Supplementary Table S1. sgRNA and target DNA sequences used for *in vitro* cleavage assay.

Name	Sequence
sgRNA20	GGAAATTAGGTGCGCTTGGCGTTTTAGTACTCTGGAAACAGAATCTACTAAAACAAGGC AAAATGCCGTGTTTATCTCGTCAACTTGTTGGCGAGAT
sgRNA21	GGGAAATTAGGTGCGCTTGGCGTTTTAGTACTCTGGAAACAGAATCTACTAAAACAAGG CAAAATGCCGTGTTTATCTCGTCAACTTGTTGGCGAGAT
sgRNA22	GGGGAAATTAGGTGCGCTTGGCGTTTTAGTACTCTGGAAACAGAATCTACTAAAACAAG GCAAAATGCCGTGTTTATCTCGTCAACTTGTTGGCGAGAT
sgRNA23	GGGGGAAATTAGGTGCGCTTGGCGTTTTAGTACTCTGGAAACAGAATCTACTAAAACAA GGCAAAATGCCGTGTTTATCTCGTCAACTTGTTGGCGAGAT
Target DNA	AGCGCCCAATACGCAAACCGCCTCTCCCCGCGCGTTGGCCGATTCATTAATGCAGCTGG CACGACAGGTTTCCCGACTGGAAAGCGGGCAGTGAGCGCAACGCAATTAATGTGAGTT AGCTCACTCATTAGGCACCCCAGGCTTTACACTTTATGCTTCCGGCTCGTATGTTGTGTG GAATTGTGAGCGGATAACAATTTCACACAGGAAACAGCTATGACCATGATTACGCCAA GCTTGCATGCCTGCAGGTCGACTCTAGAGGATCCCCGGGTACCGAGCTCGAATTCACTG GCCGTCGTTTTACAACGTCGTGACTGGGAAAACCCTGGCGTTACCCAACTTAATCGCCTT GCAGCACATCCCCCTTTCGCCAGCTGGCGTAATAGCGAAGAGGCCCGCACCGATCGCCC TTCCCAACAGTTGCGCAGCCTGAATGGCGAATGGCGCCTGATGCGGTATTTTCTCCTTAC GCATCTGTGCGGTATTTCACACCGCATACGTCAAAGCAACCATAGTACGCGCCCTGTAG CGGCGCATTAAGCGCGGCGGGTGTGGTGGTTACGCGCAGCGTGACCGCTACACTTGCCA GCGCCCTAGCGCCCGCTCCTTTCGCTTTCTTCCCTTCCTTTCTCGCCACGTTCGCCGGCTT TCCCCGTCAAGCTCTAAATCGGGGGCTCCCTTTAGGGTTCCGATTTAGTGCTTTACGGCA CCTCGACCCCAAAAAACTTGATTTGGGTGATGGTTCACGTAGTGGGCCATCGCCCTGAT AGACGGTTTTTCGCCCTTTGACGTTGGAGTCCACGTTCTTTAATAGTGGACTCTTGTTCC AAACTGGAACAACACTCAACCCTATCTCGGGCTATTCTTTTGATTTATAAGGGATTTTGC CGATTTCGGCCTATTGGTTAAAAAATGAGCTGATTTAACAAAAATTTAACGCGAATTTT AACAAAATATTAACGTTTACAATTTTATGGTGCACTCTCAGTACAATCTGCTCTGATGCC GCATAGTTAAGCCAGCCCCGACACCCGCCAACACCCGCTGACGCGCCCTGACGGGCTTG TCTGCTCCCGGCATCCGCTTACAGACAAGCTGTGACCGTCTCCGGGAGCTGCATGTGTC AGAGGTTTTCACCGTCATCACCGAAACGCGCGAGACGAAAGGGCCTCGTGATACGCCTA TTTTTATAGGTTAATGTCATGATAATAATGGTTTCTTAGACGTCAGGTGGCACTTTTCGG GGAAATGTGCGCGGAACCCCTATTTGTTTATTTTTCTAAATACGGGGGGAAATTAGGTG CGCTTGGCTTGAATTGGATTCAAATATGTATCCGCTCATGAGACAATAACCCTGATAAA TGCTTCAATAATATTGAAAAAGGAAGAGTATGAGTATTCAACATTTCCGTGTCGCCCTT ATTCCCTTTTTTGCGGCATTTTGCCTTCCTGTTTTTGCTCACCCAGAAACGCTGGTGAAAG TAAAAGATGCTGAAGATCAGTTGGGTGCACGAGTGGGTTACATCGAACTGGATCTCAAC AGCGGTAAGATCCTTGAGAGTTTTCGCCCCGAAGAACGTTTTCCAATGATGAGCACTTT TAAAGTTCTGCTATGTGGCGCGGTATTATCCCGTATTGACGCCGGGCAAGAGCAACTCG GTCGCCGCATACACTATTCTCAGAATGACTTGGTTGAGTACTCACCAGTCACAGAAAAG CATCTTACGGATGGCATGACAGTAAGAGAATTATGCAGTGCTGCCATAACCATGAGTGA TAACACTGCGGCCAACTTACTTCTGACAACGATCGGAGGACCGAAGGAGCTAACCGCTT TTTTGCACAACATGGGGGATCATGTAACTCGCCTTGATCGTTGGGAACCGGAGCTGAAT GAAGCCATACCAAACGACGAGCGTGACACCACGATGCCTGTAGCAATGGCAACAACGT TGCGCAAACTATTAACTGGCGAACTACTTACTCTAGCTTCCCGGCAACAATTAATAGAC TGGATGGAGGCGGATAAAGTTGCAGGACCACTTCTGCGCTCGGCCCTTCCGGCTGGCTG GTTTATTGCTGATAAATCTGGAGCCGGTGAGCGTGGGTCTCGCGGTATCATTGCAGCAC TGGGGCCAGATGGTAAGCCCTCCCGTATCGTAGTTATCTACACGACGGGGAGTCAGGCA ACTATGGATGAACGAAATAGACAGATCGCTGAGATAGGTGCCTCACTGATTAAGCATTG GTAACTGTCAGACCAAGTTTACTCATATATACTTTAGATTGATTTAAAACTTCATTTTTA ATTTAAAAGGATCTAGGTGAAGATCCTTTTTGATAATCTCATGACCAAAATCCCTTAACG TGAGTTTTCGTTCCACTGAGCGTCAGACCCCGTAGAAAAGATCAAAGGATCTTCTTGAG ATCCTTTTTTTCTGCGCGTAATCTGCTGCTTGCAAACAAAAAAACCACCGCTACCAGCGG TGGTTTGTTTGCCGGATCAAGAGCTACCAACTCTTTTTCCGAAGGTAACTGGCTTCAGCA GAGCGCAGATACCAAATACTGTCCTTCTAGTGTAGCCGTAGTTAGGCCACCACTTCAAG AACTCTGTAGCACCGCCTACATACCTCGCTCTGCTAATCCTGTTACCAGTGGCTGCTGCC

Supplementary Table 1

	AGTGGCGATAAGTCGTGTCTTACCGGGTTGGACTCAAGACGATAGTTACCGGATAAGGC GCAGCGGTCGGGCTGAACGGGGGGTTCGTGCACACAGCCCAGCTTGGAGCGAACGACC TACACCGAACTGAGATACCTACAGCGTGAGCTATGAGAAAGCGCCACGCTTCCCGAAGG GAGAAAGGCGGACAGGTATCCGGTAAGCGGCAGGGTCGGAACAGGAGAGCGCACGAG GGAGCTTCCAGGGGGAAACGCCTGGTATCTTTATAGTCCTGTCGGGTTTCGCCACCTCTG ACTTGAGCGTCGATTTTTGTGATGCTCGTCAGGGGGGCGGAGCCTATGGAAAAACGCCA GCAACGCGGCCTTTTTACGGTTCCTGGCCTTTTGCTGGCCTTTTGCTCACATGTTCTTTCC TGCGTTATCCCCTGATTCTGTGGATAACCGTATTACCGCCTTTGAGTGAGCTGATACCGC TCGCCGCAGCCGAACGACCGAGCGCAGCGAGTCAGTGAGCGAGGAAGCGGAAG

The target sequence and the PAM sequence are colored red and purple, respectively.

Supplementary Table S2. Plasmids used for in this study.

Name	Sequence
pSI-545-SaCas9	https://benchling.com/s/seq-jQmxNRmFxjaY3FiGqiLA?m=slm-ryLbEc2vzaLVCCrJgOsK
pSI-545-SaCas9-NNG	https://benchling.com/s/seq-oBqtHnQurOLUEdlmbiZ1?m=slm-2QIf8NyDgh78A43ptIDy
pSI-545-SaCas9-AID	https://benchling.com/s/seq-j8s4vU4JvDIqZXedQKKo?m=slm-1SiJJLsJBCEcJR4OgPOq
pSI-545-SaCas9-NNG-AID	https://benchling.com/s/seq-gmappEwyN1YSYCCPqdbj?m=slm-aP7K9IRqnlbOBwDZxX06
pSI-414-SaCas9-sgRNA	https://benchling.com/s/seq-F3EvVfqV4GtHnPS1GbJ7?m=slm-lLTlz19HlVQHr5TfMIUY
pSI-545-SaCas9-ABE8e	https://benchling.com/s/seq-IXWUzZTpj4j8I0X3YIy3?m=slm-svIW4FsGoPX7U5kAzvGp
pSI-545-SaCas9-NNG-ABE8e	https://benchling.com/s/seq-hiDaEKr5heRyZyxYd416?m=slm-SHPD5jLrvdH3jTt2mSW5
pSI-545-eSaCas9	https://benchling.com/s/seq-VAc0zaijT2aI8jQHm7PX?m=slm-81iPHzhYlILNSnEmovEI
pSI-545-eSaCas9-NNG	https://benchling.com/s/seq-rPncFkafYmBI4aqzka9K?m=slm-AzGbESfXC3Z9Nwjvz3z6
pcDNA3-eSaCas9-NNG	https://benchling.com/s/seq-GDFx8srVa0eicdWzZAqD?m=slm-1rFfqUHYIbYiNTZQWd20
pcDNA3-SpRY	https://benchling.com/s/seq-LDFoNP6UZ4QF38e9dSOl?m=slm-IbjhT9VfeOAh2AR3CkJB
pcDNA3-SpG	https://benchling.com/s/seq-Tx9KYlFMowfow5ZiVzFT?m=slm-it3yJdzrD4BSOCgL7oaD
pcDNA3-iGeoCas9	https://benchling.com/s/seq-1vCfsqFs3X8gOJCItO43?m=slm-bgLMw91owInih6EqB68w
pUC-SaCas9-sgRNA	https://benchling.com/s/seq-O3QaEzHyRw5YjFSmjaV1?m=slm-Bi2F7lJDdJVzSBAtwUby
pUC-SpCas9-sgRNA	https://benchling.com/s/seq-46sFBTzEmiYFELhDOFwp?m=slm-yC2g5S1GuaePDyBffqw1
pUC-iGeoCas9-sgRNA	https://benchling.com/s/seq-ThvEwKi3Z2XvJYw2RRY3?m=slm-XfRwHp8fQj1aZRa7xxnf
pE-SUMO-SaCas9	https://benchling.com/s/seq-qvggtaAgE4xOw4W3yQKm?m=slm-UrgA0sXhmz4L8JF1laRu

Supplementary Table 2

Supplementary Table S3. sgRNA and target DNA sequence used for cryo-EM analysis.

Name	Sequence
sgRNA	GGGAAATTAGGTGCGCTTGGCGTTTTAGTACTCTGGAAACAGAATCTACTAAAACAA GGCAAAATGCCGTGTTTATCTCGTCAACTTGTTGGCGAGAT
Target strand	ATTTGAATAGGCAAGC*C*A*A*G*C*G*CACCTAATTTCCCGTATTTAG
Non-target strand	CTAAATACGGGAAATTAGGTGC*G*C*T*T*G*G*CTTGCCTATTCAAAT

The guide sequence in the sgRNA and the target sequence in the target strand and non-target strand are underlined. The PAM sequence in the non-target strand is colored purple. * phosphorothioate bond.

Supplementary Table 3

Supplementary Table S4. Data collection, processing, model refinement, and validation.

Data collection and processing						
Sample	eSaCas9-NNG–sgRNA–DNA interrogation state	eSaCas9-NNG–sgRNA–DNA intermediate state	eSaCas9-NNG–sgRNA–DNA translocation state	eSaCas9-NNG–sgRNA–DNA active state	SaCas9–sgRNA–mismatched DNA	eSaCas9-NNG–sgRNA–mismatched DNA
EMDB ID	EMD-39941	EMD-39942	EMD-39944	EMD-39954	EMD-63767	EMD-63768
PDB ID	8ZCY	8ZCZ	8ZD0	8ZDA	9MB6	9MB7
Microscope	Titan Krios G3i					
Detector	Gatan K3 Camera					
Magnification	105,000					
Voltage (kV)	300					
Electron exposure (e^{-}/Å^2)	50					
Defocus range (μm)	−0.8 to −1.6					
Pixel size (Å)	0.83					
Symmetry imposed	C_1					
Number of movies	8,625				9,401	5,136
Initial particle images (no.)	4,900,536				4,919,604	5,775,994
Final particle images (no.)	211,623	156,154	143,305	59,818	101,789	123,595
Map resolution (Å)	3.17	2.90	2.76	3.14	3.01	2.88
FSC threshold	0.143					
3DFSC analysis						
Global resolution (Å)	3.21	2.99	2.85	3.25	3.08	2.96
Sphericity	0.797	0.912	0.983	0.967	0.956	0.963
Model building and refinement						
Map sharpening *B* factor (Å^2)	−84.9	−78.9	−83.6	−81.3	−87.0	−86.8
Model composition						
Protein atoms	5,412	6,901	7,034	8,198	8,222	8,198
Nucleic acid atoms	2,895	2,981	3,150	3,421	3,423	3,423
Other atoms	0	0	1	4	0	1
Model refinement						
Resolution (Å) by model to map FSC, threshold 0.50	3.86	2.97	2.81	3.16	3.03	2.92
Average *B* factor (Å^2) (protein/nucleotides/Other atoms)	132.2/196.95/-	77.1/70.65/-	74.4/90.25/73.9	74.4/96.6/76.6	71.8/102.1/-	57.6/82.7/38
R.m.s. deviations						
Bond lengths (Å)	0.008	0.0048	0.0055	0.0053	0.0061	0.005
Bond angles (°)	1.83	1.42	1.51	1.37	1.56	1.36
Validation						
MolProbity score	1.29	1.40	0.99	1.54	1.60	1.48
Clashscore	0.84	1.68	0.37	1.19	1.70	1.01
Rotamer outliers (%)	2.15	1.83	0.39	2.98	2.74	2.76
C_β outliers (%)	0.32	0.00	0.00	0.21	0.00	0.21
EMRinger score	1.35	3.86	4.71	3.45	4.08	4.50

Supplementary Table 4