Supplemental Information

Chimeric Guides Probe and Enhance Cas9 Biochemical Activity

Zachary J. Kartje[1], Christopher L. Barkau[2], Kushal J. Rohilla[2], Eman A. Ageely[1], and Keith T. Gagnon[1, 2,*]

[1]*Department of Chemistry & Biochemistry, Southern Illinois University*
[2]*Department of Biochemistry & Molecular Biology, School of Medicine, Southern Illinois University*
**To whom correspondence should be addressed: ktgagnon@siu.edu*

Supplemental Data ..... pg. 2-8

Methods ..... pg. 9-13

Supplemental References ..... pg. 14

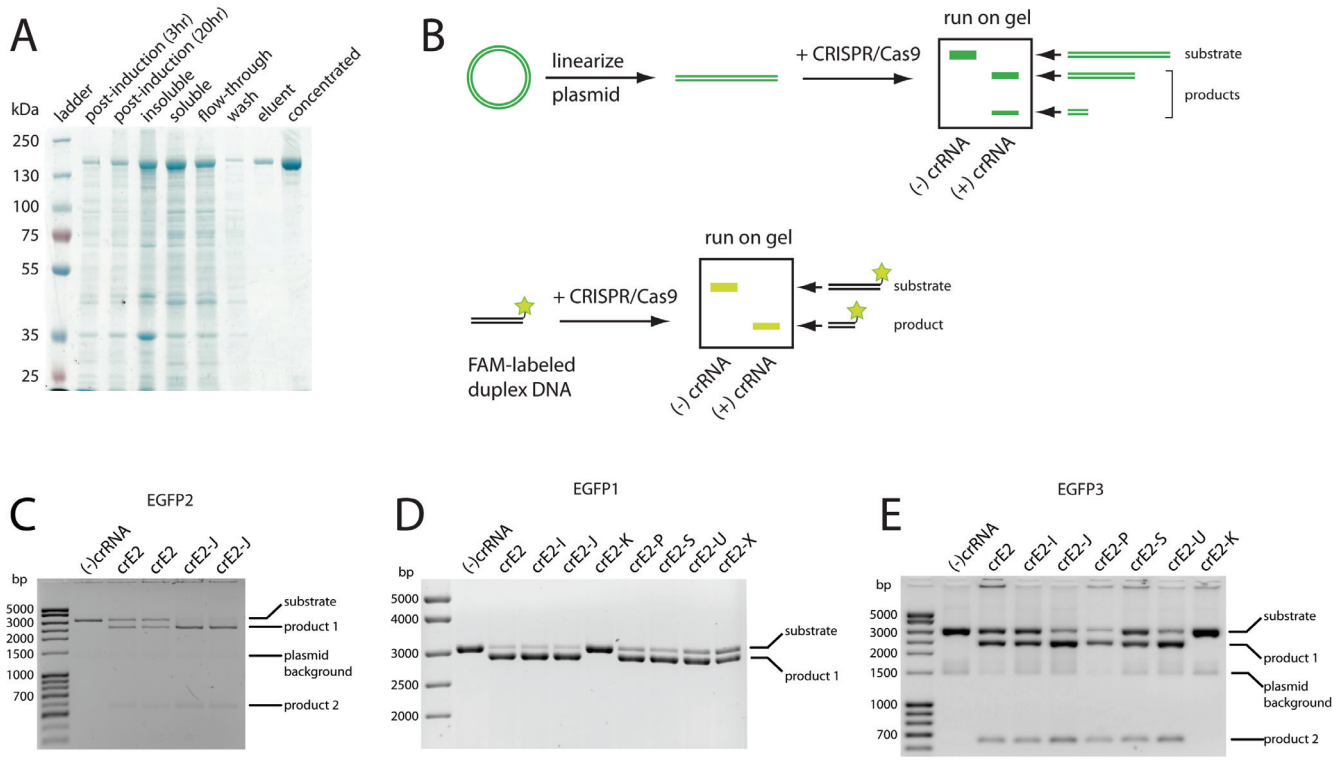

Figure S1: (**A**) Representative SDS-PAGE of *Sp*Cas9 purification by Cobalt-CMA affinity chromatography. (**B**) Experimental design to assay *in vitro* cleavage activity of CRISPR-Cas9 enzymes. Cleavage of linearized plasmid, followed by resolving products on an agarose gel and ethidium bromide staining, or cleavage of a synthetic FAM-labeled duplex followed by resolving products on a native polyacrylamide gel and imaging by fluorescence emission. (**C-E**) Representative agarose gels for *Sp*Cas9 cleavage of linearized plasmid at E1, E2 and E3 target sites. (-)crRNA indicates no crRNA present in the cleavage reaction. Gels were quantified as described in Methods.

A pSMART-EGFP-GA_Cut1c

HindIII

E1 target site

E2 target site

E3 target site

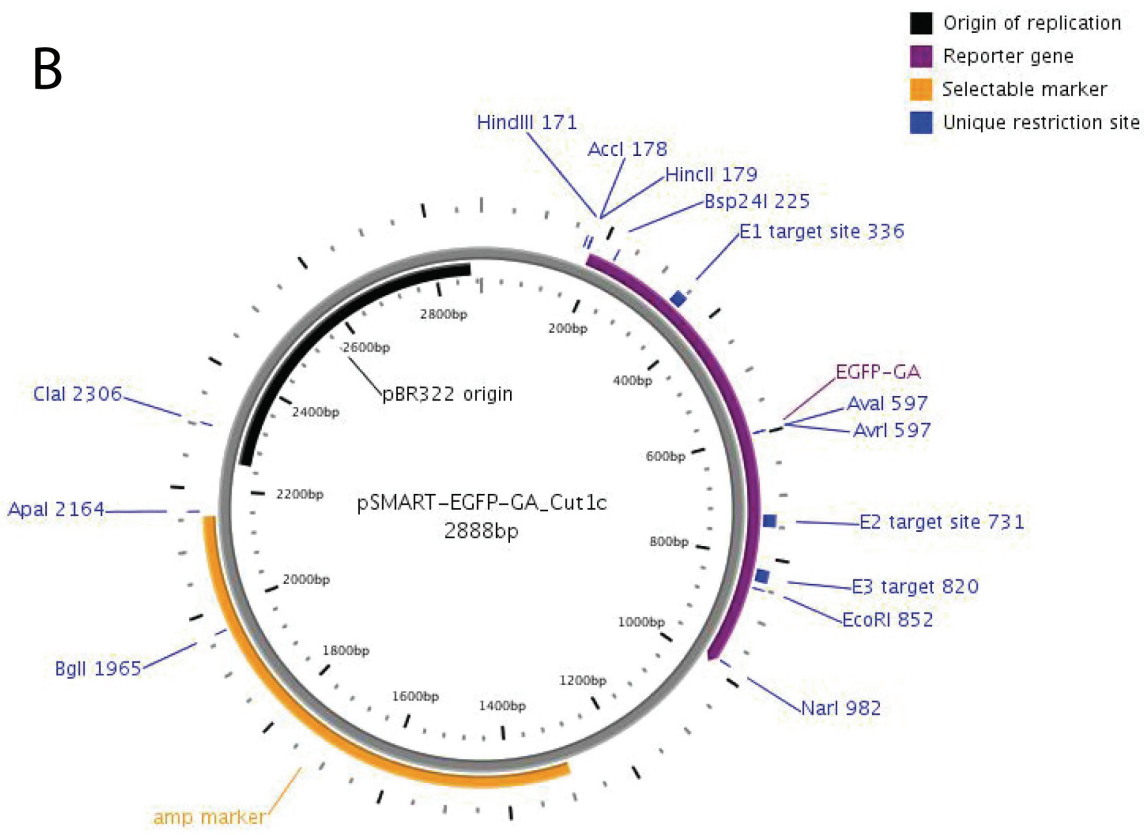

Figure S2: (**A**) Sequence of plasmid used in this study (pSMART-EGFP-GA_Cut1c) for *in vitro* cleavage assays. *Hind*III linearization site and E1, E2, and E3 target cleavage sites are indicated. (**B**) Circular map of pSMART-EGFP-GA_Cut1c generated using Plasmapper 2.0 software.

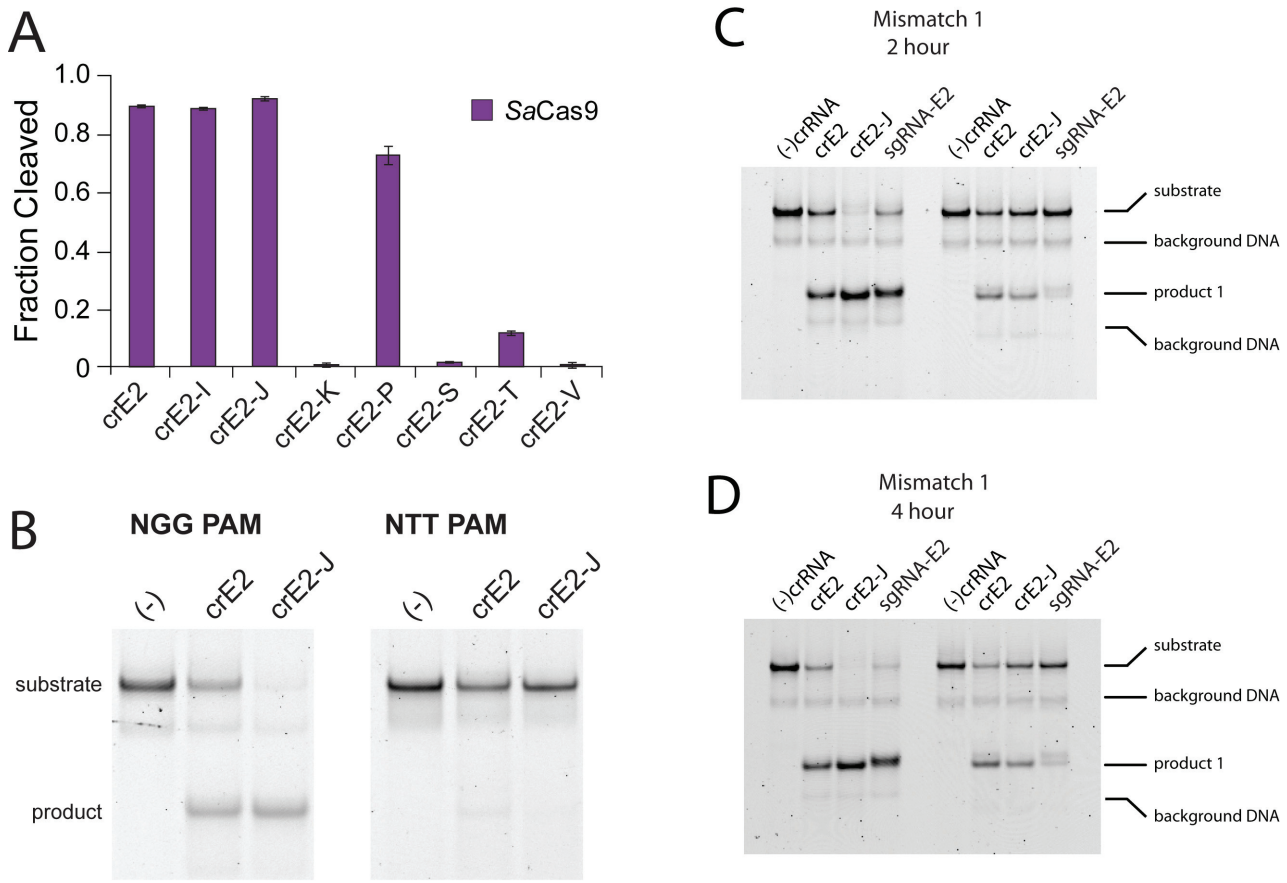

Figure S3: Chimeric crRNAs are compatible with *Sa*Cas9 and maintain substrate specificity. (**A**) *In vitro* cleavage activity for *Sa*Cas9 RNPs assembled with chimeric crRNAs targeting the E2 cleavage site of linearized pSMART-EGFP-GA_Cut1c plasmid. Naming and modification schemes of crRNAs follow the same convention presented in Figure 1B. Error bars are s.e.m. (**B**) FAM-duplex target DNA containing a CGG or CTT PAM sequence was subjected to cleavage by *Sp*Cas9 assembled with tracrRNA and crE2 or crE2-J at twice the usual final enzyme concentration in the reaction. (-) indicates no crRNA present in the cleavage reaction. (**C-D**) Cleavage of perfect match (left 4 lanes) and mismatch 1 (right 4 lanes) FAM-duplex targets with SpCas9 assembled with the indicated crRNAs at 2h (**C**) and 4 h (**D**). Reaction products were resolved by native polyacrylamide gel, imaged by fluorescence emission, and quantified as described in Methods.

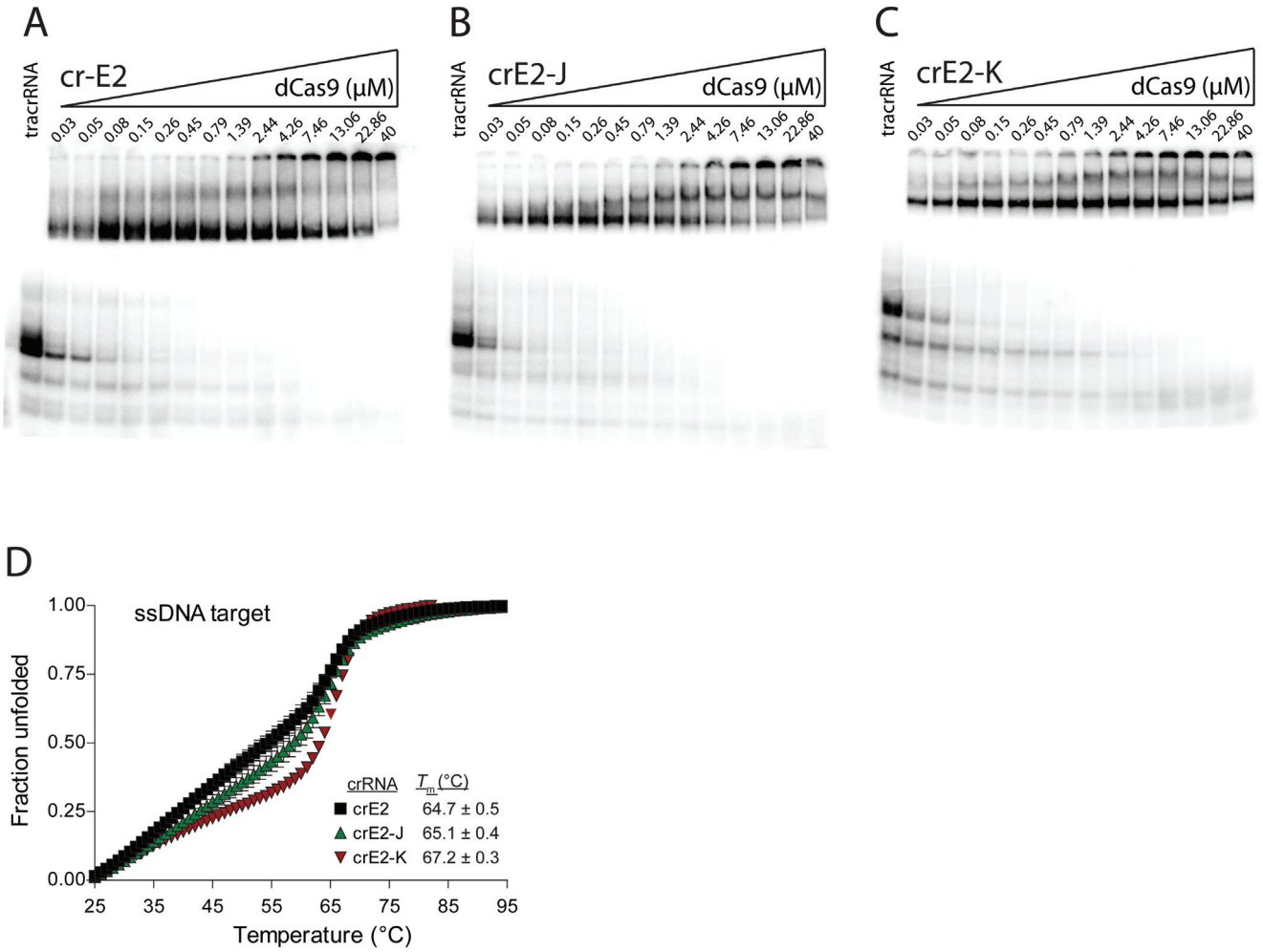

Figure S4: (**A-C**) Representative EMSA of *Sp*Cas9 RNPs with different crRNAs. Radiolabeled tracrRNA was mixed with a molar excess of the indicated crRNA and dCas9 was titrated. Resulting complexes were resolved by native PAGE and imaged by phosphorimager. Two independent experiments were performed for all three crRNAs. Very similar gel shift patterns were observed, resulting in very similar binding affinities as reported in Figure 4. (**D**) Thermal denaturation monitored by absorbance at 280 nm for the indicated crRNAs when annealed to the antisense (complementary) strand of FAM-duplex target DNA. T_m values were calculated as described in Methods.

Comparison	Sample 1	Sample 2	Figure	Detail	p value
1	crE2	crE2-J	1B	plasmid/FAM	0.0007638/0.000899
2	crE2	crE2-L	1B	plasmid/FAM	0.008657/0.009553
3	crE2	crE2-O	1B	plasmid/FAM	0.001239/0.002955
4	crE2	crE2-P	1B	plasmid/FAM	0.001956/0.002614
5	crE2	crE2-Z	1B	plasmid/FAM	0.9717 / 0.7847
6	crE2-J (perfect match)	crE2-J (mismatch 1)	2B	45 mins	0.02943
7	crE2-J (perfect match)	crE2-J (mismatch 1)	2B	2 hours	0.00133
8	crE2-J (perfect match)	crE2-J (mismatch 1)	2B	4 hours	0.0006761
9	crE2-J (perfect match)	crE2-J (mismatch 1)	2B	8 hours	0.0008463
10	crE2-J (perfect match)	crE2-J (mismatch 1)	2B	16 hours	0.0003718
11	crE2	crE2-J	4A	2 min	0.0002252
12	crE2	crE2-J	4A	5 min	0.0002366
13	crE2	crE2-J	4B	$k_{d\,app}$	0.4208
14	crE2	crE2-K	4B	$k_{d\,app}$	0.9978
15	crE2	crE2-J	4C	$k_{d\,app}$	0.6206
16	crE2	crE2-J	4D	T_m	0.02982
17	crE2	crE2-K	4D	T_m	0.002446

Table S1. Statistical analysis of selected data. Reported p values are result of two sample unpaired t-tests. Comparisons 1-5 were calculated for both plasmid target cleavage data and FAM target cleavage data. Boxes highlighted in gray denote no statistical significance (p values > 0.05). P values highlighted in green are not significant.

Calculation for statistical significance

Data points from specific experiments were compared and analyzed with an unpaired t-test in R Studio®. Tests were performed with a 95% confidence interval with equal variance. P values are reported in Table S1.

Table S2: Sequences, Substitutions and Modifications of Oligonucleotides

Oligonucleotides	Sequence (lowercase = RNA, uppercase = DNA, FAM = 3' fluorescein label)
crE1	caggaaagcugccagugccaguuuuagagcuaugcuguuuug
crE1_I	caggaaagcugcCAGTGCCAguuuuagagcuaugcuguuuug
crE1_J	caggaaagcugccagugccaGTTTTAGAGCTATGCTGTTTTG
crE1_K	CAGGAAAGCTGCCAGTGCCAGTTTTAGAGCTATGCTGTTTTG
crE1_P	CAGGAAagcugccagugccaGTTTTAGAGCTATGCTGTTTTG
crE1_S	CAGGAAAGCTGCcagugccaGTTTTAGAGCTATGCTGTTTTG
crE1_T	CAGGaaagCTGCcaguGCCAGTTTTAGAGCTATGCTGTTTTG
crE1_V	CAGGAAAGCTGCcaGugCcaGTTTTAGAGCTATGCTGTTTTG
E2_target_anti	TTCGCACGTCAGTGACGTCCGATCGGAGTGTTTTGCTGGTACATTGCTCGTGAGTCGCTT
E2_FAM-duplex_sense	AAGCGACTCACGAGCAATGTACCAGCAAAACACTCCGATCGGACGTCACTGACGTGCGAA
E2_FAM-duplex_anti	TTCGCACGTCAGTGACGTCCGATCGGAGTGTTTTGCTGGTACATTGCTCGTGAGTCGCTT - (FAM)
E2_FAM-duplex-mm1_sense	AAGCGACTCACGAGCAATGTACCAGCAAAACACTGCGATCGGACGTCACTGACGTGCGAA
E2_FAM-duplex-mm1_anti	TTCGCACGTCAGTGACGTCCGATCGCAGTGTTTTGCTGGTACATTGCTCGTGAGTCGCTT - (FAM)
E2_FAM-duplex-mm2_sense	AAGCGACTCACGAGCAATGTACCAGCAAAAGACTGCGATCGGACGTCACTGACGTGCGAA
E2_FAM-duplex-mm2_anti	TTCGCACGTCAGTGACGTCCGATCGCAGTCTTTTGCTGGTACATTGCTCGTGAGTCGCTT - (FAM)
E2_FAM-duplex-mm2-5'_sense	AAGCGACTCACGAGCAATGTACCACCAAAACACTGCGATCGGACGTCACTGACGTGCGAA
E2_FAM-duplex-mm2-5'_anti	TTCGCACGTCAGTGACGTCCGATCGCAGTGTTTTGGTGGTACATTGCTCGTGAGTCGCTT - (FAM)
FAM-duplex_NTT PAM_sense	AAGCGACTCACGAGCAATGTACCAGCAAAACACTCCGATCTTACGTCACTGACGTGCGAA
FAM-duplex_NTT PAM_anti	TTCGCACGTCAGTGACGTAAGATCGGAGTGTTTTGCTGGTACATTGCTCGTGAGTCGCTT - (FAM)
crE2	uaccagcaaaacacuccgauguuuuagagcuaugcuguuuug
crE2_A	TACcagcaaaacacuccgauguuuuagagcuaugcuguuuug
crE2_B	TACCAGCaaaacacuccgauguuuuagagcuaugcuguuuug
crE2_C	TACCAGCAAaacacuccgauguuuuagagcuaugcuguuuug
crE2_D	TACCAGCAAAACacuccgauguuuuagagcuaugcuguuuug
crE2_E	TACCAGCAAAACACTccgauguuuuagagcuaugcuguuuug
crE2_F	TACCAGCAAAACACTCCGauguuuuagagcuaugcuguuuug
crE2_G	TACCAGCAAAACACTCCGATguuuuagagcuaugcuguuuug
crE2_H	uaccagcaaaacacuccgaTguuuuagagcuaugcuguuuug
crE2_I	uaccagcaaaacACTCCGATguuuuagagcuaugcuguuuug
crE2_J	uaccagcaaaacacuccgauGTTTTAGAGCTATGCTGTTTTG
crE2_K	TACCAGCAAAACACTCCGATGTTTTAGAGCTATGCTGTTTTG
crE2_L	uaccagcaaaacacucCGATGTTTTAGAGCTATGCTGTTTTG
crE2_M	uaccagcaaaacACTCCGATGTTTTAGAGCTATGCTGTTTTG
crE2_N	uaccagcaAAACACTCCGATGTTTTAGAGCTATGCTGTTTTG
crE2_O	TACcagcaaaacacuccgauGTTTTAGAGCTATGCTGTTTTG
crE2_P	TACCAGcaaaacacuccgauGTTTTAGAGCTATGCTGTTTTG
crE2_Q	TACCAGcaaaacacucCGATGTTTTAGAGCTATGCTGTTTTG
crE2_R	TACCAGcaaaacacTCCGATGTTTTAGAGCTATGCTGTTTTG
crE2_S	TACCAGCAAAACacuccgauGTTTTAGAGCTATGCTGTTTTG
crE2_T	TACCagcaAAACacucCGATGTTTTAGAGCTATGCTGTTTTG
crE2_U	TACCAGCAAAACAcuccgaTGTTTTAGAGCTATGCTGTTTTG
crE2_V	TACCAGCAAAACacTccGauGTTTTAGAGCTATGCTGTTTTG
crE2_W	TACCAGCAAAACaCTcCGaTGTTTTAGAGCTATGCTGTTTTG
crE2_X	TACCAGCAAAACAcTCCGaTGTTTTAGAGCTATGCTGTTTTG
crE2_Y	TACCAGCAAAACACucCGaTGTuuuAGAGCTATGCTGTTTTG
crE2_Z	uaccagcaaaacacTCcgAuguTTTagagcuaugcuguuuug
crE3	aaacgagaaacgagaucauaguuuuagagcuaugcuguuuug
crE3_I	aaacgagaaacgAGATCATAguuuuagagcuaugcuguuuug
crE3_J	aaacgagaaacgagaucauaGTTTTAGAGCTATGCTGTTTTG
crE3_K	AAACGAGAAACGAGATCATAGTTTTAGAGCTATGCTGTTTTG
crE3_P	AAACGAgaaacgagaucauaGTTTTAGAGCTATGCTGTTTTG
crE3_S	AAACGAGAAACGagaucauaGTTTTAGAGCTATGCTGTTTTG
crE3_T	AAACgagaAACGagauCATAGTTTTAGAGCTATGCTGTTTTG
crE3_V	AAACGAGAAACGagAucAuaGTTTTAGAGCTATGCTGTTTTG
T7_tracrRNA_as	AAGCACCGACTCGGTGCCACTTTTTCAAGTTGATAACGGACTAGCCTTATTTTAACTTGCTATGCTGTTTCCCTATAGTGAGTCGTATTA
tracrRNA	aaacagcauagcaaguuaaaauaaggcuaguccguuaucaacuugaaaaaguggcaccgagucggugcuu
tracrDNA	AAACAGCATAGCAAGTTAAAATAAGGCTAGTCCGTTATCAACTTGAAAAAGTGGCACCGAGTCGGTGCTT
tracr-A	AAACAGCATAGCAAGTTAAAATaaggcuaguccguuaucaacuugaaaaaguggcaccgagucggugcuu
tracr-B	acagcaTAGCAAguuAaAauaaGGcuagTcCguuaTCaACTTgaaaaaGTggCACCgagucggTGC
tracr-C	ACagcauagcAAguuaaaauAAgGCTAgTcCguuATCaACTTGAAAaagTGgCACCgAguCggTGC
tracr-D	ACagcauagcAAguuaaaauAAGGCTAGTCCGTTATCAACTTGAAAAAGTGGCACCGAGTCGGTGC
Sa_tracrRNA_T7anti	ATCTCGCCAACAAGTTGACGAGATAAACACGGCATTTTGCCTTGTTTTAGTAGATTCTGTAATTTCCTATAGTGAGTCGTATTA
Sa_crE2	uaccagcaaaacacuccgauguuuuaguacucuguaauuu
Sa_crE2_I	uaccagcaaaacACTCCGATguuuuaguacucuguaauuu
Sa_crE2_J	uaccagcaaaacacuccgauGTTTTAGTACTCTGTAATTT
Sa_crE2_K	TACCAGCAAAACACTCCGATGTTTTAGTACTCTGTAATTT
Sa_crE2_P	TACCAGcaaaacacuccgauGTTTTAGTACTCTGTAATTT
Sa_crE2_S	TACCAGCAAAACacuccgauGTTTTAGTACTCTGTAATTT
Sa_crE2_T	TACCagcaAAACacucCGATGTTTTAGTACTCTGTAATTT
Sa_crE2_V	TACCAGCAAAACacTccGauGTTTTAGTACTCTGTAATTT
T7 promoter oligo	TAATACGACTCACTATA

Methods:

RNA Synthesis

DNA oligonucleotides, FAM-labeled DNA, crRNAs, DNA-RNA chimeric crRNAs, and DNA-RNA chimeric tracrRNAs (see **Table S2**) were synthesized by Integrated DNA Technologies (IDT). Some tracrRNAs were prepared by T7 *in vitro* transcription with DNA templates synthesized by IDT. Single-stranded DNA templates were annealed to T7 promoter oligo to generate double-stranded promoter regions, which support *in vitro* transcription by T7 RNA polymerase[1]. In vitro transcriptions were performed by standard protocols for 2 hours. Briefly, reactions contained purified T7 RNA polymerase, 30 mM Tris (at pH 7.9), 12.5 mM NaCl, 40 mM $MgCl_2$, 2% PEG8000, 0.05% Triton X-100, 2 mM spermidine, and 2.5 μM T7-DNA template. Afterward, the DNA template was degraded by the addition of 1 unit of DNase I for every 20 μL of reaction and incubated at 37°C for 15 min. Reactions were phenol-chloroform extracted and gel-purified from denaturing polyacrylamide gels. Purified RNA was quantified by measuring absorbance at 260 nm and calculated extinction coefficients using nearest neighbor approximations and Beer's Law.

Preparation of Cas9 Enzymes

Plasmid encoding an *Sp*Cas9 with a C-terminal fusion of a nuclear localization signal (NLS) and a 6x-Histidine tag (pET-Cas9-NLS-6xHis)[2] was obtained from Addgene (62933). A dead Cas9 (dCas9) version was prepared by performing site-directed mutagenesis on this plasmid to generate H840A and D10A mutations (pET-dCas9-NLS-6xHis). Plasmid encoding mammalian codon-optimized *Sa*Cas9 fused to an NLS (BPK2139)[3] was obtained from Addgene (65776). The *Sa*Cas9-NLS reading frame was cloned into a pET28a vector in-frame with an N-terminal 6x-Histidine tag in the vector backbone (pET28-His-SaCas9-NLS) and has been deposited with Addgene.

Cas9 proteins were prepared similar to that previously described[4] with several changes. Briefly, protein expression was induced in Rosetta (DE3) cells with 0.4 mM IPTG at 18°C for 16 h. Cell pellets were resuspended in 6 mL of chilled binding buffer (20 mM Tris-HCl, pH 8.0, 250 mM NaCl, 1 mM PMSF, 5 mM imidazole) per 0.5 L of culture. Resuspended cells were sonicated and clarified by centrifugation. His-Pur Cobalt-CMA resin (Thermo Scientific) was equilibrated with binding buffer and the supernatant added to the

equilibrated resin and incubated at 4°C for 1 h. The supernatant was washed sequentially with increasing concentrations of NaCl in 50 mL volumes of wash buffer (Tris-HCl, pH 8, 0.25/0.5/0.75/1.0 M NaCl, 10 mM imidazole). Protein was eluted with 15 mL elution buffer (Tris-HCl, pH 8, 250mM NaCl, 130 mM imidazole). For *Sp*Cas9, the eluent was further purified over a Superdex 200 10/300 GL column equilibrated with degassed 2x Cas9 storage buffer (40 mM HEPES-KOH, pH 7.5, 300 mM KCl, 2 mM EDTA, 2 mM DTT). Purified Cas9 enzyme was concentrated with Vivaspin 15 centrifugal concentrators (Sartorius, 30K MWCO). Concentration was determined by UV absorbance at 280 nm using a calculated extinction coefficient (120,450 M^{-1} cm^{-1})[4] and Beer's law. One volume of glycerol was added to a final of 50% and purified Cas9 stored as aliquots at -80°C.

Radiolabeling of RNA and DNA

The 5' phosphate on T7-transcribed tracrRNA was removed using alkaline phosphatase following the manufacturer's recommended protocol. Synthetic duplex target DNA and crRNA lacks a 5' phosphate and was directly labeled. 100 pmols of tracrRNA, crRNA, or antisense DNA target strand was radiolabeled with [γ-^{32}P]-ATP using T4 polynucleotide kinase following the manufacturer's recommended enzyme protocol. Reactions were phenol-chloroform extracted and radiolabeled RNA or DNA was gel-purified on 15% denaturing polyacrylamide gels (1x TBE, 7 M urea) by the crush-and-soak method. Gel-purified radiolabeled RNA and DNA was quantified by scintillation counting.

In Vitro Cas9 Cleavage Activity Assays

Plasmid DNA containing the EGFP target gene (pSMART-EGFP, custom-made minigene plasmid) was linearized by digestion with *Hind*III restriction endonuclease and then purified by phenol-chloroform extraction and ethanol precipitation. The Cas9 ribonucleoprotein complex was assembled (final concentrations: 0.75 μM Cas9, 0.25 μM tracrRNA, 0.3 μM crRNA) in a 1x cleavage buffer (20 mM Tris-HCl, pH 7.5, 100 mM KCl, 5% glycerol, 1 mM DTT, 0.5 mM EDTA, 2 mM $MgCl_2$) supplemented with 0.1 mg/mL of purified yeast tRNA. The concentration of tracrRNA was purposely set as the limiting component of the RNP complex and used to predict final RNP concentration. Molar excess of Cas9 and crRNA will ensure complete assembly of tracrRNA into RNP complexes. The target supplied in the final reaction was either a

target duplex with the antisense (complementary to the crRNA guide sequence) strand 5' FAM-labeled (30 nM) or a linearized plasmid DNA (100 ng).

Standard reaction conditions were 37°C for 2 h in a final reaction volume of 40 μL. The mixture was treated with 5 μg of RNase A for 15 min followed by 10 μg of proteinase K for 20 min at room temperature, followed by precipitation with 10 volumes of 2% $LiClO_4$ in acetone for >1 h at -20˚C. Precipitated reactions were centrifuged and washed with acetone, air dried, and re-suspended in 1x loading dye (10% glycerol, 1x TBE, orange G dye) and resolved on TBE-buffered 1.2% agarose gels or by native polyacrylamide TBE-buffered gel electrophoresis (FAM-duplex target, 15% acrylamide). Agarose gels were stained with ethidium bromide and visualized by UV imager. Acrylamide gels were visualized using a Typhoon FLA 9500 (GE Healthcare Life Sciences).

For determining mismatch discrimination of Cas9 RNP complexes, the same reaction conditions described above were used. Targets were either a perfect complement FAM-duplex or mismatch-containing FAM-duplexes. Reactions were incubated for 45 min, 2 h, 4 h, 8 h, or 16 h at 37°C then reactions stopped by addition of 10 volumes of 2% $LiClO_4$ in acetone for >1 h at -20˚C. Precipitated reactions were centrifuged and washed with acetone, air dried, and re-suspended in 1x loading dye (10% glycerol, 1x TBE, orange G dye). To remove RNA and protein before resolving on a gel, 5 μg of RNase A was added for 15 min followed by 10 μg of proteinase K for 20 min at room temperature. Samples were resolved by polyacrylamide gel electrophoresis and imaged using a Typhoon FLA 9500 as described above.

For time-course experiments Cas9 RNP complexes were first assembled and equilibrated at 37°C for 10 min prior to initiating reactions. Linearized target plasmid was added at 37°C and reactions stopped at specified time points by addition of 2% $LiClO_4$ in acetone and placed on ice. Samples were centrifuged, washed with acetone, then resuspended in 1x loading dye (10% glycerol, 1x TBE, orange G dye). To remove RNA and protein RNase A and proteinase K were added as described above. Cleavage products were resolved on TBE-buffered 1.2% agarose gels.

For site-specific cleavage assays using radiolabeled DNA duplex, 100,000 cpms of radiolabeled antisense E2 DNA target was annealed to 5 pmols of sense E2 DNA target at 95°C for 3 min, followed by cooling at room temperature for 15 min. A standard cleavage assay was setup where radiolabeled duplex (50,000 cpm) was used instead of FAM-duplex. Reactions were RNase A and proteinase K treated and precipitated as described above. Pellets were resuspended in denaturing loading dye (1x TBE, 85% formamide, xylene cyanol, bromophenol blue), boiled for 3 min, then loaded onto a 15% denturing polyacrylamide sequencing gel. After electrophoresis, the gel was dried and imaged by phosphorimager.

The fraction of target plasmid or FAM-duplex cleaved was quantified using ImageJ software. The band intensity for the cleavage product band was divided by the combined intensity of cleavage product 1 and uncut substrate bands and reported as fraction cleaved (i.e. "cut" / "cut + uncut"). Time course cleavage assay results were plotted using Prism (Graphpad) software and fit to a one-site binding hyperbola by non-linear regression. Error bars for all quantified data represent experimental replicates, not technical replicates. Samples size was selected based on the expectation that 3 or more replicates will be representative of typical *in vitro* assay conditions.

Dot-Blot Filter Binding Assays for Duplex Target Binding

For target binding by Cas9 RNP complexes, radiolabeled duplex target DNA (1,500 cpm/reaction) was combined with increasing concentrations of a pre-assembled dCas9-tracrRNA-crRNA complex in a final reaction of 20 μL 1x cleavage buffer and 0.1 mg/mL tRNA. After incubation at 37°C for 15 min, reactions were vacuum filtered over nitrocellulose membrane (Protran Premium NC, Amersham) using a 96-well dot blot apparatus. Wells were washed twice with 200 μL of 1x cleavage buffer. Membrane was then removed and washed with 1x PBS solution and air dried at RT. Binding of radioactive crRNA was then visualized by phosphorimager. Spots were quantified with ImageQuant software, plotted in Prism (GraphPad) and fit to a one-site binding hyperbola equation. Error bars for all quantified data represent experimental replicates, not technical replicates. Samples size was selected based on the expectation that 3 or more replicates will be representative of typical *in vitro* assay conditions.

Electrophoretic Mobility Shift Assays (EMSAs)

Radiolabeled tracrRNA (5,000 cpm/reaction) were mixed with crRNA (0.6 μM final), and dCas9 protein in 1x Cas9 cleavage buffer, supplemented with 5 μg BSA, 5 μg yeast tRNA, 10U SUPERase-in (ThermoFisher) in 20 μL reactions. Reactions were incubated at 37°C for 15 min. Resultant RNP complexes were then resolved on a TBE-buffered 6% native polyacrylamide gel at 4˚C. Gels were vacuum-dried and exposed to phosphorimager screen and visualized with a Typhoon FLA 9500. Gel shifts were quantified using ImageJ software, plotted in Prism (GraphPad) and fit to a one-site binding hyperbola equation, and K_d values calculated by non-linear regression.

Thermal Denaturation Monitored by UV Absorbance

Cas9 alone or complexed to tracrRNA and crRNA at 1 μM final concentration (equimolar concentrations of all components) was incubated at room temperature for 10 min in degassed 1x UV melt buffer (20 mM Cacodylate, pH 7.5, 150 mM KCl, 1 mM $MgCl_2$). Samples were melted in a Cary 400 UV/Vis spectrophotometer at a ramp rate of 1°C/min while UV absorbance at 280 nm was collected every 1 min. Experiments were repeated in duplicate. For crRNA annealed to FAM-duplex antisense DNA, 1 μM final concentration of both crRNA and target DNA were melted under the same conditions described above, except UV absorbance was measured at 260 nm and multiple heating and cooling ramps were used. Melting temperatures were determined using Van't Hoff calculations and error determined by standard error of the mean using two or more experimental replicates for each sample.

Supplemental References:

[1] Milligan, J. F., Groebe, D. R., Witherell, G. W., and Uhlenbeck, O. C. (1987) Oligoribonucleotide synthesis using T7 RNA polymerase and synthetic DNA templates, *Nucleic acids research 15*, 8783-8798.

[2] Zuris, J. A., Thompson, D. B., Shu, Y., Guilinger, J. P., Bessen, J. L., Hu, J. H., Maeder, M. L., Joung, J. K., Chen, Z. Y., and Liu, D. R. (2015) Cationic lipid-mediated delivery of proteins enables efficient protein-based genome editing in vitro and in vivo, *Nat Biotechnol 33*, 73-80.

[3] Kleinstiver, B. P., Prew, M. S., Tsai, S. Q., Topkar, V. V., Nguyen, N. T., Zheng, Z., Gonzales, A. P., Li, Z., Peterson, R. T., Yeh, J. R., Aryee, M. J., and Joung, J. K. (2015) Engineered CRISPR-Cas9 nucleases with altered PAM specificities, *Nature 523*, 481-485.

[4] Anders, C., and Jinek, M. (2014) In vitro enzymology of Cas9, *Methods in enzymology 546*, 1-20.