4A	This study
pYZEJS023	pGCC2/protospacer 9 2T3A	Zhang *et al*., 2013
pYZEJS147	pGCC2/protospacer 9 seed 19	This study
pYZEJS148	pGCC2/protospacer 9 seed 20	This study
pYZEJS149	pGCC2/protospacer 9 seed 21	This study
pYZEJS150	pGCC2/protospacer 9 seed 22	This study
pYZEJS151	pGCC2/protospacer 9 seed 23	This study
pYZEJS152	pGCC2/protospacer 9 seed 24	This study
pYZEJS153	pGCC2/protospacer 9 seed 25	This study
pYZEJS154	pGCC2/protospacer 9 seed 26	This study
pYZEJS155	pGCC2/protospacer 9 seed 27	This study
pYZEJS156	pGCC2/protospacer 9 seed 28	This study
pYZEJS157	pGCC2/protospacer 9 seed 29	This study
pYZEJS158	pGCC2/protospacer 9 seed 30	This study
pYZEJS020	pGCC2/protospacer 9 seed 15+16	Zhang *et al*., 2013
pYZEJS181	pGCC2/protospacer 9 seed 19+20	This study
pYZEJS182	pGCC2/protospacer 9 seed 20+21	This study
pYZEJS183	pGCC2/protospacer 9 seed 21+22	This study
pYZEJS184	pGCC2/protospacer 9 seed 22+23	This study
pYZEJS185	pGCC2/protospacer 9 seed 23+24	This study
pYZEJS186	pGCC2/protospacer 9 seed 24+25	This study
pYZEJS187	pGCC2/protospacer 9 seed 25+26	This study
pYZEJS188	pGCC2/protospacer 9 seed 26+27	This study
pYZEJS021	pGCC2/protospacer 9 seed 27+28	Zhang *et al*., 2013
pYZEJS189	pGCC2/protospacer 9 seed 28+29	This study
pYZEJS190	pGCC2/protospacer 9 seed 29+30	This study

Table S3. Plasmids used in this study for interference assays, related to Figures 5, 6, S5, and S7.

Plasmids	Relevant characteristics	Source
Plasmids for interference assays in Fig. 5B and Fig. S5C		
pYZEJS040	pSTblue-1/*siaA+CAT+ctrA*	Zhang *et al.*, 2013
pYZEJS101	pYZEJS040-protospacer25 wt (short)	This study
pYZEJS102	pYZEJS040-protospacer25 1A	This study
pYZEJS103	pYZEJS040-protospacer25 1T	This study
pYZEJS104	pYZEJS040-protospacer25 1C	This study
pYZEJS105	pYZEJS040-protospacer25 2T	This study
pYZEJS106	pYZEJS040-protospacer25 2C	This study
pYZEJS107	pYZEJS040-protospacer25 2G	This study
pYZEJS108	pYZEJS040-protospacer25 3A	This study
pYZEJS109	pYZEJS040-protospacer25 3C	This study
pYZEJS110	pYZEJS040-protospacer25 3G	This study
pYZEJS111	pYZEJS040-protospacer25 4A	This study
pYZEJS112	pYZEJS040-protospacer25 4C	This study
pYZEJS113	pYZEJS040-protospacer25 4G	This study
pYZEJS114	pYZEJS040-protospacer25 2T3A	This study
pYZEJS115	pYZEJS040-protospacer25 1C2T3A4A	This study
pYZEJS116	pYZEJS040-protospacer25 2C3C	This study
pYZEJS117	pYZEJS040-protospacer25 2T4A	This study
pYZEJS118	pYZEJS040-protospacer25 2C4C	This study
pYZEJS119	pYZEJS040-protospacer25 3A4A	This study
pYZEJS120	pYZEJS040-protospacer25 3C4C	This study
pYZEJS121	pYZEJS040-protospacer25 2T3A4A	This study
pYZEJS122	pYZEJS040-protospacer25 2C3C4C	This study
pYZEJS125	pYZEJS040-protospacer25 2T3C	This study
pYZEJS126	pYZEJS040-protospacer25 2T3G	This study
pYZEJS127	pYZEJS040-protospacer25 2C3A	This study
pYZEJS128	pYZEJS040-protospacer25 2C3G	This study
pYZEJS129	pYZEJS040-protospacer25 2G3A	This study
pYZEJS130	pYZEJS040-protospacer25 2G3C	This study
pYZEJS131	pYZEJS040-protospacer25 2G3G	This study
pYZEJS132	pYZEJS040-protospacer25 2T4C	This study
pYZEJS133	pYZEJS040-protospacer25 2T4G	This study
pYZEJS134	pYZEJS040-protospacer25 2C4A	This study
pYZEJS135	pYZEJS040-protospacer25 2C4G	This study
pYZEJS136	pYZEJS040-protospacer25 2G4A	This study
pYZEJS137	pYZEJS040-protospacer25 2G4C	This study
pYZEJS138	pYZEJS040-protospacer25 2G4G	This study
pYZEJS139	pYZEJS040-protospacer25 3A4C	This study
pYZEJS140	pYZEJS040-protospacer25 3A4G	This study
pYZEJS141	pYZEJS040-protospacer25 3C4A	This study
pYZEJS142	pYZEJS040-protospacer25 3C4G	This study
pYZEJS143	pYZEJS040-protospacer25 3G4A	This study
pYZEJS144	pYZEJS040-protospacer25 3G4C	This study
pYZEJS145	pYZEJS040-protospacer25 3G4G	This study
pYZEJS146	pYZEJS040-protospacer25 2G3G4G	This study
Plasmids for interference assay and *in vitro* cleavage assays in Fig. 6		
pYZEJS241	pYZEJS040-protospacer23-lk1	This study
pYZEJS242	pYZEJS040-protospacer23-lk2	This study
pYZEJS243	pYZEJS040-protospacer23-lk3	This study
pYZEJS244	pYZEJS040-protospacer23-lk4	This study
pYZEJS245	pYZEJS040-protospacer23-lk5	This study
pYZEJS246	pYZEJS040-protospacer23-lk6	This study
pYZEJS247	pYZEJS040-protospacer23-lk7	This study

pYZEJS248	pYZEJS040-protospacer23-lk8	This study
Plasmids for interference assay in Fig. S5B		
pYZEJS070	pYZEJS040-protospacer24 wt	This study
pYZEJS081	pYZEJS040-protospacer24 1A	This study
pYZEJS082	pYZEJS040-protospacer24 1T	This study
pYZEJS083	pYZEJS040-protospacer24 1C	This study
pYZEJS084	pYZEJS040-protospacer24 2T	This study
pYZEJS085	pYZEJS040-protospacer24 2C	This study
pYZEJS086	pYZEJS040-protospacer24 2G	This study
pYZEJS087	pYZEJS040-protospacer24 3A	This study
pYZEJS088	pYZEJS040-protospacer24 3C	This study
pYZEJS089	pYZEJS040-protospacer24 3G	This study
pYZEJS090	pYZEJS040-protospacer24 4A	This study
pYZEJS091	pYZEJS040-protospacer24 4C	This study
pYZEJS092	pYZEJS040-protospacer24 4G	This study
pYZEJS094	pYZEJS040-protospacer24 1C2T3A4A	This study
Plasmids for interference assays in Fig. S7		
pYZEJS192	pYZEJS040-protospacer25 seed 19	This study
pYZEJS193	pYZEJS040-protospacer25 seed 20	This study
pYZEJS194	pYZEJS040-protospacer25 seed 21	This study
pYZEJS195	pYZEJS040-protospacer25 seed 22	This study
pYZEJS196	pYZEJS040-protospacer25 seed 23	This study
pYZEJS197	pYZEJS040-protospacer25 seed 24	This study
pYZEJS198	pYZEJS040-protospacer25 seed 25	This study
pYZEJS199	pYZEJS040-protospacer25 seed 26	This study
pYZEJS200	pYZEJS040-protospacer25 seed 27	This study
pYZEJS201	pYZEJS040-protospacer25 seed 28	This study
pYZEJS202	pYZEJS040-protospacer25 seed 29	This study
pYZEJS203	pYZEJS040-protospacer25 seed 30	This study
pYZEJS208	pYZEJS040-protospacer25 seed 19+20	This study
pYZEJS209	pYZEJS040-protospacer25 seed 20+21	This study
pYZEJS210	pYZEJS040-protospacer25 seed 21+22	This study
pYZEJS211	pYZEJS040-protospacer25 seed 22+23	This study
pYZEJS212	pYZEJS040-protospacer25 seed 23+24	This study
pYZEJS213	pYZEJS040-protospacer25 seed 24+25	This study
pYZEJS214	pYZEJS040-protospacer25 seed 25+26	This study
pYZEJS215	pYZEJS040-protospacer25 seed 26+27	This study
pYZEJS216	pYZEJS040-protospacer25 seed 27+28	This study
pYZEJS217	pYZEJS040-protospacer25 seed 28+29	This study
pYZEJS218	pYZEJS040-protospacer25 seed 29+30	This study

Table S4. Oligonucleotides used in this study for in vitro cleavage/binding assays, related to Figures 2-6 and S2-S4.

Oligo Name	[a]	Sequence, 5'-3'[b]	Purpose
For generating *in vitro* transcription templates by annealing (T7 promoter in bold)			
T7-tracrRNA Forw	F	**TAATACGACTCACTATA**GAAATGAGAACCGTTGCTAC AATAAGGCCGTCTGAAAAGATGTGCCGCAACGCTCTG CCCCTTAAAGCTTCTGCTTTAAGGGGCATCGTTTA	TracrRNA (Hou et al., 2013)
T7-tracrRNA AS	R	TAAACGATGCCCCTTAAAGCAGAAGCTTTAAGGGGCA GAGCGTTGCGGCACATCTTTTCAGACGGCCTTATTGT AGCAACGGTTCTCATTTC**TATAGTGAGTCGTATTA**	
T7-Sp25-R Forw	F	**TAATACGACTCACTATA**GGGTGCGCGGCGCATTACCT TTACGTTGTAGCTCCCTTTCTCATTTCG	CrRNA-sp25
T7-Sp25-R AS	R	CGAAATGAGAAAGGGAGCTACAACGTAAAGGTAATGC GCCGCGCACCC**TATAGTGAGTCGTATTA**	
T7-Sp9-R Forw	F	**TAATACGACTCACTATA**GGGTCCTCAGATTTAGTATTC AGA GTTGTAGCTCCCTTTCTCATTTCG	CrRNA-sp9
T7-Sp9-R AS	R	CGAAATGAGAAAGGGAGCTACAACTCTGAATACTAAAT CTGAGGACCC**TATAGTGAGTCGTATTA**	
T7-Sp23-R Forw	F	**TAATACGACTCACTATA**GGGTTTCATGGCGCGTTCTT GCTG GTTGTAGCTCCCTTTCTCATTTCG	CrRNA-sp23
T7-Sp23-R AS	R	CGAAATGAGAAAGGGAGCTACAACCAGCAAGAACGC GCCATGAAACCC**TATAGTGAGTCGTATTA**	
T7-Sp25-12nt R Forw	F	**TAATACGACTCACTATA**GGGTGCGCGGCGCATTACCT TTACGTTGTAGCTCCC	CrRNA-sp25 12nt R
T7-Sp25-12nt R AS	R	GGGAGCTACAACGTAAAGGTAATGCGCCGCGCACCC **TATAGTGAGTCGTATTA**	
T7-Sp25-8nt R Forw	F	**TAATACGACTCACTATA**GGGTGCGCGGCGCATTACCT TTACGTTGTAGC	CrRNA-sp25 8nt R
T7-Sp25-8nt R AS	R	GCTACAACGTAAAGGTAATGCGCCGCGCACCC**TATAG TGAGTCGTATTA**	
T7-Sp25-4nt R Forw	F	**TAATACGACTCACTATA**GGGTGCGCGGCGCATTACCT TTACGTTG	CrRNA-sp25 4nt R
T7-Sp25-4nt R AS	R	CAACGTAAAGGTAATGCGCCGCGCACCC**TATAGTGAG TCGTATTA**	
T7-Sp25-0nt R Forw	F	**TAATACGACTCACTATA**GGGTGCGCGGCGCATTACCT TTAC	CrRNA-sp25 0nt R
T7-Sp25-0nt RAS	R	GTAAAGGTAATGCGCCGCGCACCC**TATAGTGAGTCGT ATTA**	
T7-Sp25-Sp.R Forw	F	**TAATACGACTCACTATA**GGGTGCGCGGCGCATTACCT TTACGTTTTAGAGCTATGCTGTTTTG	CrRNA-sp25 Sp.R
T7-Sp25-Sp.R AS	R	CAAAACAGCATAGCTCTAAAACGTAAAGGTAATGCGC CGCGCACCC**TATAGTGAGTCGTATTA**	
T7-Sp25-0nt R+3'Δ5nt F	F	**TAATACGACTCACTATA**GGGTGCGCGGCGCATTACC	CrRNA-sp25 0nt R+3'Δ5nt
T7-Sp25-0nt R+3'Δ5nt AS	R	GGTAATGCGCCGCGCACCC**TATAGTGAGTCGTATTA**	
T7-Sp25-0nt R+3'Δ5nt+5' 2nt extension F	F	**TAATACGACTCACTATA**GGGCATGCGCGGCGCATTAC C	CrRNA-sp25 0nt R+3'Δ5nt+5' 2nt extension
T7-Sp25-0nt R+3'Δ5nt+5' 2nt extension AS	R	GGTAATGCGCCGCGCATGCCC**TATAGTGAGTCGTATT A**	
T7-Sp25-0nt R+3'Δ5nt+5' 3nt extension F	F	**TAATACGACTCACTATA**GGGGCATGCGCGGCGCATT ACC	CrRNA-sp25 0nt R+3'Δ5nt+5' 3nt extension
T7-Sp25-0nt R+3'Δ5nt+5' 3nt extension AS	R	GGTAATGCGCCGCGCATGCCCC**TATAGTGAGTCGTAT TA**	
T7-Sp25-0nt R+5' 5nt extension F	F	**TAATACGACTCACTATA**GGGCCGCATGCGCGGCGCA TTACCTTTAC	CrRNA-sp25 0nt R+5' 5nt extension
T7-Sp25-0nt R+5' 5nt extension AS	R	GTAAAGGTAATGCGCCGCGCATGCGGCCC**TATAGTG AGTCGTATTA**	
T7-Sp25-0nt R+3' 10nt extension F	F	**TAATACGACTCACTATA**GGGTGCGCGGCGCATTACCT TTACATATGATTAT	CrRNA-sp25 0nt R+3' 10nt extension
T7-Sp25-0nt R+3' 10nt extension AS	R	ATAATCATATGTAAAGGTAATGCGCCGCGCACCC**TAT AGTGAGTCGTATTA**	
T7- C RNA target 25 Forw	F	CAAGTC**TAATACGACTCACTATA**GGGTCAGCACGGCC AGCAATCCGGCGTAAAGGTAATGCGCCGCGCATGAG GAATAAAAATCTG	To generate ssRNA bearing sequence antisense to sp25.
T7- C RNA target 25 AS	R	CAGATTTTTATTCCTCATGCGCGGCGCATTACCTTTAC GCCGGATTGCTGGCCGTGCTGACCC**TATAGTGAGTC**	

		GTATTAGACTTG	
For *in vitro* cleavage/binding assays (Mutations, in red; PAMs, highlighted in grey,)			
Sp25-NC (Non Complementary strand)	F	CAGATTTTTATTCCTCATGCGCGGCGCATTACCTTTAC GCCGGATTGCTGGCCGTGCTGA	For Figs. 2C, 3B, 3D, 5C, & S2.
Sp25-C (Complementary strand)	R	TCAGCACGGCCAGCAATCCGGCGTAAAGGTAATGCG CCGCGCATGAGGAATAAAAATCTG	
Sp25-NC PAM Mut	F	CAGATTTTTATTCCTCATGCGCGGCGCATTACCTTTAC GCCGCTAAGCTGGCCGTGCTGA	For Figs. 5C
Sp25-C PAM Mut	R	TCAGCACGGCCAGCTTAGCGGCGTAAAGGTAATGCG CCGCGCATGAGGAATAAAAATCTG	
FAM- sp25-C	R	/56-FAM/ATTCAGCACGGCATATAATCATATGTAAAGGT AATGCGCCGCGCATGCGG	For Figs. 3C, 4A-D, 5D, & S4.
FAM- sp25-C NO PAM	R	/56-FAM/ATTCAGCACGGCATATATAGATATGTAAAGGT AATGCGCCGCGCATGCGG	
CrDNA25	F	TCATGCGCGGCGCATTACCTTTACGTTGTAGCTCCCTT TCTCATTTCG	For Fig. 4D.
FAM-extended crDNA25	F	/56-FAM/CCACCTGCTGAAGGAATAGTGCGCGGCGCAT TACCTTTACGTTGTAGCTCCCTTTCTCATTTCG	
Control RNA	-	AAGTTTGAAGGTGATACCCTTGTTAATAGAATCGAGTT AA	
Sp25-LK6-NC	F	CAGATTTTTATTCCTCATGCGCGGCGCATTACCTTTAC ATATATGATTGCTGGCCGTGCT	For Figs. 6C & 6D.
Sp25-LK6-C	R	AGCACGGCCAGCAATCATATATGTAAAGGTAATGCGC CGCGCATGAGGAATAAAAATCTG	
Sp25-LK5-NC	F	CAGATTTTTATTCCTCATGCGCGGCGCATTACCTTTAC ATATAGATTGCTGGCCGTGCTG	
Sp25-LK5-C	R	CAGCACGGCCAGCAATCTATATGTAAAGGTAATGCGC CGCGCATGAGGAATAAAAATCTG	
Sp25-LK4-NC	F	CAGATTTTTATTCCTCATGCGCGGCGCATTACCTTTAC ATATGATTGCTGGCCGTGCTGA	
Sp25-LK4-C	R	TCAGCACGGCCAGCAATCATATGTAAAGGTAATGCGC CGCGCATGAGGAATAAAAATCTG	
Sp25-LK3-NC	F	CAGATTTTTATTCCTCATGCGCGGCGCATTACCTTTAC ATAGATTGCTGGCCGTGCTGAA	
Sp25-LK3-C	R	TTCAGCACGGCCAGCAATCTATGTAAAGGTAATGCGC CGCGCATGAGGAATAAAAATCTG	
Sp25-LK2-NC	F	CAGATTTTTATTCCTCATGCGCGGCGCATTACCTTTAC ATGATTGCTGGCCGTGCTGAAG	
Sp25-LK2-C	R	CTTCAGCACGGCCAGCAATCATGTAAAGGTAATGCGC CGCGCATGAGGAATAAAAATCTG	
37nt NCsp25	F	CAGATTTTTATTCCTCATGCGCGGCGCATTACCTTTA	Size markers for oligo cleavage assays
36nt NCsp25	F	CAGATTTTTATTCCTCATGCGCGGCGCATTACCTTT	
35nt NCsp25	F	CAGATTTTTATTCCTCATGCGCGGCGCATTACCTT	
34nt NCsp25	F	CAGATTTTTATTCCTCATGCGCGGCGCATTACCT	
33nt NCsp25	F	CAGATTTTTATTCCTCATGCGCGGCGCATTACCT	
25nt Csp25	R	TCAGCACGGCCAGCAATCCGGCGTA	
24nt Csp25	R	TCAGCACGGCCAGCAATCCGGCGT	
22nt Csp25	R	TCAGCACGGCCAGCAATCCGGC	
[a] F, Forward; R, Reverse.			
[b] **Bold**, T7 promoter; highlighted in grey, PAMs; red, mutations.			

Table S5. Oligonucleotides used in this study for protein purification, strain creation and interference assay, related to Figures 1, 5-7, and S5-S7.

Oligo Name	[a]	Sequence, 5'-3'	Purpose
To construct plasmids for strain creation and protein expression			
CR US Forw	F	GCCTGCATTAGGCTTGTTTCATAG	For pYZEJS067
CR US AS+SalISpeI	R	ACTAGTATAGTCGACACTTCGACGGGAAATCCTTATTTC	
SalISpeI +CR DS Forw	F	GTCGACTATACTAGTCAGCCGTTGCGATAAGCGAAC	
CR DS AS	R	TGGTGCAATTTCTGTGTTGGACGG	
AatII+TracrUPForw	F	TATTGACGTCCTCCGTAAACAACGTTGCAAATAATG	For pYZEJS079
TracrPro+Sp25 AS	R	GTAAAGGTAATGCGCCGCGCATGAGTTTGGGATTCTAGCCGTTGTGAG	
Sp25+R Forw	F	TCATGCGCGGCGCATTACCTTTACGTTGTAGCTCCCTTTCTCATTTCGG	
PacI+tracrend AS	R	TCGCTTAATTAATAAACGATGCCCCTTAAAGCAGAAGC	
PacI+trdel1 AS	R	TCGCTTAATTAAAAGCTTTAAGGGGCAGAGCGTTG	For pYZEJS162
PacI+trdel2 AS	R	TCGCTTAATTAACGGCACATCTTTTCAGACGGCC	For pYZEJS163
PacI+trdel3 AS	R	TCGCTTAATTAATCAGACGGCCTTATTGTAGCAAC	For pYZEJS164
PacI+trdel4 AS	R	TCGCTTAATTAAATTGTAGCAACGGTTCTCATTTC	For pYZEJS165
PacI+trdel6 AS	R	TCGCTTAATTAATAAAGCAGAAGCTTTAAGGGGCAG	For pYZEJS176
Hinddel5endPacI Forw	F	AGCTTCTGCTTTAAGGGGCATTAAT	For pYZEJS175
Hinddel5endpacI AS	R	TAATGCCCCTTAAAGCAGA	
Int 1 AS	R	GGGAGCTACAACGTAAAGGTAATGCG	For pYZEJS177
OL+int1F	F	CCTTTACGTTGTAGCTCCCCGTTGCTACAATAAGGCCGTCTG	
Int 2 AS	R	ATTGTAGCAACGGTTCTCATTTC	For pYZEJS178
OL+int2F	F	ATGAGAACCGTTGCTACAATCAACGCTCTGCCCCTTAAAGCTTC	
Int 3 AS	R	TTTCGGGAGCTACAACGTAAAGGTAATGCG	For pYZEJS179
OL+int3F	F	CCTTTACGTTGTAGCTCCCGAAACGTTGCTACAATAAGGCCGTCTG	
NmeCas9 Forw-RR	F	TACTTCCAATCCAATGCCATGGCTGCCTTCAAACC	For pYZEJS265
NmeCas9 AS-RR	R	TTATCCACTTCCAATGTTTTAACGGACAGGCGG	
PGCC2 FwbeforeMCS	F	GCTCGAATTCCGATCATATTCAATAACCC	PGCC2 based sequencing
PGCC2 ASafterMCS	R	AGAACCATCCGTTCTGCTCTATACCCTCG	
To construct plasmids with protospacer mutants and NmeCas9 mutants			
All pGCC2-, pYZEJS040- and pMCSG7- based plasmids carrying point mutations in the protospacers, PAM or NmeCas9 were created by QuickChange mutagenesis and confirmed by sequencing. The oligos used are not listed.			
[a] F, Forward; R, Reverse.			

SUPPLEMENTAL EXPERIMENTAL PROCEDURES

Bacterial Strains and Growth Conditions

N. meningitidis 8013 (MC8013) and mutant derivatives used in this study are listed in Supplementary Table S1. Meningococcal strains were grown on GC Medium Base (GCB) (Difco) plates with appropriate antibiotics and Kellogg's supplements I and II (Sigma). All solid cultures were incubated at 37˚C in a 5% CO_2 humidified atmosphere.

Mutant Strain Construction

All mutant strains were confirmed by PCR and DNA sequencing. To create the unmarked Δ*crispr* strain, we first transformed a streptomycin-resistant (Sm^R) strain with plasmid pSmartHCAmp/Δ*crispr*/*CAT-rpsL*, in which a dual-marker cassette [*CAT* (chloramphenicol acetyltransferase, conferring chloramphenicol resistance) (Cm^R) and wild-type *rpsL*] replaced the *crispr* locus. The resulting Sm^S Cm^R transformants were transformed with plasmid pSmartHCAmp/Δ*crispr*/SalI+SpeI. Sm^R Cm^S colonies were screened by PCR to confirm replacement of the dual marker cassette with the unmarked *crispr* deletion.

To generate complementation strains expressing sgRNAs, we generated sp 25 sgRNA variants and cloned them into plasmid pGCC2, transformed the resulting plasmids into the parental Δ*crispr*Δ*tracr* strain, and selected erythromycin-resistant (Erm^R) transformants.

Recombinant NmeCas9 expression and purification

The NmeCas9 gene of MC8013 was cloned into the pMCSG7 vector using Ligation Independent Cloning (Stols et al., 2002). The resulting NmeCas9 protein contains an N-terminal His_6-tag, followed by a Tobacco Etch Virus (TEV) protease site to remove the His-tag by TEV

cleavage. For protein expression, the plasmid was transformed into BL21(DE3) Rosetta cells. The cells were grown in terrific broth medium at 36 ºC to an OD600 of ~0.8, transferred to ice to reduce the temperature to around 16ºC, induced with 0.5 mM β-D-1-thiogalactopyranoside (IPTG), and grown overnight at 16ºC for protein expression. Cell pellets were re-suspended in re-suspension buffer (50 mM TRIS pH 8, 500 mM NaCl, 10% glycerol, and 5 mM Imidazole) and stored at -80ºC. For purification, cells were thawed and protease inhibitors [Benzimidine (1 mM), Pepstatin (1µg/ml), and PMSF (1 mM)] were added to the cell suspension. The cells were then incubated while sequentially adding Lysozyme (0.625 mg/ml), Brij58 (0.1%), and DNase (0.02 mg/mL, along with 10 mM $MgCl_2$), with 30 minutes of gentle rocking at 4ºC for each step. This was followed by a gentle sonication of the cells. The cell lysate was spun at 35,000 rpm for 30 minutes and the supernatant was filtered through a 0.2 µm filter. The salt concentration of the supernatant was adjusted to 1M NaCl, and 0.2% Polyethyleneimine (PEI) was added slowly to the supernatant while stirring at 4ºC, for a total period of 30 minutes. The solution was spun at 18,000 rpm at 4ºC for 30 minutes, during which NmeCas9 separated into the supernatant. Solid ammonium sulfate was added to the supernatant to a final concentration of 50% (29.1 g for a 100 ml solution) and stirred at 4ºC for 30 minutes. The solution was spun at 18,000 rpm for 30 minutes and the majority of NmeCas9 settled in the pellet. The pellet was solubilized in Buffer A (re-suspension buffer supplemented with salt to a final 1 M NaCl concentration, along with 1 mM PMSF). The protein solution was loaded onto a Ni-NTA column that was equilibrated with the same buffer. After loading, the column was washed with around 500 mL of Buffer A to remove any unbound or loosely bound contaminating protein followed by 100 mL Buffer A supplemented with 20 mM Imidazole, and finally with Buffer A with 350 mM Imidazole to elute the protein. Protein was dialyzed into S-column buffer (20 mM HEPES pH 7.5, 250 mM KCl, 10% glycerol, 1 mM DTT, and 1 mM PMSF) and simultaneously incubated with TEV protease [(1:50 (mg/ml)] to cleave the His-tag. The protein was passed through a Ni-NTA column to deplete the His-tag and the uncleaved NmeCas9. The flow-through from the Ni-NTA column was loaded

onto a MonoS column and eluted by a salt gradient from 250 mM to 1.5 M KCl. The pure fractions were pooled and dialyzed into S200-column buffer (20 mM HEPES pH 7.5, 500 mM KCl, 10% glycerol, 1 mM DTT, and 1 mM PMSF). The protein was concentrated and loaded onto a gel filtration column (S200). Pure NmeCas9 protein fractions were pooled, concentrated to around 20 mg/ml, and stored at -80ºC.

Plasmid Construction

Plasmids used in this study are listed in Supplementary Table S2. *E. coli* Top10 competent cells (Invitrogen) were used for cloning. All plasmids were verified by DNA sequencing. Regular and overlapping PCRs for cloning were done using Platinum Pfx DNA Polymerase (Invitrogen). Protospacer plasmids [pGCC2 or pYZEJS040 (Zhang et al., 2013) derivatives] for in vitro cleavage and *N. meningitidis* transformation assays were constructed as described previously (Zhang et al., 2013). All PAM and seed mutants of the protospacer plasmids were created by QuikChange (Agilent) mutagenesis. Plasmids for creating *N. meningitidis ΔcrisprΔtracrRNA* strains with sgRNA complementation were constructed by overlapping PCR or regular PCR and ligation as previously described (Zhang et al., 2013). The wild type NmeCas9 gene was cloned into the pMCSG7 vector to create the plasmid for overexpression of NmeCas9 protein, and derivative plasmids for mutant NmeCas9 proteins were constructed by QuikChange (Agilent) mutagenesis.

Binding of SSB and RecA proteins to ssDNA substrates

FAM-labeled ssDNA oligonucleotide (IDT, Coralville IA) was incubated in standard cleavage buffer, with increasing concentrations of SSB (0.05, 0.5, 5, 50 ng/μL) or RecA (2, 20, 200 ng/μL) proteins (NEB) at 37°C for 10 min. For cleavage assays, small RNAs (500 nM) and NmeCas9 (500 nM) were then added to the reaction, incubated at 37°C for 30 min, and analyzed as in Fig.

4A. For EMSAs, half of the binding reaction was supplied with 10% glycerol and immediately analyzed as in Fig. 3C.

Electrophoretic Mobility Shift Assay (Fig. S3)

RNAs (500 nM) were incubated with NmeCas9 (500nM) in standard cleavage buffer (without Mg^{2+}) at room temperature for 8-10 min. The reactions were resolved by 6% Native PAGE at 4°C. RNAs were stained by SYBR Gold (Life Technologies) and visualized with an ImageQuant LAS 4000 imager. The “Control RNA” (kindly provided by W. Tang and C. C. Mello) is a 40-nt ssRNA complementary to a *C. elegans*-derived piRNA.

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