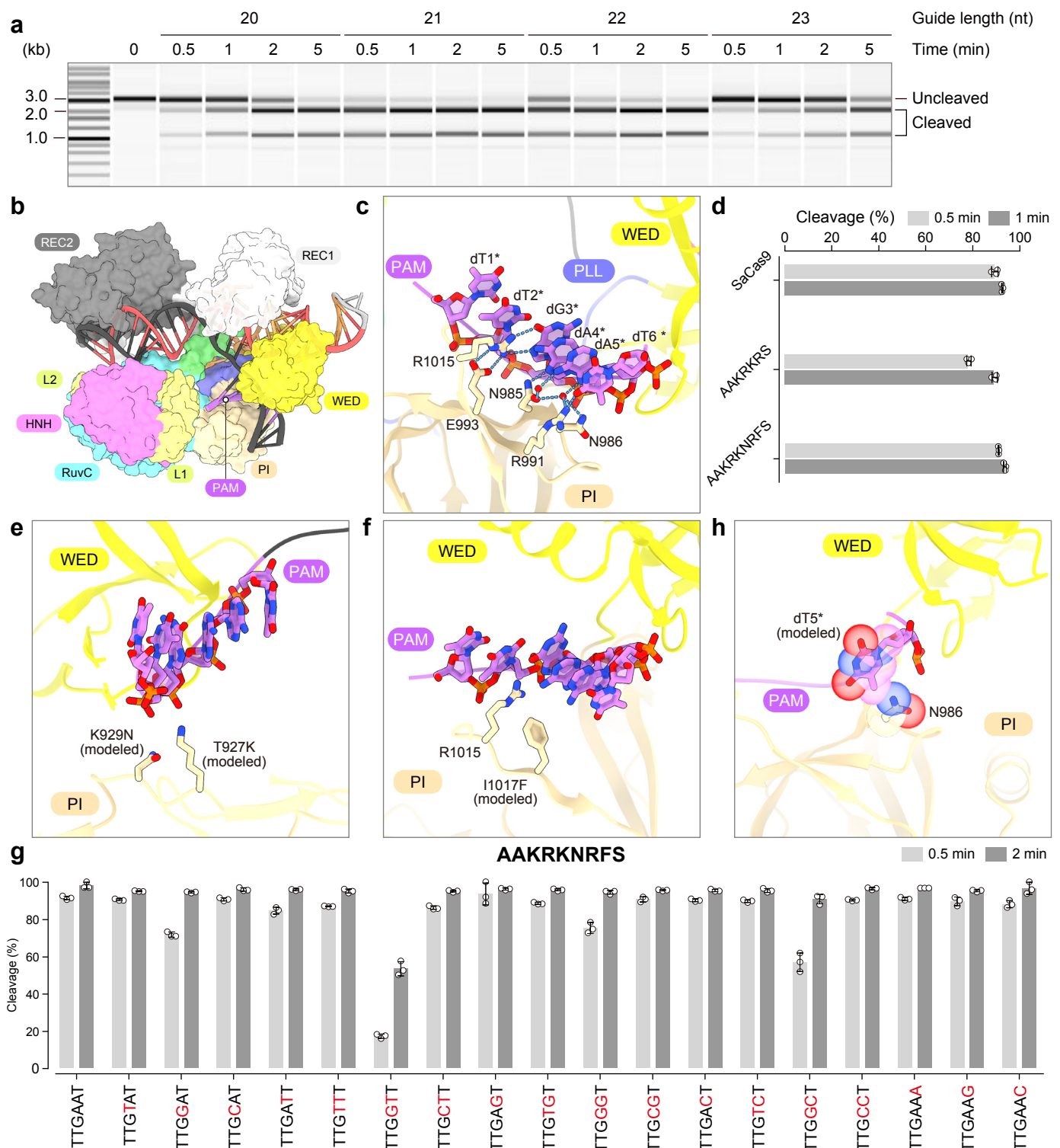




Supplementary Fig. 1 | Engineering of the SaCas9-NGG variant.

(a) In vitro DNA cleavage activities of SaCas9 with 20–23-nt guide sgRNAs. The linearized plasmid target bearing the TTGAAT PAM was incubated with the SaCas9–sgRNA complex at 37°C for 0.5, 1, 2, and 5 min. The cleavage products were then analyzed by a MultiNA microchip electrophoresis system.

(b) Crystal structure of SaCas9 in complex with its sgRNA and target DNA (PDB: 5CZZ).

(c) PAM recognition by SaCas9. dG3* is recognized by Arg1015, while dA4*/dA5* and dT6* are recognized by Asn985 and Arg991, respectively.

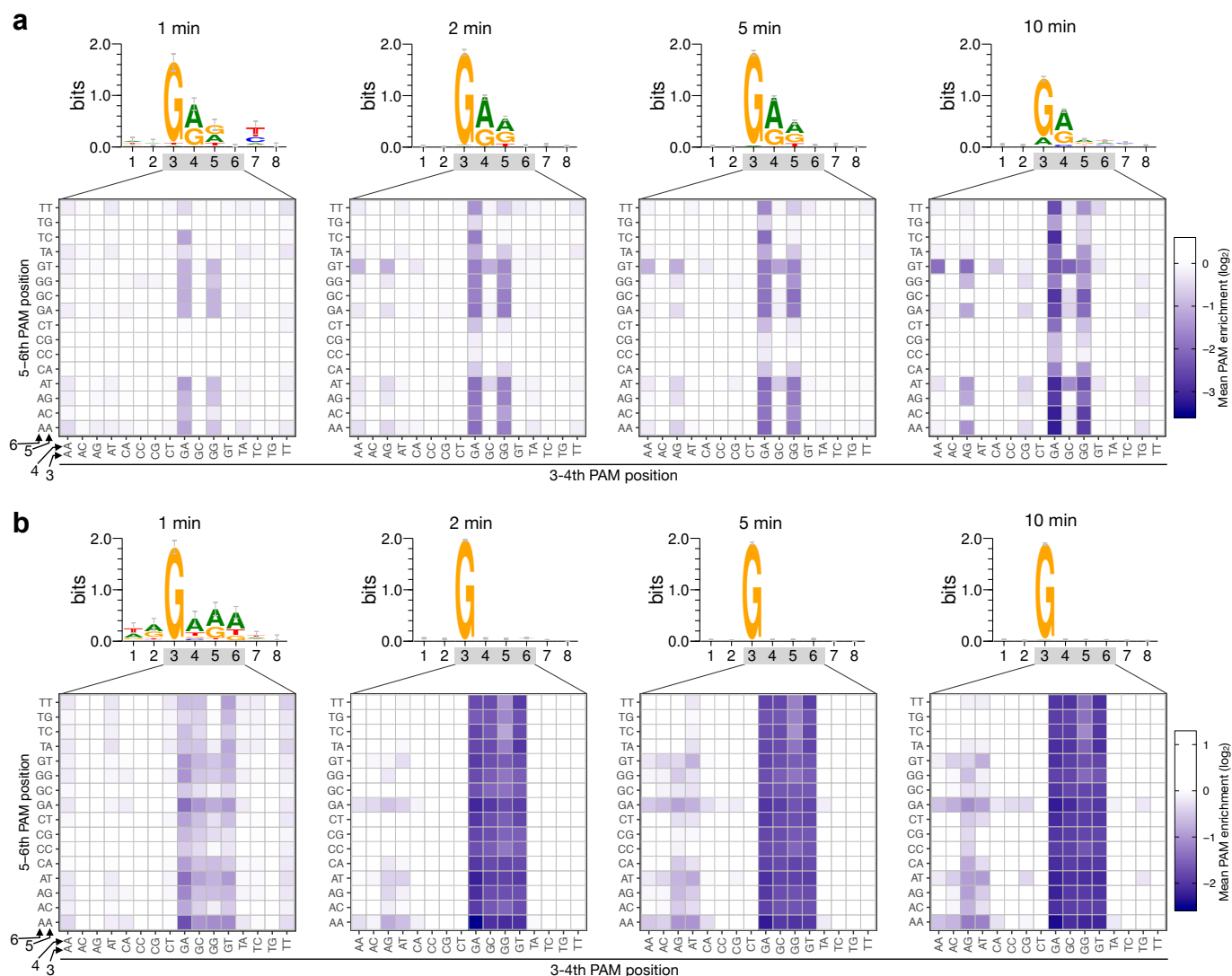
(d) In vitro cleavage activities of SaCas9, AAKRKRS, and AAKRKNRFS toward the target DNA bearing the TTGAAT PAM. Data are mean $\pm$ s.d. from $n = 3$ biologically independent experiments.

(e and f) Structural modeling of the T927K/K929N (e) and I1017F (f) mutations. The mutated residues were manually modeled using the Coot software.

(g) In vitro DNA cleavage activities of AAKRKNRFS toward DNA targets with different PAMs. The linearized plasmid targets were incubated with the SaCas9–sgRNA complex at 37°C for 0.5 and 2 min. Data are mean $\pm$ s.d. from $n = 3$ biologically independent experiments.

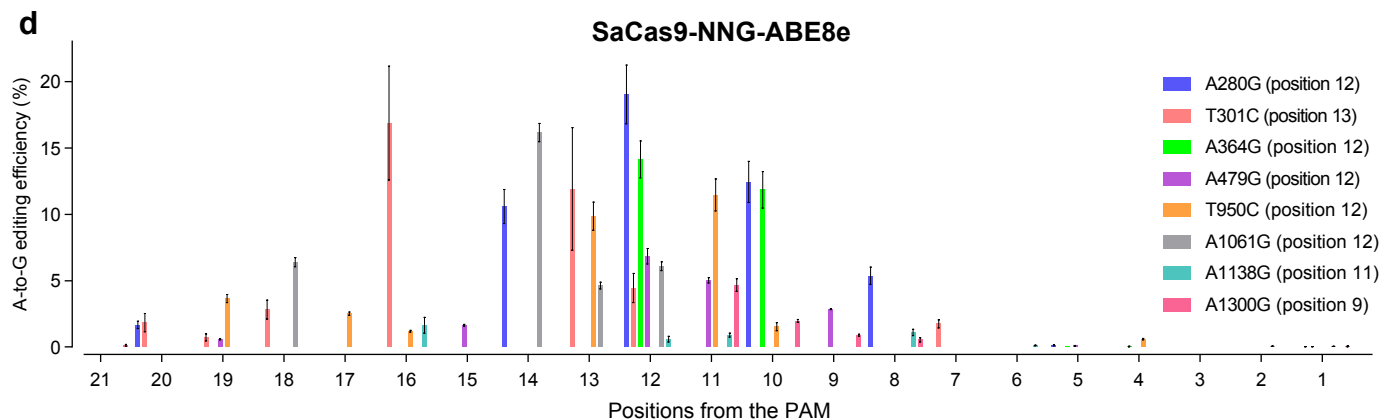
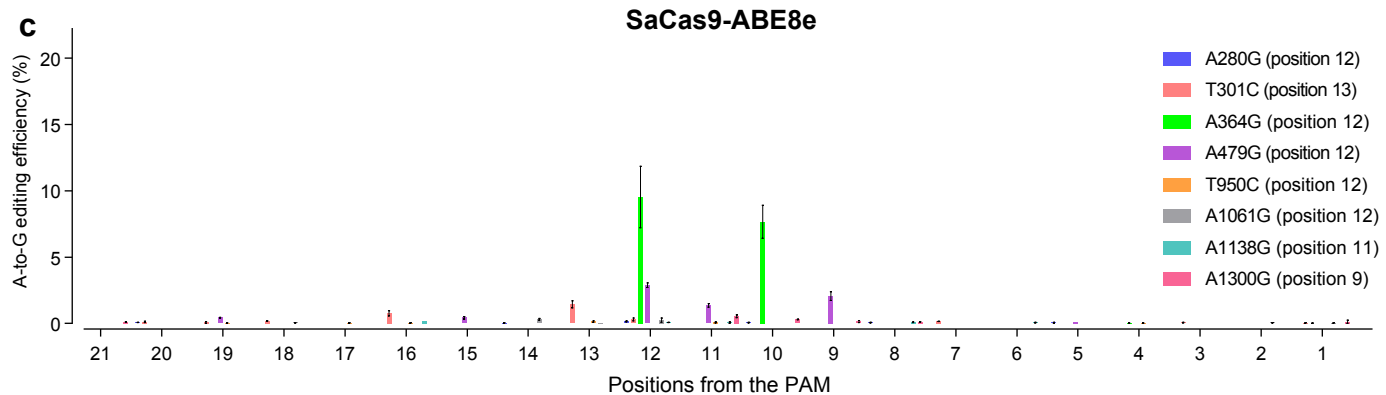
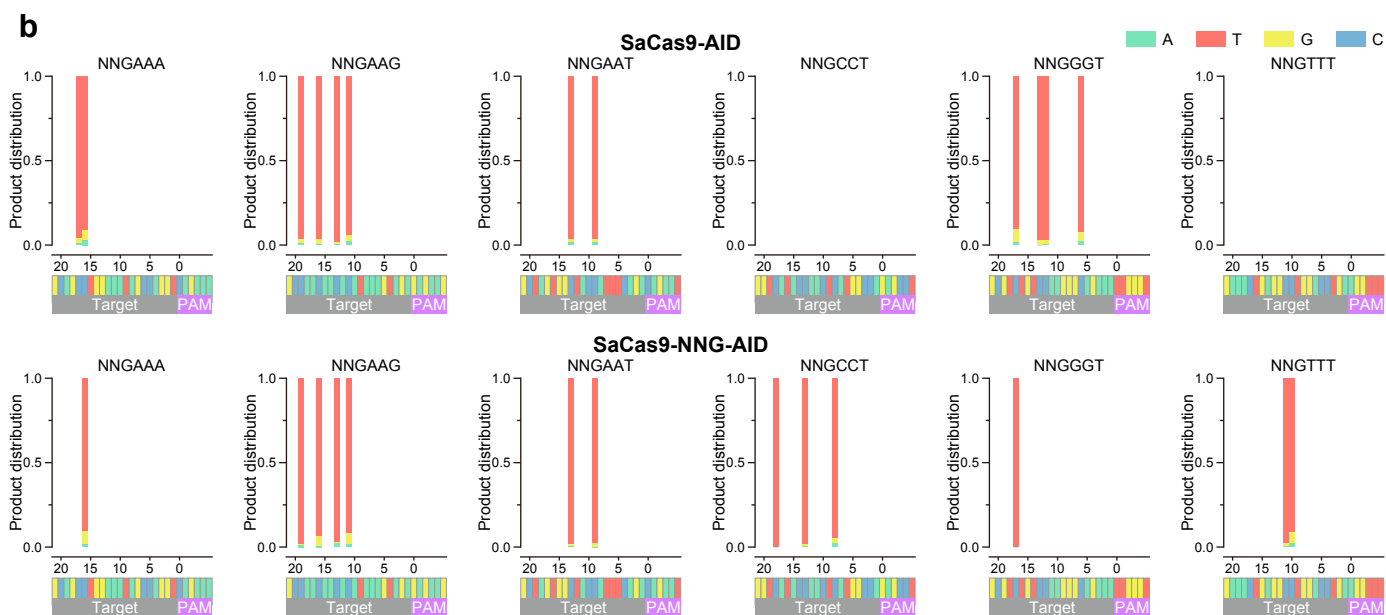
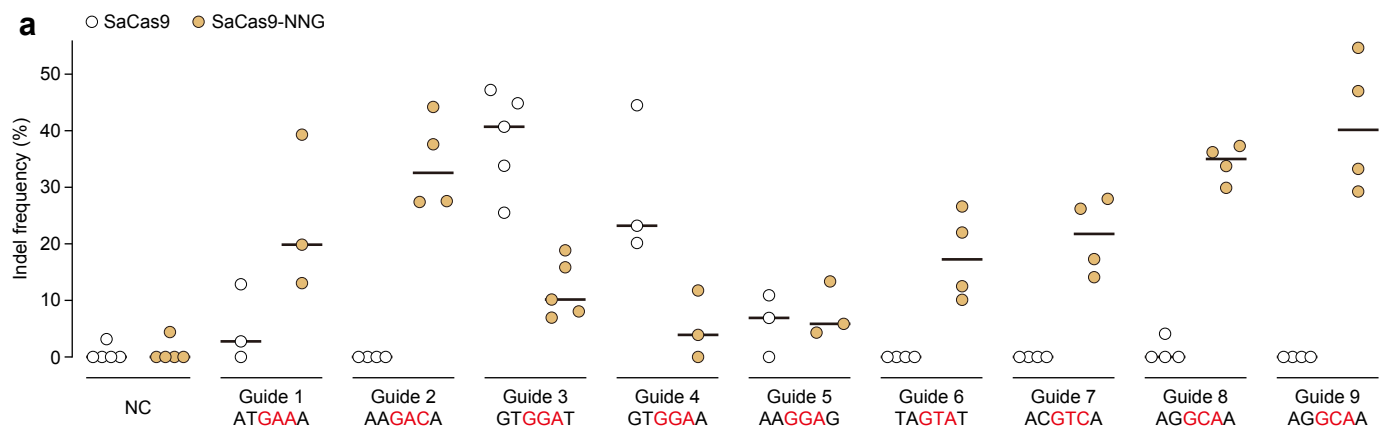
(h) Potential steric clash between the fifth T (dT5*) and Asn986. The side chain of Asn986 clashes with the methyl group of dT5*, resulting in the weak activity of AAKRKNRFS toward the TTGGTT target. dT5* was manually modeled using the Coot software.

All molecular graphics figures were prepared with UCSF ChimeraX-1.7.1⁶⁶.



Supplementary Fig. 2 | Comprehensive PAM discovery analysis.

(a and b) PAM preference analysis of SaCas9 and SaCas9-NGG. Sequence logos and corresponding heatmaps summarize PAM enrichment derived from the in vitro PAM discovery assay at 1, 2, 5, and 10 min for SaCas9 (a) and SaCas9-NGG (b). Data are mean $\pm$ s.d. from $n = 3$ biologically independent experiments.

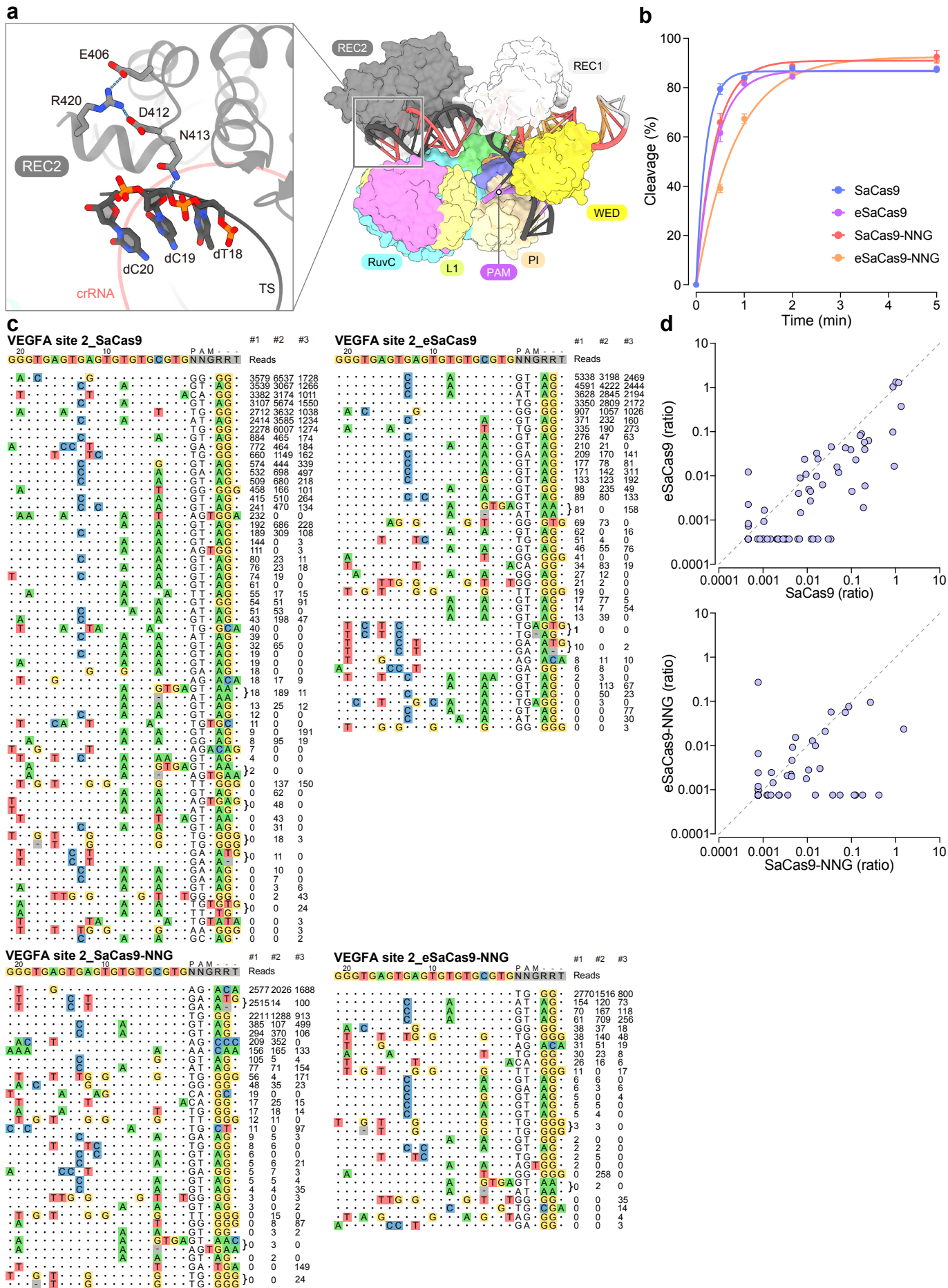


Supplementary Fig. 3 | Genome- and base-editing activities in mammalian cells.

(a) Indel efficiencies of SaCas9 (white) and SaCas9-NNG (orange) at nine endogenous target loci in murine immortalized liver (TLR3) cells. Data are mean $\pm$ s.d. from n = 5 (NC), n = 3 (Guide 1 and 4), and n = 4 (Guide 2, 3, 5, 6, 7, 8, and 9) biologically independent experiments.

(b) Representative product distributions at the VEGFA locus for SaCas9-AID (top) and SaCas9-NNG-AID (bottom) across different PAM contexts. The y-axis indicates the fractional distribution of base substitutions observed at each nucleotide position within the protospacer. Schematics below each panel illustrate the corresponding target sequence and PAM.

(c and d) A-to-G conversions in HEK293 cells by SaCas9-ABE8e (c) and SaCas9-NNG-ABE8e (d) at various point mutations known to cause hemophilia B. The A-to-G base conversions were mainly observed between the 7th to 20th positions from the PAM within the protospacer. The positions of individual pathogenic adenine residues are indicated according to their distance from the PAM. Data are mean $\pm$ s.d. from n = 3 biologically independent experiments.



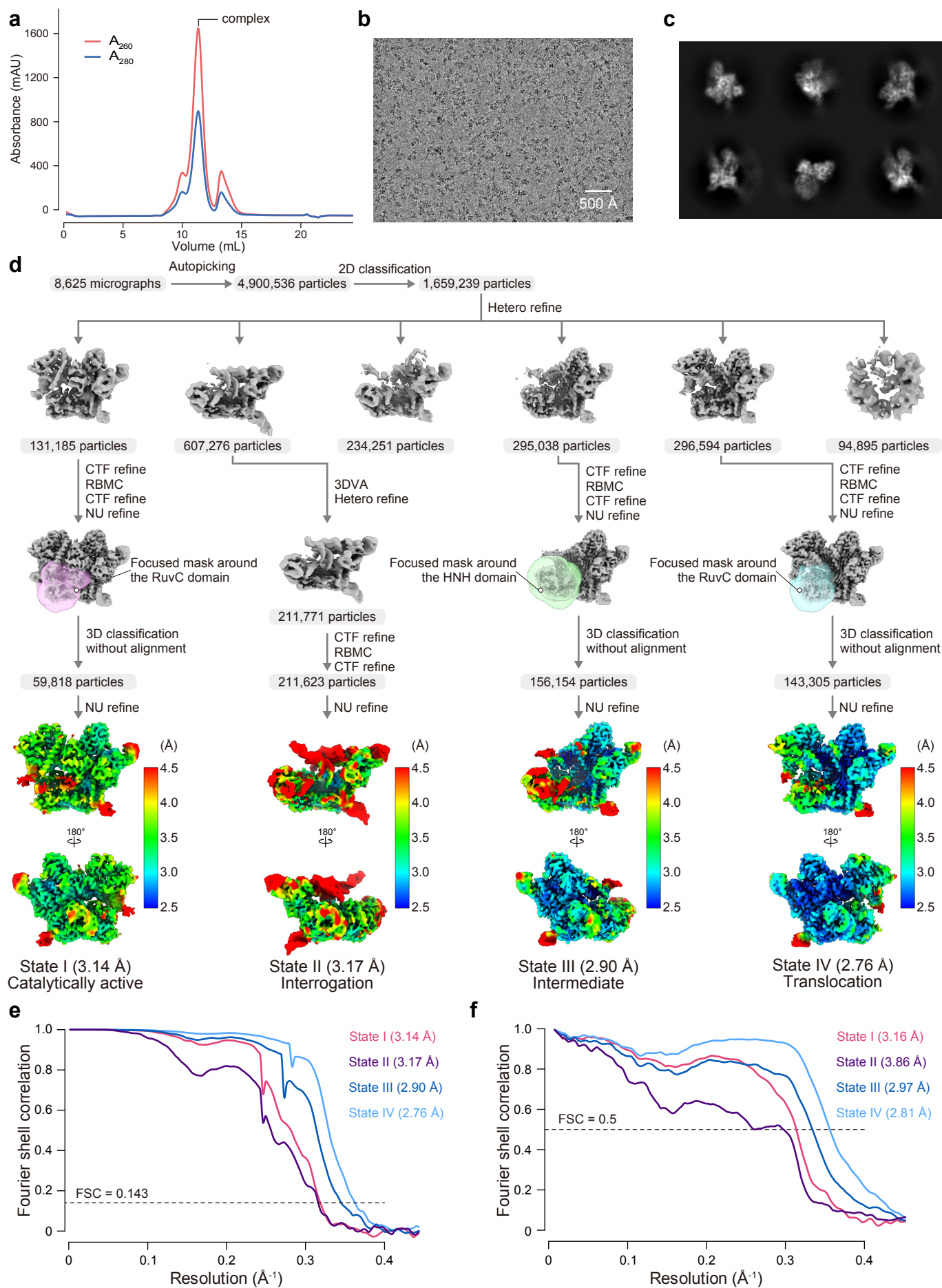
Supplementary Fig. 4 | Engineering of the eSaCas9-NNG variant.

(a) Close-up view of the PAM-distal end of the heteroduplex and the REC2 domain in the SaCas9 structure (PDB: 5CZZ). Asn413 interacts with the dC19 ribose moiety in the target DNA, while Arg420 forms salt bridges with Glu406 and Asp412 within the REC2 domain. Molecular graphics figures were prepared with UCSF ChimeraX-1.7.1⁶⁶.

(b) In vitro DNA cleavage kinetics of SaCas9, eSaCas9, SaCas9-NNG, and eSaCas9-NNG. The linearized plasmid target bearing the TTGAAT PAM was incubated with the SaCas9–sgRNA complexes at 37°C for 0.5, 1, 2, and 5 min. The cleavage products were then analyzed by a MultiNA microchip electrophoresis system. Data are mean $\pm$ s.d. from $n = 3$ biologically independent experiments.

(c) Off-target cleavage at the VEGFA site, identified by GUIDE-seq. The on-target sequence is shown in the top line, with detected cleaved sites shown underneath. The read counts are shown to the right of each site. Data are mean $\pm$ s.d. from $n = 3$ biologically independent experiments.

(d) Summarized on- and off-target activities for GUIDE-seq2 results from (c) plotted as a scatter plot. Off-target activities are calculated as the ratio between read counts for the off-target and the corresponding on-target read count.



Supplementary Fig. 5 | Cryo-EM analysis of the eSaCas9-NNG–guide RNA–target DNA complex.

(a) Size-exclusion chromatography profile of the eSaCas9-NNG–guide RNA–target DNA complex. The peak fraction indicated as a complex was used for cryo-EM analysis.

(b) Representative cryo-EM micrograph, recorded on a 300 kV Titan Krios electron microscope with a K3 camera.

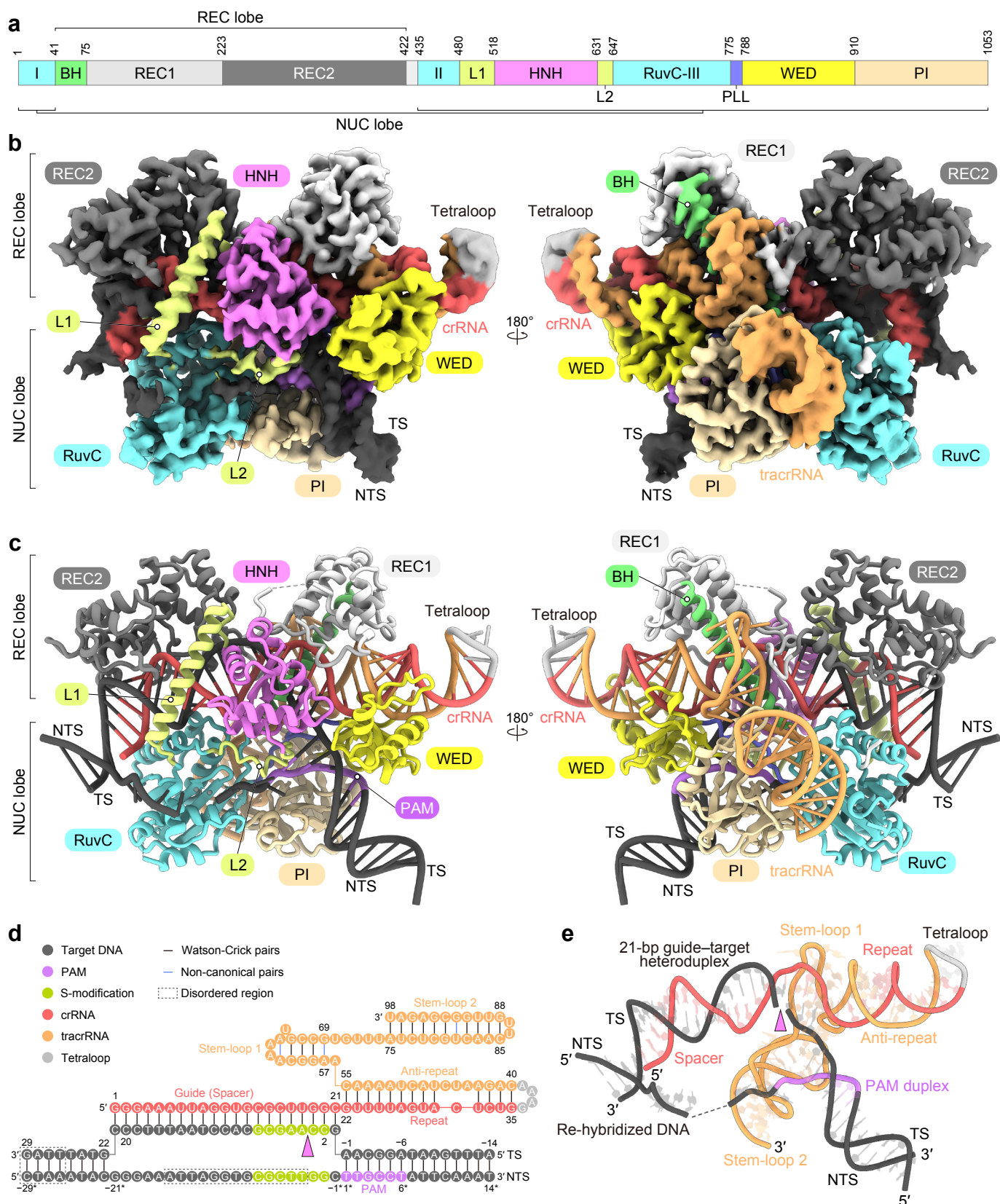
(c) Representative 2D class average images.

(d) Single-particle cryo-EM image processing workflow. Cryo-EM density maps according to the local resolution are shown at the bottom. Data were processed using the cryoSPARC v4.4.0 software platform.

(e) Fourier shell correlation curves for the 3D reconstructions.

(f) Fourier shell correlation curves calculated between the refined models and the cryo-EM density maps.

All molecular graphics figures were prepared with UCSF ChimeraX-1.7.1⁶⁶.



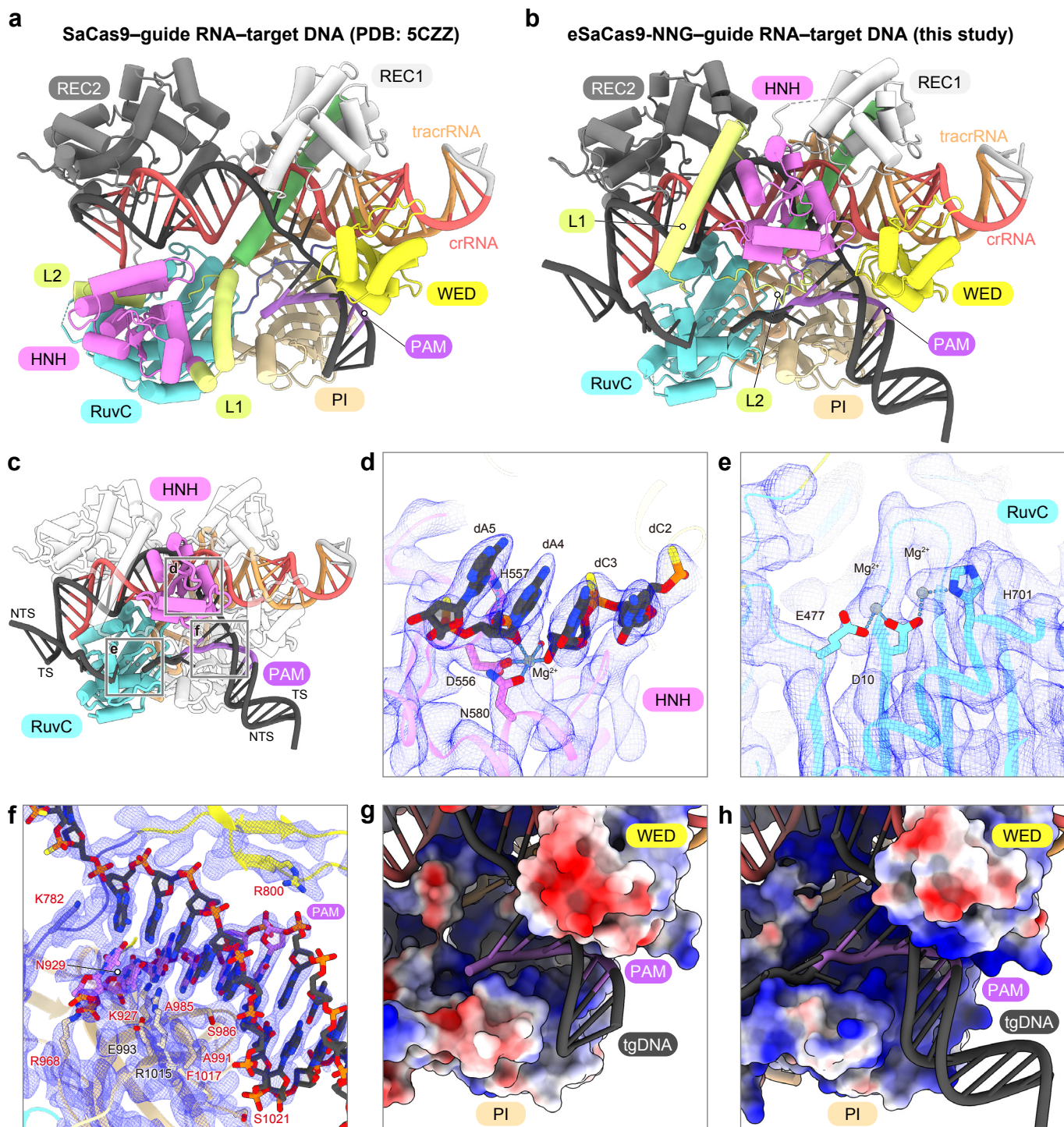
Supplementary Fig. 6 | Cryo-EM structure of the eSaCas9-NNG-guide RNA-target DNA complex.

(a) Domain structure of eSaCas9-NNG. I, RuvC-I; II, RuvC-II; BH, bridge helix; PLL, phosphate lock loop.

(b and c) Cryo-EM density maps (b) and ribbon models (c) of the eSaCas9-NNG-guide RNA-target DNA complex. TS, target DNA strand; NTS, non-target DNA strand.

(d) Schematic of the sgRNA and the target DNA. The cleavage site in the TS is indicated by a magenta triangle. The disordered regions are enclosed by dashed boxes. Phosphorothioate-modified DNA nucleotides (S-modification) are colored green.

(e) Structure of the sgRNA and target DNA complex. The cleavage site in the TS is indicated by a magenta triangle. All molecular graphics figures were prepared with UCSF ChimeraX-1.7.1⁶⁶.



Supplementary Fig. 7 | Structural comparison between SaCas9 and eSaCas9-NNG.

(a) Crystal structure of the SaCas9–guide RNA–target DNA complex (PDB: 5CZZ).

(b) Cryo-EM structure of the eSaCas9-NNG–guide RNA–target DNA complex.

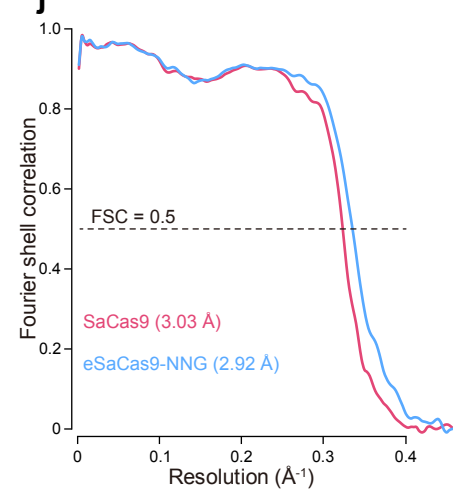
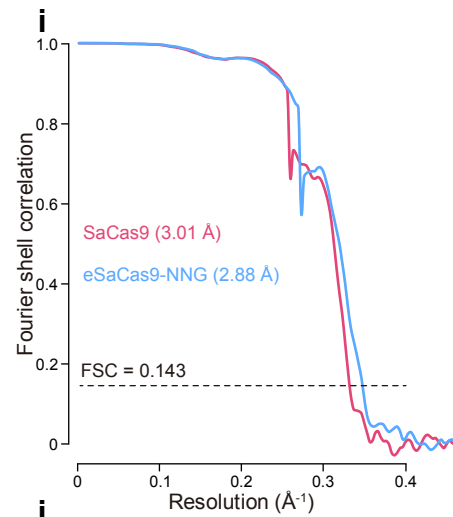
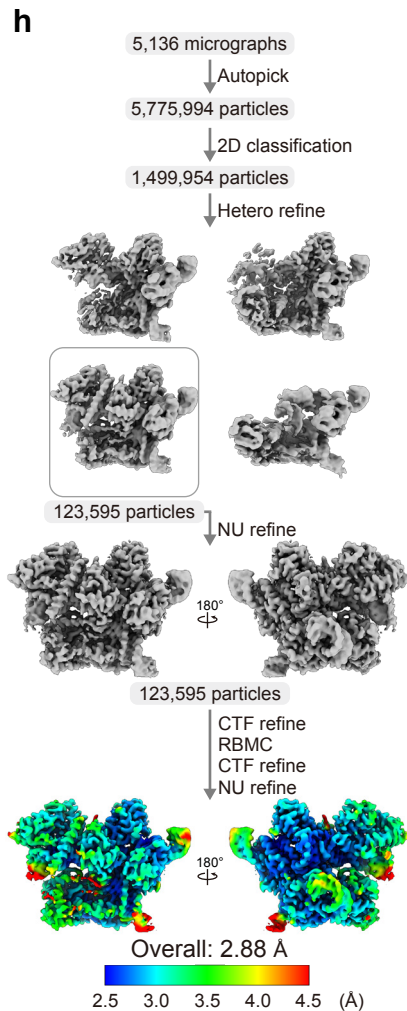
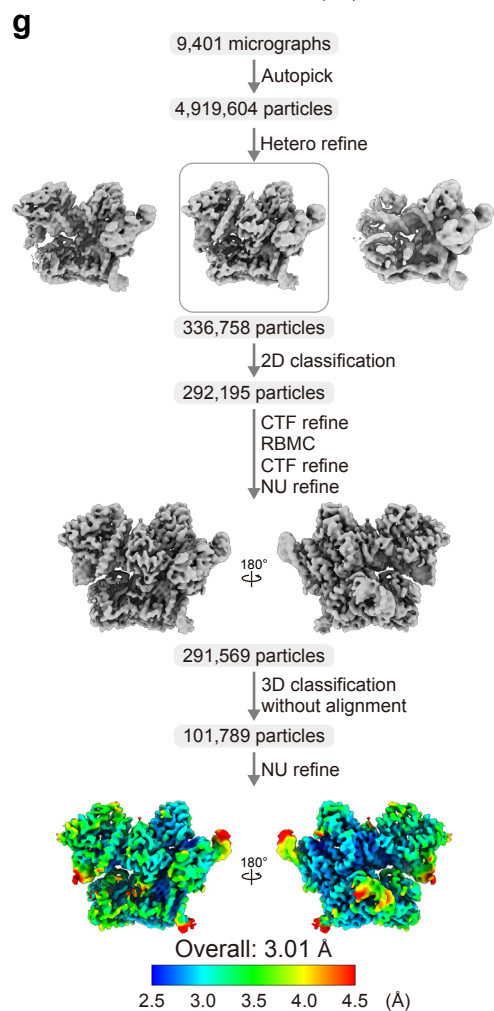
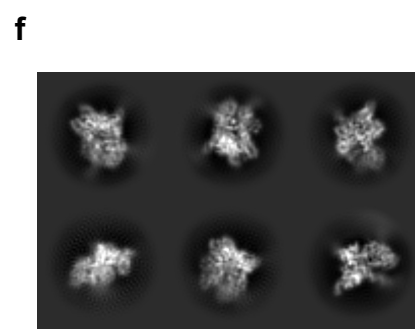
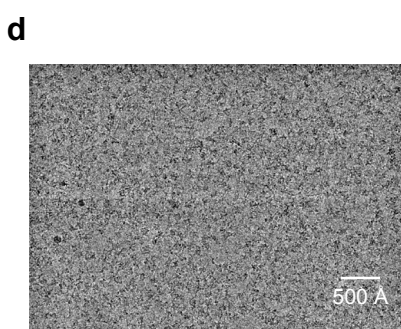
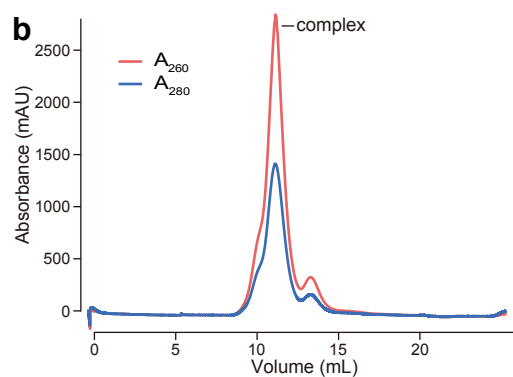
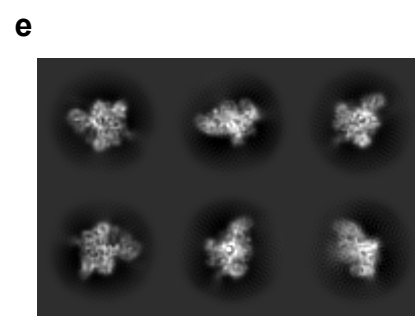
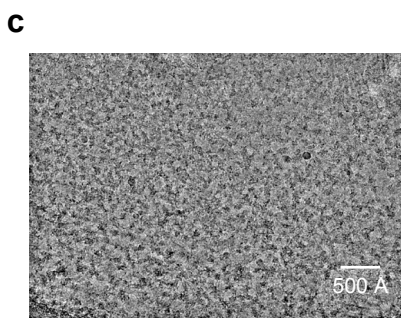
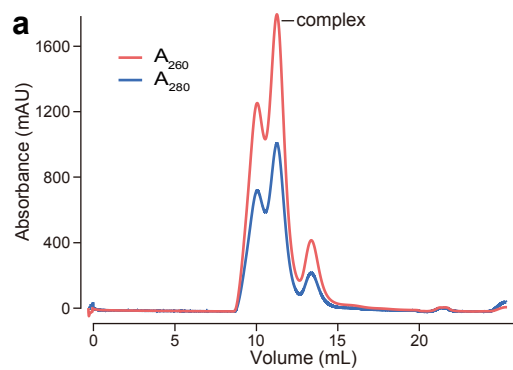
(c) Locations of the RuvC and HNH domains of eSaCas9-NNG in the active state.

(d and e) Close-up views of the HNH (d) and RuvC (e) active sites in eSaCas9-NNG. Cryo-EM density maps are shown as blue meshes. The bound Mg^{2+} ions and water molecules are depicted as gray and red spheres, respectively.

(f) Cryo-EM density map of the eSaCas9-NNG–guide RNA–target DNA complex around the PAM-containing DNA duplex. Mutated residues are highlighted in red. Cryo-EM density maps are shown as blue meshes.

(g and h) Surface electrostatic potential maps around the WED and PI domains of SaCas9 (g) and eSaCas9-NNG (h), highlighting the differences in electrostatic landscapes surrounding the PAM-binding region.

All molecular graphics figures were prepared with UCSF ChimeraX-1.7.1⁶⁶.



Supplementary Fig. 8 | Cryo-EM analysis of SaCas9 and eSaCas9-NNG bound to their cognate sgRNA and the mismatched target DNA.

(**a** and **b**) Size-exclusion chromatography profiles of the SaCas9–guide RNA–mismatched target DNA (**a**) and eSaCas9-NNG–guide RNA–mismatched target DNA (**b**) complexes. The peak fractions indicated as a complex were used for cryo-EM analysis.

(**c** and **d**) Representative cryo-EM micrographs of the SaCas9–guide RNA–mismatched target DNA (**c**) and eSaCas9-NNG–guide RNA–mismatched target DNA (**d**) complexes, recorded on a 300 kV Titan Krios electron microscope with a K3 camera.

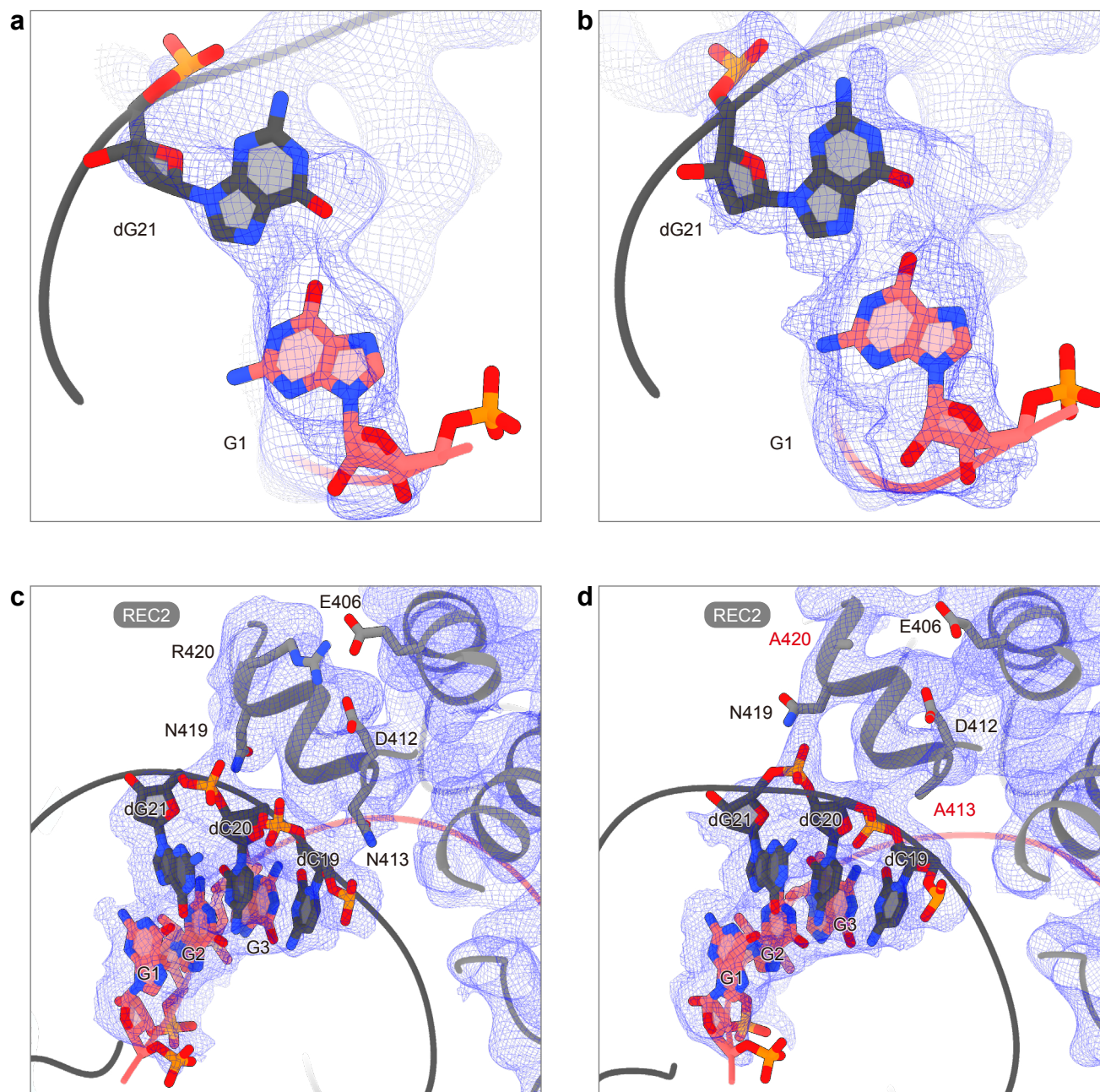
(**e** and **f**) Representative 2D class average images of the SaCas9–guide RNA–mismatched target DNA (**e**) and eSaCas9-NNG–guide RNA–mismatched target DNA (**f**) complexes.

(**g** and **h**) Single-particle cryo-EM image processing workflows of the SaCas9–guide RNA–mismatched target DNA (**g**) and eSaCas9-NNG–guide RNA–mismatched target DNA (**h**) complexes. Cryo-EM density maps according to the local resolution are shown at the bottom. Data were processed using the cryoSPARC v4.4.0 software platform.

(**i**) Fourier shell correlation curves for the 3D reconstructions.

(**j**) Fourier shell correlation curves calculated between the refined models and the cryo-EM density maps.

All molecular graphics figures were prepared with UCSF ChimeraX-1.7.1⁶⁶.



Supplementary Fig. 9 | Representative cryo-EM densities.

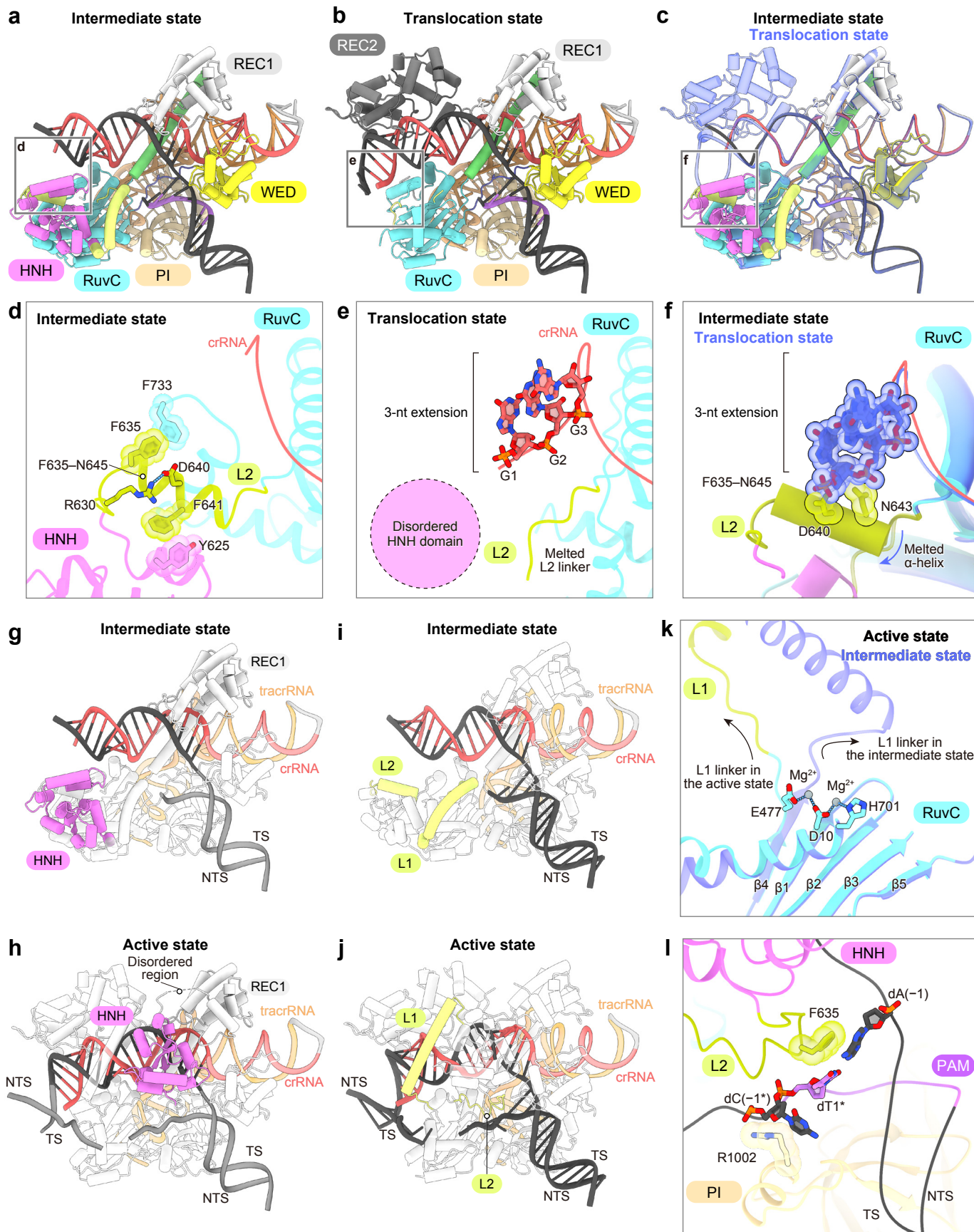
(a) Cryo-EM density map of the SaCas9-guide RNA-mismatched target DNA complex around the G1:dG21 mismatch at the PAM-distal end. Cryo-EM density maps are shown as blue meshes.

(b) Cryo-EM density map of the eSaCas9-NNG-guide RNA-mismatched target DNA complex around the G1:dG21 mismatch at the PAM-distal end. Cryo-EM density maps are shown as blue meshes.

(c) Cryo-EM density map of the SaCas9-guide RNA-mismatched target DNA complex around the REC2 domain and PAM-distal guide-target heteroduplex. Cryo-EM density maps are shown as blue meshes.

(d) Cryo-EM density map of the eSaCas9-NNG-guide RNA-mismatched target DNA complex around the REC2 domain and PAM-distal guide-target heteroduplex. Mutated residues are highlighted in red. Cryo-EM density maps are shown as blue meshes.

All molecular graphics figures were prepared with UCSF ChimeraX-1.7.1⁶⁶.



Supplementary Fig. 10 | Conformational changes in eSaCas9-NNG coupled with the guide–target heteroduplex formation.

(a and b) Structures of the eSaCas9-NNG–guide RNA–target DNA complexes in the intermediate (a) and translocation (b) states.

(c) Superimposition of the intermediate (colored as in a) and translocation (light blue) states.

(d and e) Close-up views around the L2 linker and the RuvC domain in the intermediate (d) and translocation (e) states. In the intermediate state, residues 635–645 adopt an α helix structure and interact with both the RuvC and HNH domains, whereas in the translocation state, the L2 linker is melted and the HNH domain is disordered. Hydrogen bonds are shown as dashed lines.

(f) Structural comparison of the PAM-distal region between the intermediate and translocation states. The two structures are superimposed based on their RuvC domains. The PAM-distal heteroduplex in the translocation state clashes with the L2 linker in the intermediate state.

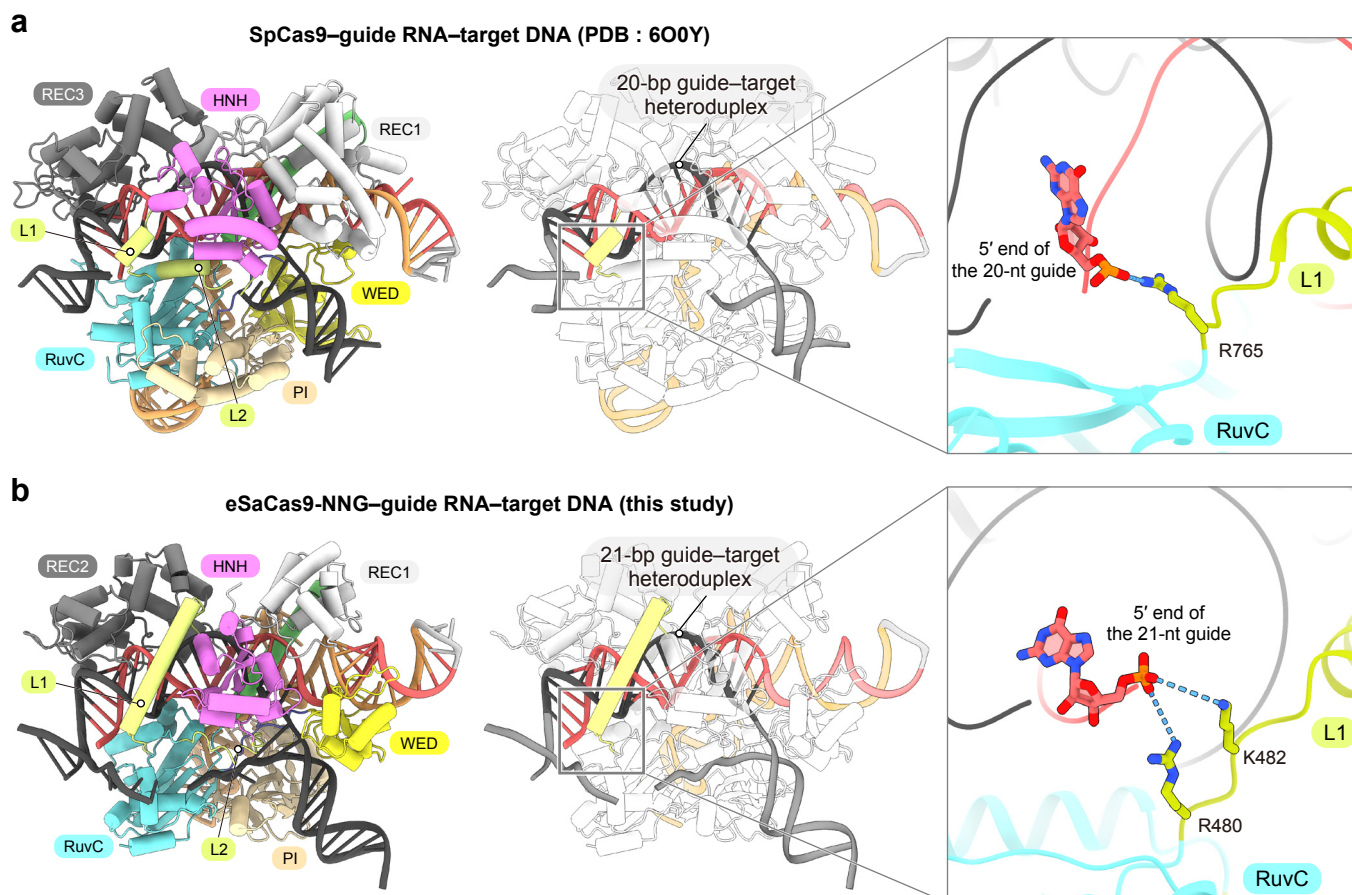
(g and h) Structures of the eSaCas9-NNG–guide RNA–target DNA complexes in the intermediate (g) and active (h) states. The HNH domains are highlighted in magenta. During the transition from the intermediate state to the active state, the HNH domain undergoes an approximately 140° rotation, docking onto the TS cleavage site. Upon docking of the HNH domain, residues 126–146 in the REC1 domain become disordered due to steric clashes with the HNH domain.

(i and j) Surface models of the eSaCas9-NNG–guide RNA–target DNA complexes in the intermediate (i) and active (j) states. The L1 and L2 linkers are highlighted in light green. During the transition from the intermediate state to the active state, the L1 linker undergoes an approximately 180° rotation and binds to the minor groove of the PAM-distal heteroduplex, while the L2 linker adopts a loop conformation and forms an NTS-binding pathway toward the RuvC active site in the active state.

(k) Superimposition of the RuvC domains in the intermediate (light blue) and active (colored as in a) states. The catalytic residues in the RuvC active site and the bound Mg^{2+} ions in the active state are shown as sticks and gray spheres, respectively. In the active state, the C-terminal region of the $\beta 4$ strand is melted.

(l) Recognition of the dA(–1)–dT1* base pair and the displaced non-target DNA strand.

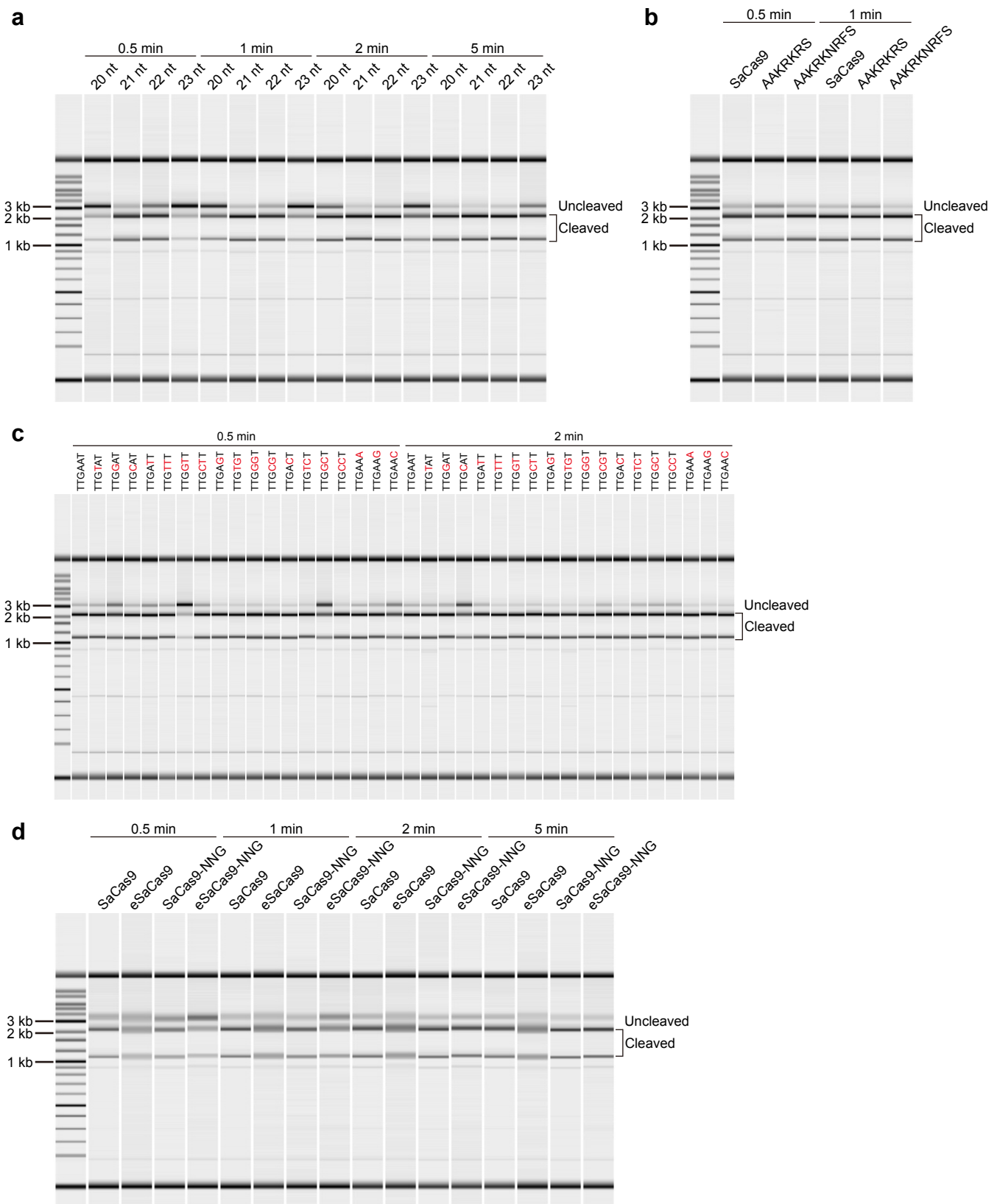
All molecular graphics figures were prepared with UCSF ChimeraX-1.7.1⁶⁶.



Supplementary Fig. 11 | Conformational changes in SpCas9 and eSaCas9-NNG.

(a and b) Recognition of the PAM-distal region of the guide–target heteroduplex by SpCas9 (PDB: 6O0Y) (a) and eSaCas9-NNG (b).

All molecular graphics figures were prepared with UCSF ChimeraX-1.7.1⁶⁶.



Supplementary Fig. 12 | Uncropped electropherograms.

(a–d) Uncropped electropherogram images used in Supplementary Fig. 1a (a), Supplementary Fig. 1d (b), Supplementary Fig. 1g (c), and Supplementary Fig. 4b (d).

Name	Sequence
sgRNA20	GGAAATTAGGTGCGCTTGGCGTTTTAGTACTCTGGAAACAGAATCTACTAAAACAAGGC AAAATGCCGTGTTTATCTCGTCAACTTGTTGGCGAGAT
sgRNA21	GGGAAATTAGGTGCGCTTGGCGTTTTAGTACTCTGGAAACAGAATCTACTAAAACAAGG CAAAATGCCGTGTTTATCTCGTCAACTTGTTGGCGAGAT
sgRNA22	GGGGAAATTAGGTGCGCTTGGCGTTTTAGTACTCTGGAAACAGAATCTACTAAAACAAG GCAAAATGCCGTGTTTATCTCGTCAACTTGTTGGCGAGAT
sgRNA23	GGGGGAAATTAGGTGCGCTTGGCGTTTTAGTACTCTGGAAACAGAATCTACTAAAACAA GGCAAAATGCCGTGTTTATCTCGTCAACTTGTTGGCGAGAT
Target DNA	AGCGCCCAATACGCAAACCGCCTCTCCCCGCGCGTTGGCCGATTCAATATGCAGCTGGCAGCAGC AGGTTTTCCCGACTGGAAAGCGGGCAGTGAGCGCAACGCAATTAATGTGAGTTAGCTCACTCATT GGCAGCCCGAGGCTTTACACTTTATGCTTCCGGCTCGTATGTTGTGTGGAATTGTGAGCGGATAACA ATTTACACACAGGAAACAGCTATGACCATGATTACGCCAAGCTTGCATGCCTGCAGGTGACTCTA GAGGATCCCCGGGTACCGAGCTCGAATTCAGTGGCCGTCGTTTTACAACGTCGTGACTGGGAAAA CCCTGGCGTTACCCAACCTAATCGCCTTGCAGCACATCCCCCTTCGCCAGCTGGCGTAATAGCGA AGAGGCCCCGACCGATCGCCCTTCCCAACAGTTGCGCAGCCTGAATGGCGAATGGCGCCTGATGC GGTATTTTCTCCTTACGCATCTGTGCGGTATTTACACCCGCATACGTCAAAGCAACCATAGTACGC GCCCTGTAGCGGCGCATTAAAGCGCGGCGGGTGTGGTGGTTACGCGCAGCGTGACCGCTACACTTG CCAGCGCCCTAGCGCCCGCTCCTTTCGCTTTCTTCCCTTCTTCGCCACGTTCCGCCGGCTTTCC CCGTCAAGCTCTAAATCGGGGGCTCCCTTTAGGGTTCGATTTAGTGCTTTACGGCACCTCGACCC CAAAAACTTGATTTGGGTGATGGTTCACGTAGTGGGCCATCGCCCTGATAGACGGTTTTTCGCCC TTTGACGTTGGAGTCCACGTTCTTTAATAGTGGACTCTTGTTCCAAACTGGAACAACACTCAACCC TATCTCGGGCTATTTCTTTGATTTATAAGGGATTTTGCCGATTTCCGGCCTATTGGTTAAAAAATGA GCTGATTTAACAAAAATTTAACGCGAATTTTAAACAAATATTAACGTTTACAATTTTATGGTGCAC TCTCAGTACAATCTGCTCTGATGCCGCATAGTTAAGCCAGCCCCGACACCCGCCAACACCCCGCTG ACGCGCCCTGACGGGCTTGTCTGCTCCCGGCATCCGCTTACAGACAAGCTGTGACCGTCTCCGGG AGCTGCATGTGTCAGAGGTTTTACCGTCATACCCGAAACGCGCGAGACGAAAGGGCCTCGTGAT ACGCCTATTTTATAGGTAAATGTCATGATAATAATGGTTTCTTAGACGTCAGGTGGCACTTTTCG GGGAAATGTGCGCGGAACCCCTATTTGTTTATTTTCTAAATACGGGGGAAATTAGGTGCGCTTGG GCTTGAATTGGATTCAAATATGTATCCGCTCATGAGACAATAACCCTGATAAATGCTTCAATAATA TTGAAAAAGGAAGAGTATGAGTATTCAACATTTCCGTGTCGCCCTTATTCCTTTTTTTCGGGCAT TTGCCTTCTCTGTTTTTGTCTACCCAGAAACGCTGGTGAAAGTAAAAGATGCTGAAGATCAGTTGG GTGCACGAGTGGGTACATCGAACTGGATCTCAACACGCGTAAGATCCTTGAGAGTTTTTCGCCCC GAAGAACGTTTTTCCAATGATGAGCACTTTTAAAGTTCTGCTATGTGGCGCGGTATTATCCCGTATT GACGCCGGGCAAGAGCAACTCGGTCCGCCCATACACTATTCTCAGAATGACTTGGTTGAGTACTC ACCAAGTCACAGAAAAGCATCTTACGGATGGCATGACAGTAAGAGAATTATGCAGTGCTGCCATAA CCATGAGTGATAAACACTGCGGCCAACTTACTTCTGACAACGATCGGAGGACCGAAGGAGCTAACC GCTTTTTTGCACAACATGGGGGATCATGTAACCTCGCCTTGATCGTTGGGAACCGGAGCTGAATGA AGCCATACCAAACGACGAGCGTGACACCACGATGCCTGTAGCAATGGCAACAACGTTGCGCAAA CTATTAACCTGGCGAACTACTTACTCTAGCTTCCCGGCAACAATTAATAGACTGGATGGAGGCGGA TAAAGTTGCAGGACCACTTCTGCGCTCGGCCCTTCCGGCTGGCTGGTTTATTGCTGATAAATCTGG AGCCGGTGAGCGTGGGTCTCGCGGTATCATTGCAGCACTGGGGCCAGATGGTAAGCCCTCCCGTA TCGAGTTATCTACACGACGGGGAGTCAGGCAACTATGGATGAACGAAATAGACAGATCGCTGA GATAGGTGCCTCACTGATTAAGCATTGGTAAGTGTACAGACCAAGTTTACTCATATATACTTTAGAT TGATTTAAACTTCATTTTTAATTTAAAGGATCTAGGTGAAGATCCTTTTTGATAATCTCATGAC CAAAATCCCTTAACGTGAGTTTTTCGTTCCACTGAGCGTCAGACCCCGTAGAAAAGATCAAAGGAT CTTCTTGAGATCCTTTTTTCTGCGCGTAATCTGCTGCTTGCAAACAAAAAAACCACCGCTACCAG CGGTGGTTTGTGTTGCCGGATCAAGAGCTACCAACTCTTTTTCCGAAGGTAAGTGGCTTCAGCAGAG CGCAGATACCAAATACTGTCCTTCTAGTGTAGCCGTAGTTAGGCCACCACTTCAAGAACTCTGTAG CACCOCCTACATACCTCGCTCTGCTAATCCTGTTACCAGTGGCTGCTGCCAGTGGCGATAAGTCGT GTCTTACCGGGTTGGACTCAAGACGATAGTTACCGGATAAGGCGCAGCGTCCGGGCTGAACGGG GGGTTCTGTGCACACAGCCAGCTTGGAGCGAACGACCTACACCGAACTGAGATACCTACAGCGTG AGCTATGAGAAAGCGCCACGCTTCCCGAAGGGAGAAAGGCGGACAGGTATCCGGTAAGCGGCAG GGTCGGAACAGGAGAGCGCACGAGGGAGCTTCCAGGGGGAAACGCCTGGTATCTTTATAGTCTT GTCGGGTTTTCGCCACCTCTGACTTGAGCGTCGATTTTTGTGATGCTCGTCAGGGGGGGCGGAGCCTA TGGA AAAACGCCAGCAACGCGGCCTTTTACGGTTCCTGGCCTTTTGCTGGCCTTTTGCTCACATG TTCTTTCTGCGTTATCCCCTGATTCTGTGGATAACCGTATTACCGCCTTTGAGTGAGCTGATACCG CTCGCCGACGCCAAGCAGCGCAGCGAGTCAGTGAGCGAGGAAGCGGAAG

Supplementary Table 1 | sgRNA and target DNA sequences used for in vitro cleavage assay.
The target sequence and the PAM sequence are colored red and purple, respectively.

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-1SiJLsJBCEcJR4OgPOq
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-ILtLz19HIVQHr5TfMIUY
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-81iPHzhYIILNSnEmovEI
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-LDFoNP6UZ4QF38e9dSOI?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-Bi2F7IJDdJVzSBAtwUby
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-UrgA0sXhzmz4L8JF1laRu

Supplementary Table 2 | Plasmids used for in this study.

Mutation	Candidate PAM Sites	NGG PAM	NNGRRT PAM	NNNRRT PAM	NNG PAM
G280>A	A G AGATCAGTGTGAGTCCAATCCATG	0	1	3	7
C301>T	TGATGGAGATCAGTGTGAGTCCAAT T	1	0	1	3
G364>A	A G ATTGAAGGAAAGAACTGTGAATT	1	1	1	7
G479>A	AATATCGACTTGCAGAAAACCAGAAG	0	0	0	5
C950>T	ATTAATAAGTACAACCATGACATTG T	1	0	3	4
G1061>A	A T GGCTGGGGAAGAGTCTTCCACAAA	4	1	1	8
G1138>A	A C CACATGTCTTCGATCTACAAAGTT	0	0	2	3
G1300>A	A A GTGTGCAATGAAAGGCAAAATATGG	2	0	3	8

Supplementary Table 3 | Distribution of candidate PAM sequences near pathogenic pointmutations associated with hemophilia B.

Distribution of candidate PAM sequences proximal to pathogenic point mutations associated with hemophilia B. Pathogenic G-to-A substitutions (or C-to-T substitutions on the reverse strand) are highlighted in red within the Candidate PAM Sites column.

Name	Sequence
sgRNA	<u>GGGAAATTAGGTGCGCTTGGC</u> GTTTTAGTACTCTGGAAACAGAATCTACTAAAACAA GGCAAAATGCCGTGTTTATCTCGTCAACTTGTTGGCGAGAT
Target strand	ATTGAATAGGCAAGC* <u>C*A*A*G*C*G</u> *CACCTAATTTCCCGTATTAG
Non-target strand	CTAAATACGGGAAATTAGGTGC* <u>G*C*T*T*G*G</u> *CTTGCCTATTCAAAT

Supplementary Table 4 | sgRNA and target DNA sequence used for cryo-EM analysis.

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.

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 <i>B</i> 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 <i>B</i> 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
Ramachandran plot						
Favored (%)	95.96	95.87	94.76	95.09	95.10	94.99
Allowed (%)	4.04	4.00	5.24	4.91	4.90	5.01
Outliers (%)	0.00	0.12	0.00	0.00	0.00	0.00

Supplementary Table 5 | Data collection, processing, model refinement, and validation.