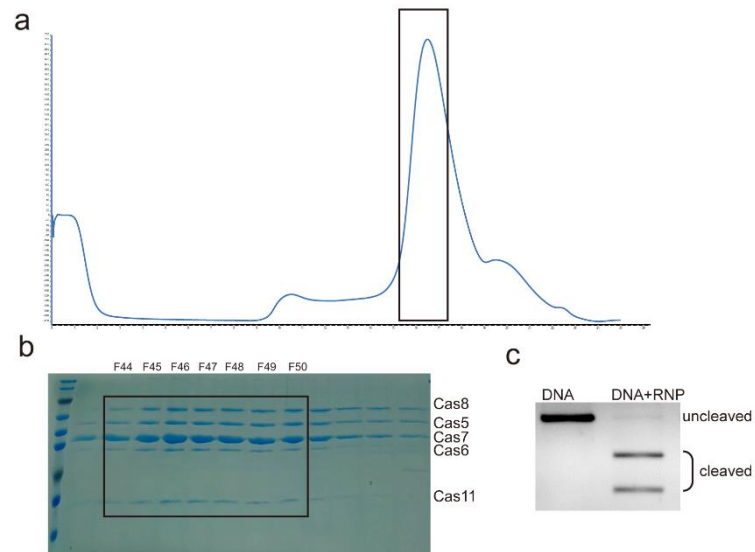
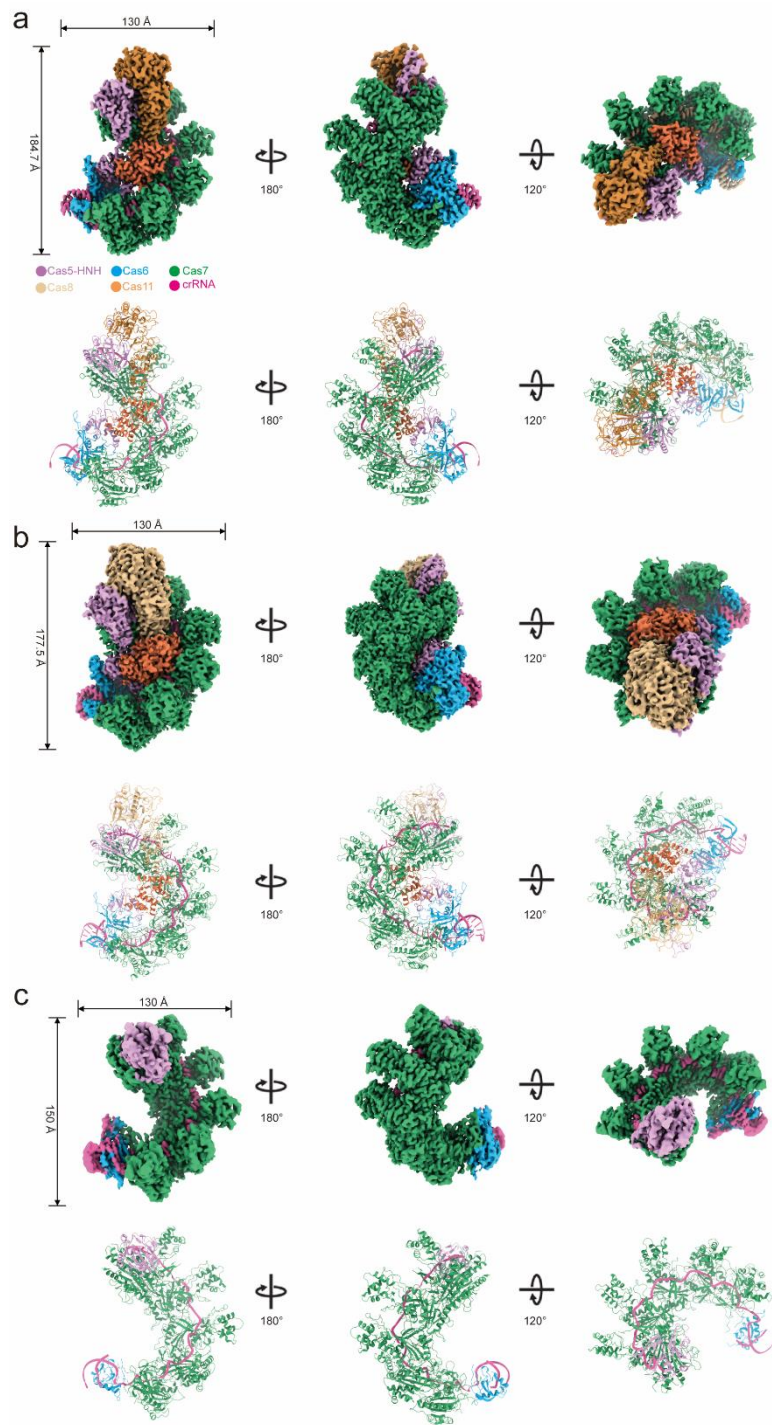
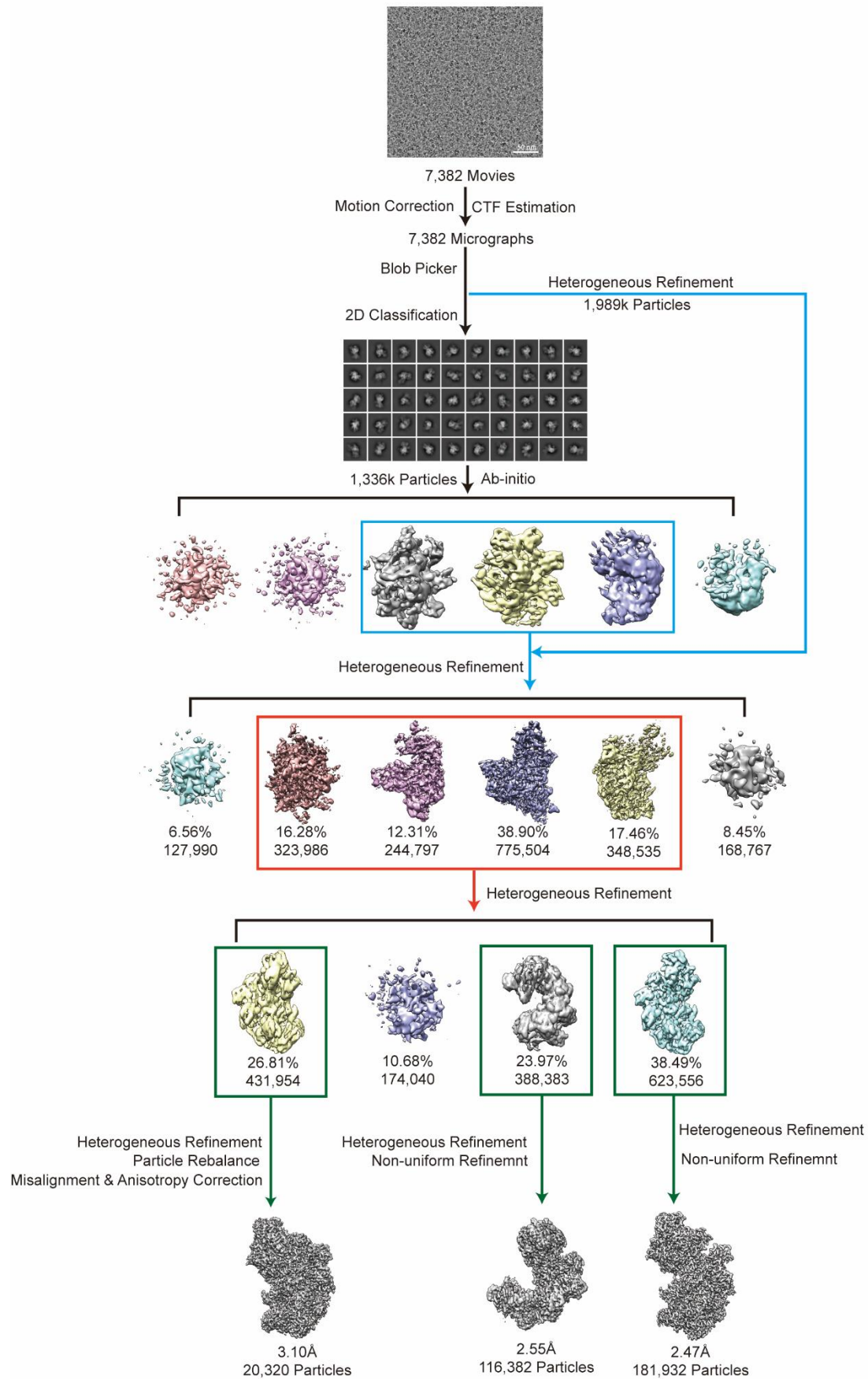




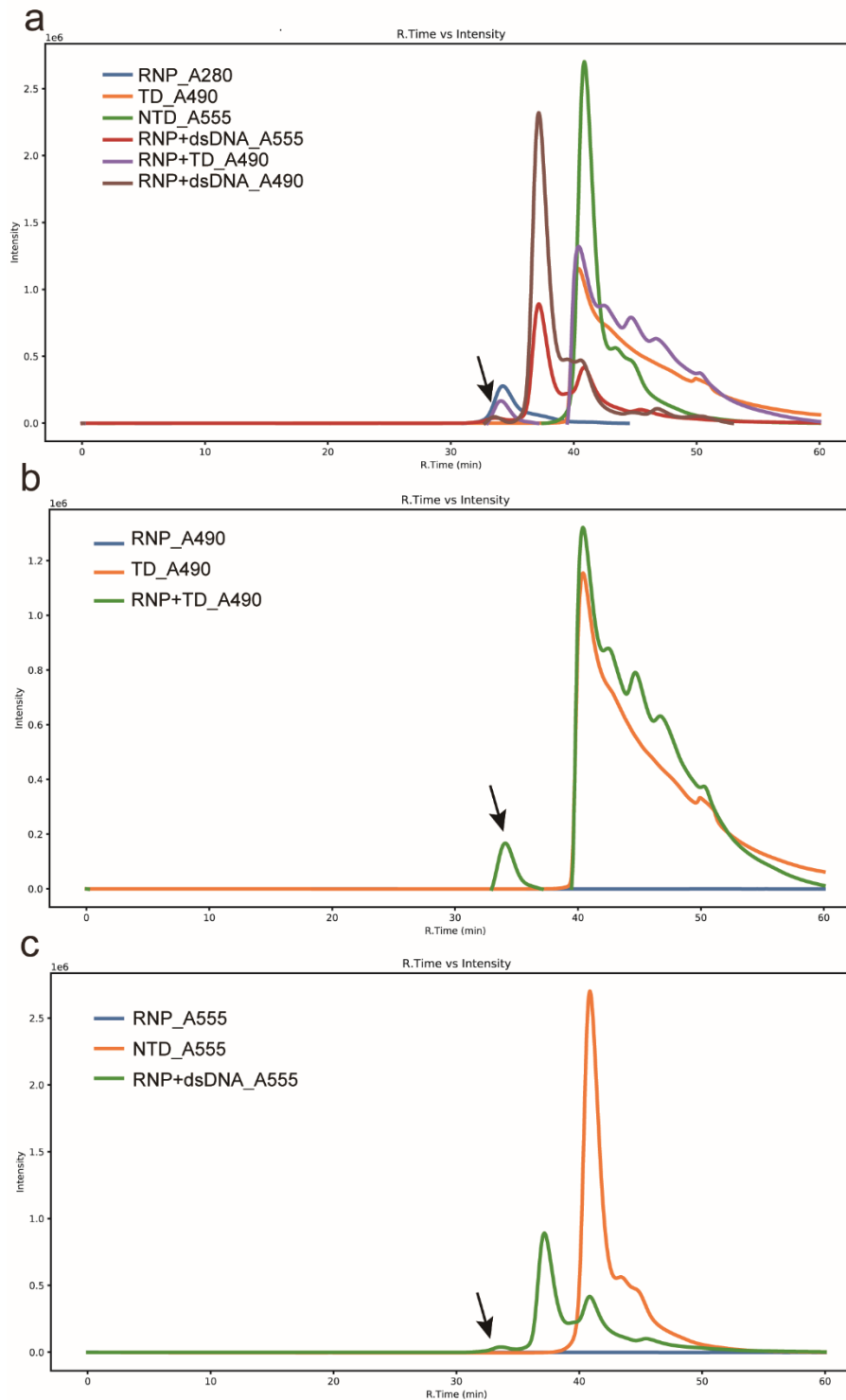
Supplementary Fig. 1 Purification of Cas5-HNH/Cascade complex. **a**, The SEC trace showed a mono dispersed sample. **b**, The components of the SEC-peak were confirmed by SDS-PAGE. **c**, The cleavage activity of wild-type complex was verified.



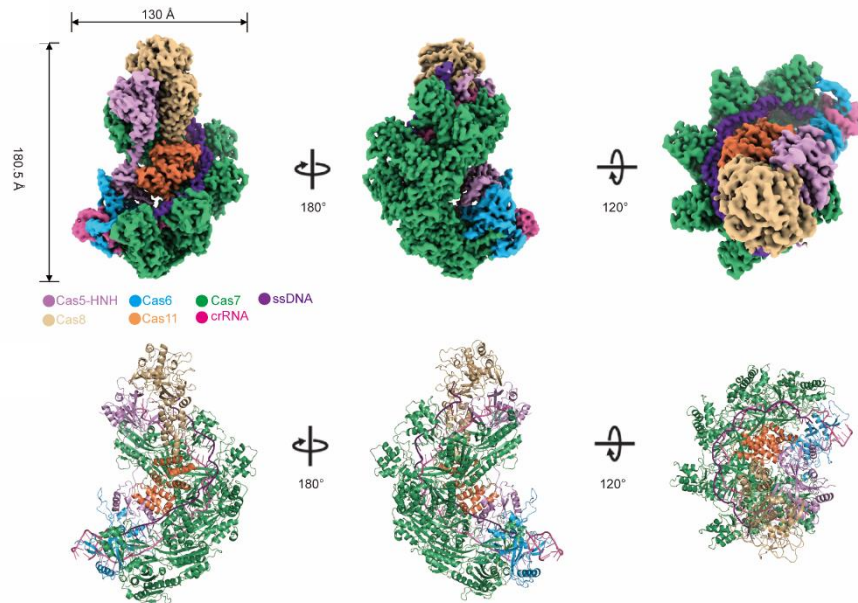
Supplementary Fig. 2 Cryo-EM maps and model of apo complex. Structure of apo_Conf1 (a), apo_con2 (b) and apo_Conf3 (c). The color code is the same to Fig.1.



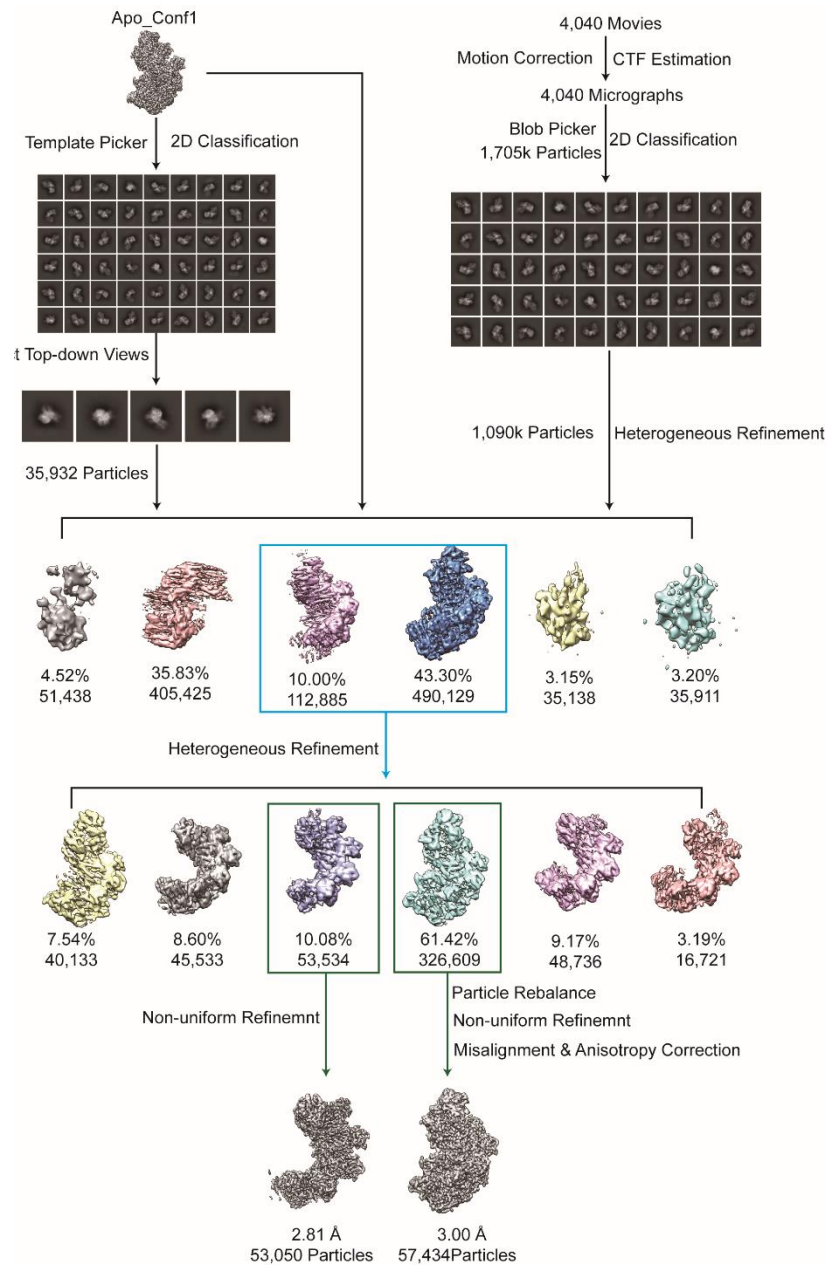
Supplementary Fig. 3 Flow chart for apo-Cas5-HNH/Cascade complex.



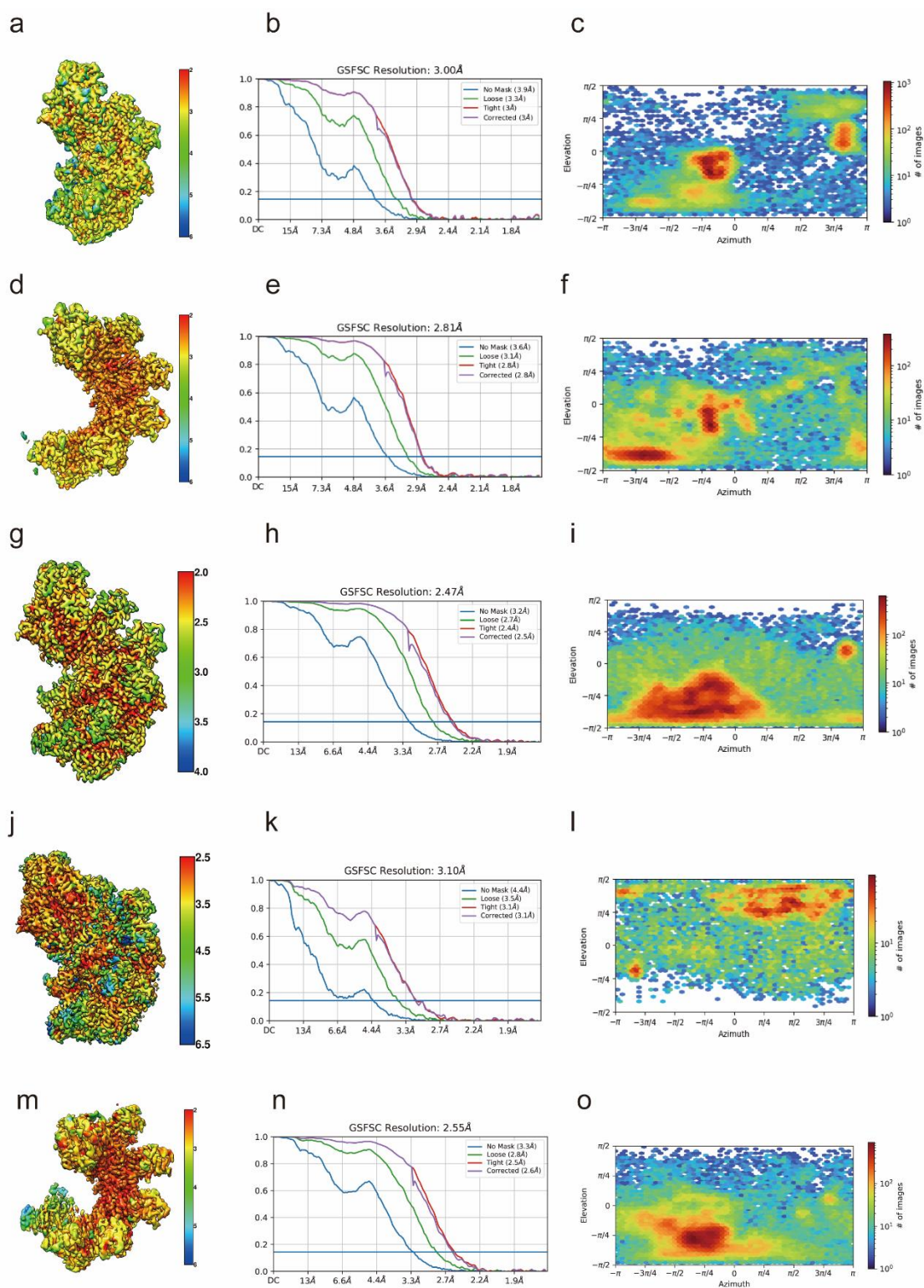
Supplementary Fig. 4 FSEC analysis showed that Cas5-HNH/Cascade complex can bind to both ssDNA (**a**, **b**) and dsDNA (**a**, **c**). The arrow marks the peak of the complex. The target strand was labeled with FAM (absorbance at 490 nm), while the non-target strand was labeled with Cy3 (absorbance at 555 nm). Source data are provided as a Source Data file.



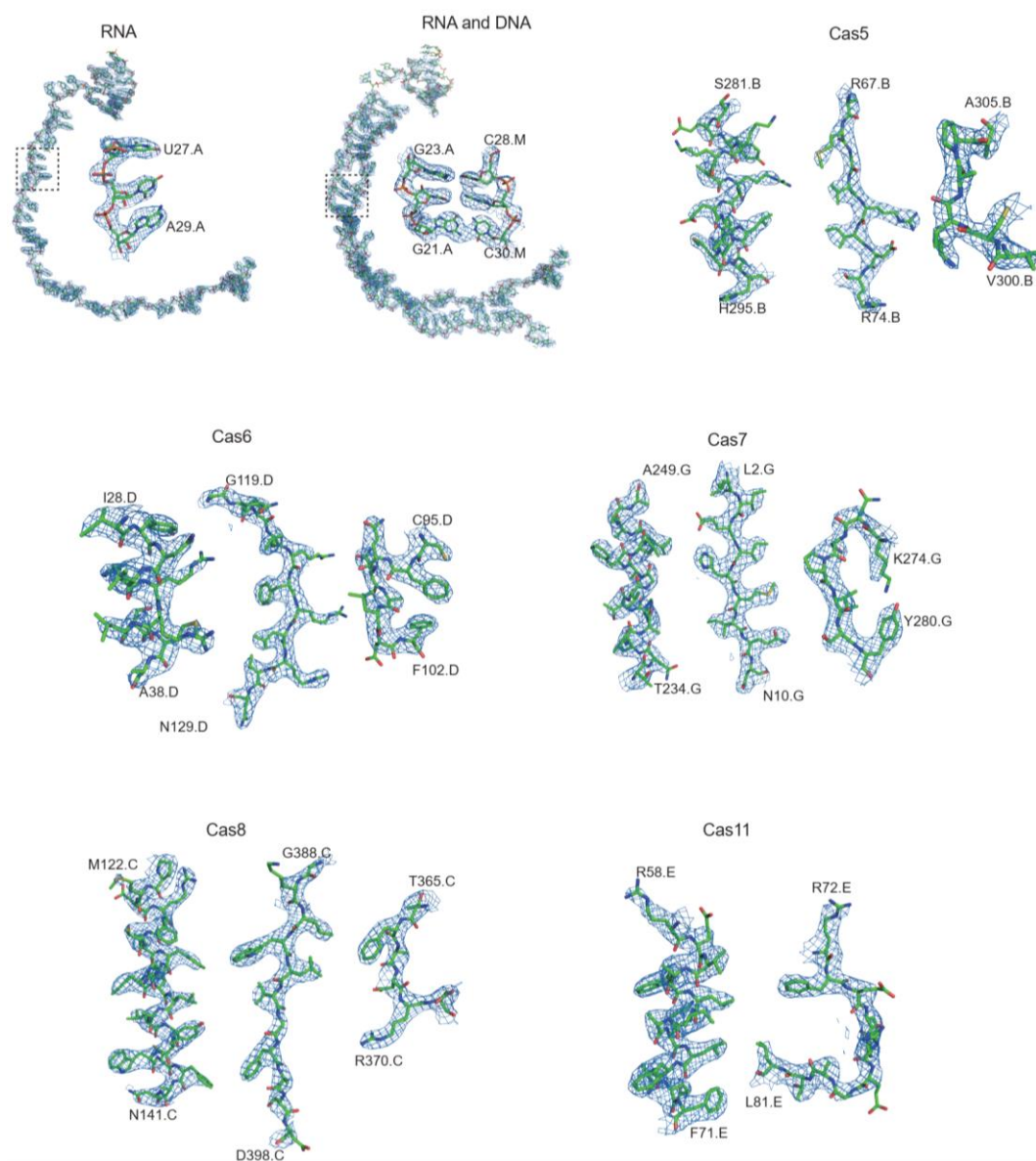
Supplementary Fig. 5 Cryo-EM maps and model of the complex bound to ssDNA, with the ssDNA colored in purple.



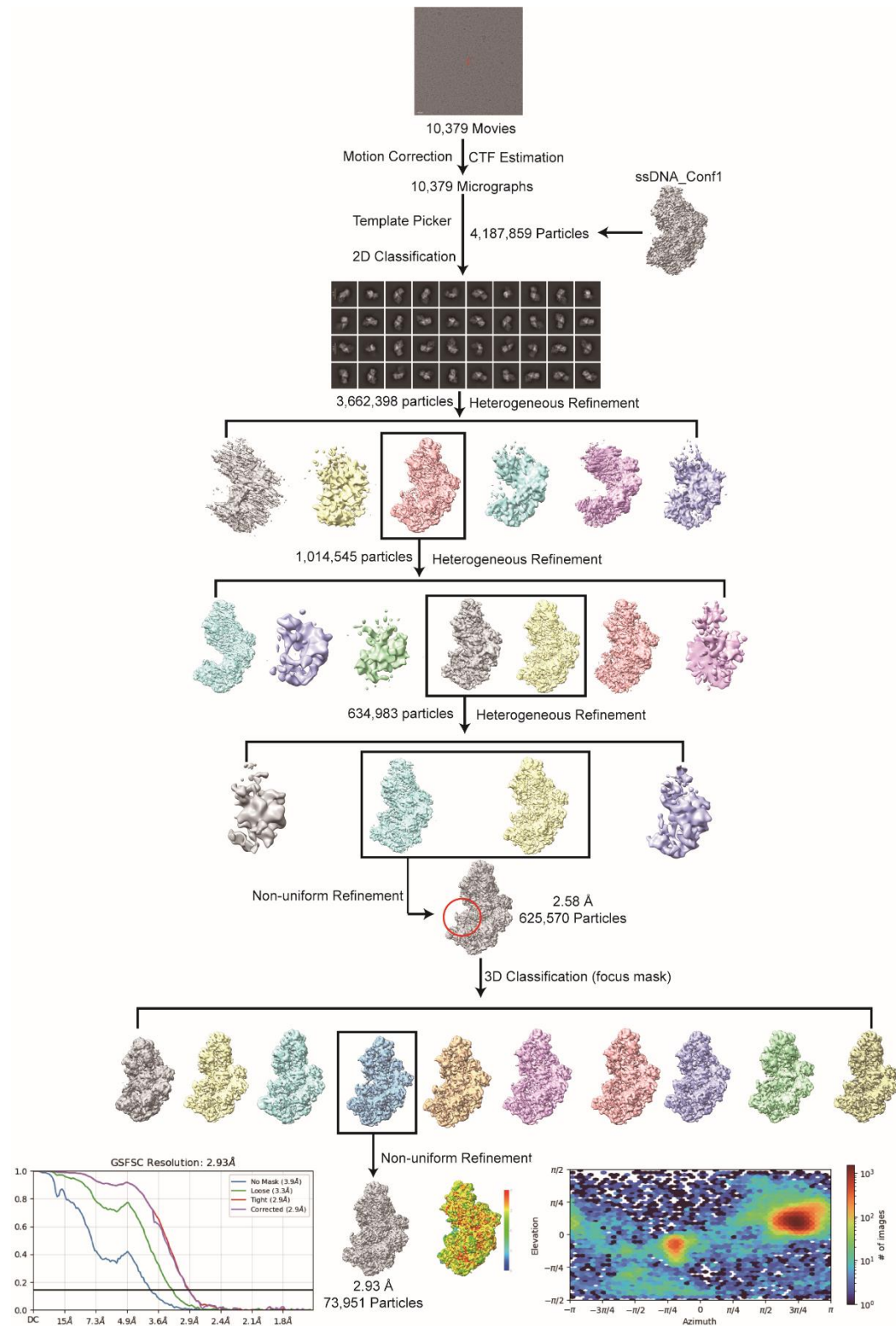
Supplementary Fig. 6 Flow chart for Cas5-HNH/Cascade complex bound with target ssDNA.



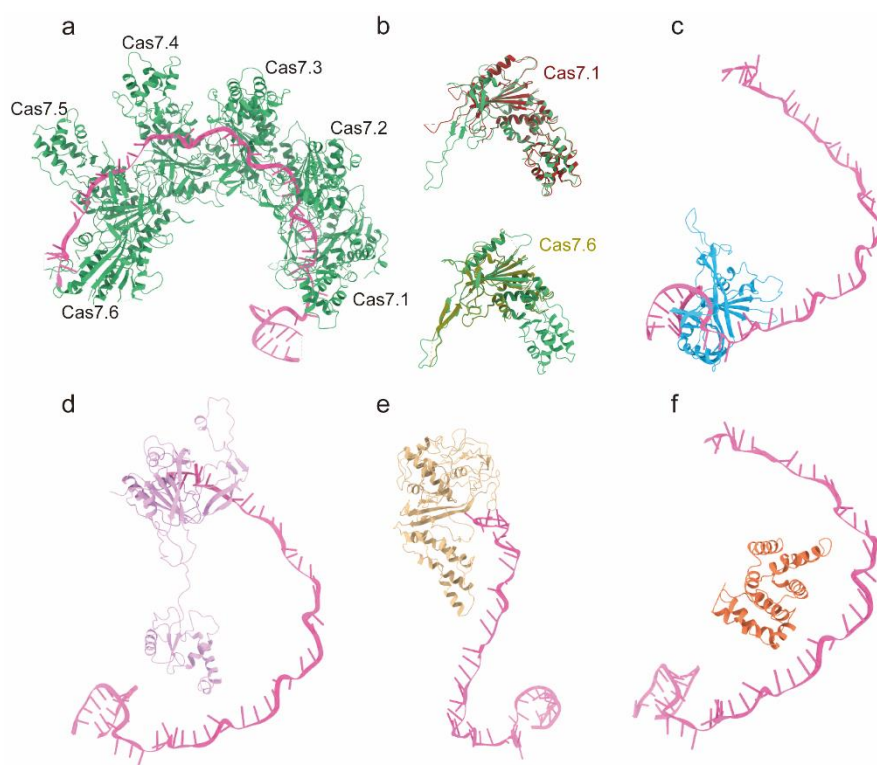
Supplementary Fig. 7 3D reconstruction of the cryo-EM maps. Local resolution, FSC curves, and particle distributions for intact Cas5-HNH/Cascade complex with target ssDNA (**a-c**), incomplete complex with ssDNA (**d-f**), apo_Conf1(**g-i**), compact apo_Conf2 (**j-l**), and incomplete apo_Conf3 (**m-o**), respectively.



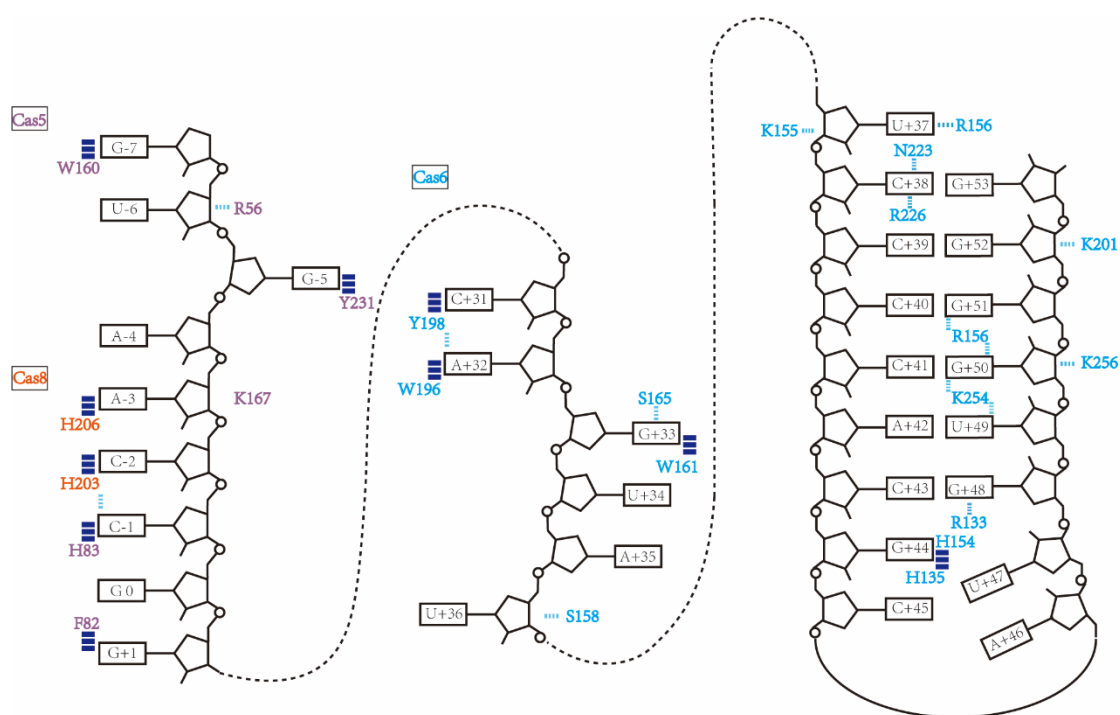
Supplementary Fig. 8 Representative densities of nucleotides and subunits.

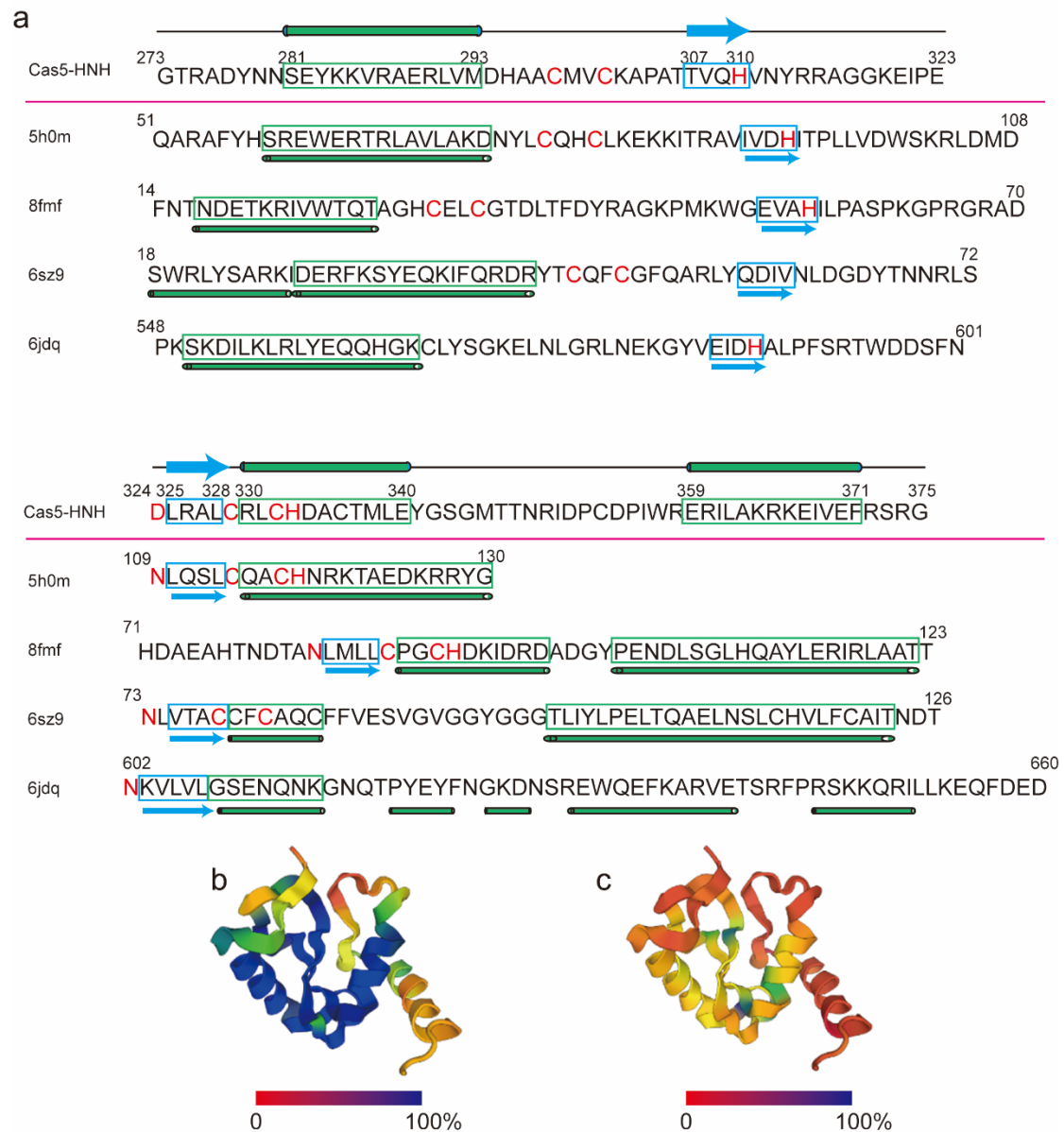


Supplementary Fig. 9 Flow chart for Cas5-HNH/Cascade complex bound with target dsDNA (dsDNA_Conf).

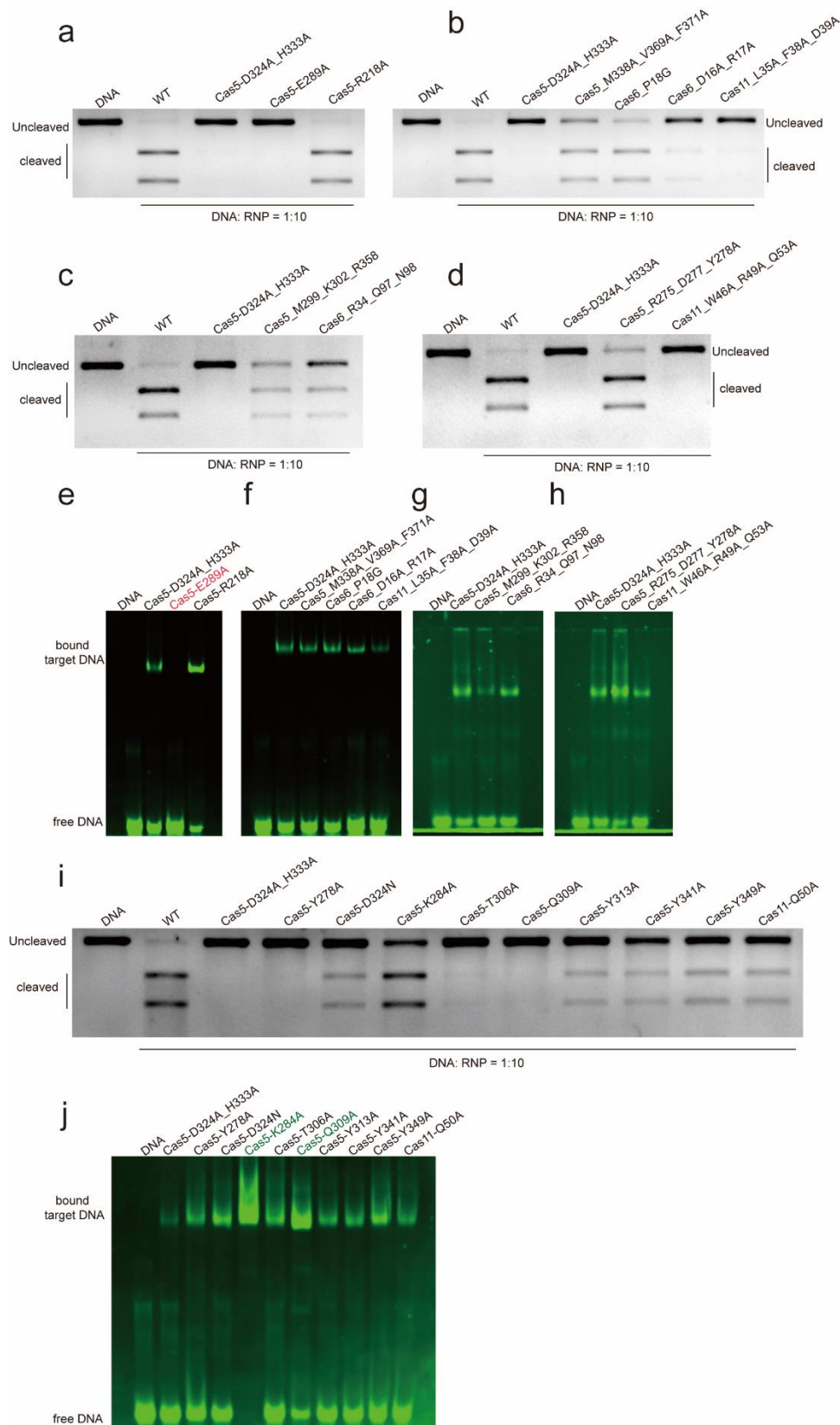


Supplementary Fig. 10 Subunits in Cas5-HNH/Cascade complex. **a**, Six Cas7 subunits assemble with crRNA to form the backbone of the complex. **b**, Comparison of classic Cas7 subunit (e.g. Cas7.5) with Cas7.1 (brown) and Cas7.6 (olive), respectively. **c**, Cas6 is clamped by a step-loop structure of 3'-handle crRNA. **d**, Cas5 caps 5'-handle of the crRNA. **e**, Cas8 is positioned at the 5'-end of crRNA, where it interacts weakly with crRNA. **f**, The Cas11 small subunit is located at the center of the complex, without direct interaction with crRNA.



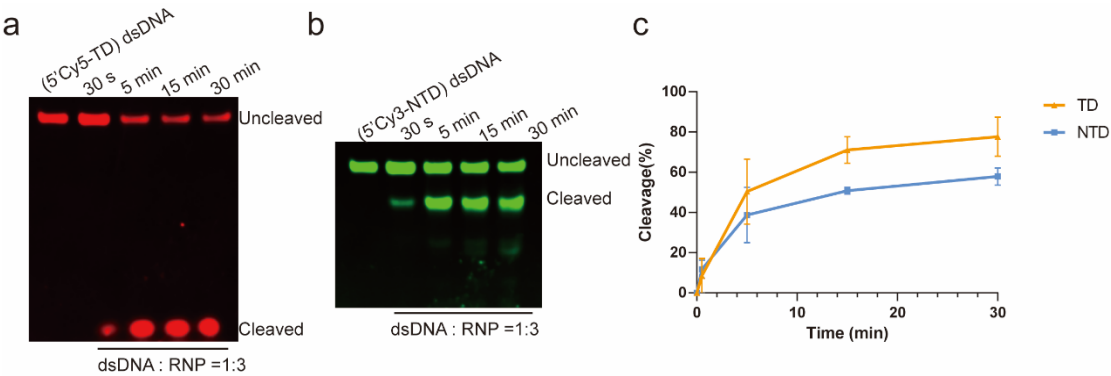


Supplementary Fig. 13 Sequence alignment of HNH domain. **a**, Structural and sequence alignment of the HNH domains of Cas5 with the HNH endonuclease from the deep-sea thermophilic bacteriophage GVE2 (PDB: 5h0m), *Pseudomonas syringae* Cap5 (PDB: 8fmf), the Type IV Coupling Complex (T4CC) from *Legionella pneumophila* (PDB: 6sz9), and the HNH domain of type II nuclease Cas9 (PDB: 6jdq). **b-c**, Diagrams illustrating structural similarities (**b**) and sequence conservation (**c**), where conservation gradually decreases from blue (100%) to red (0).

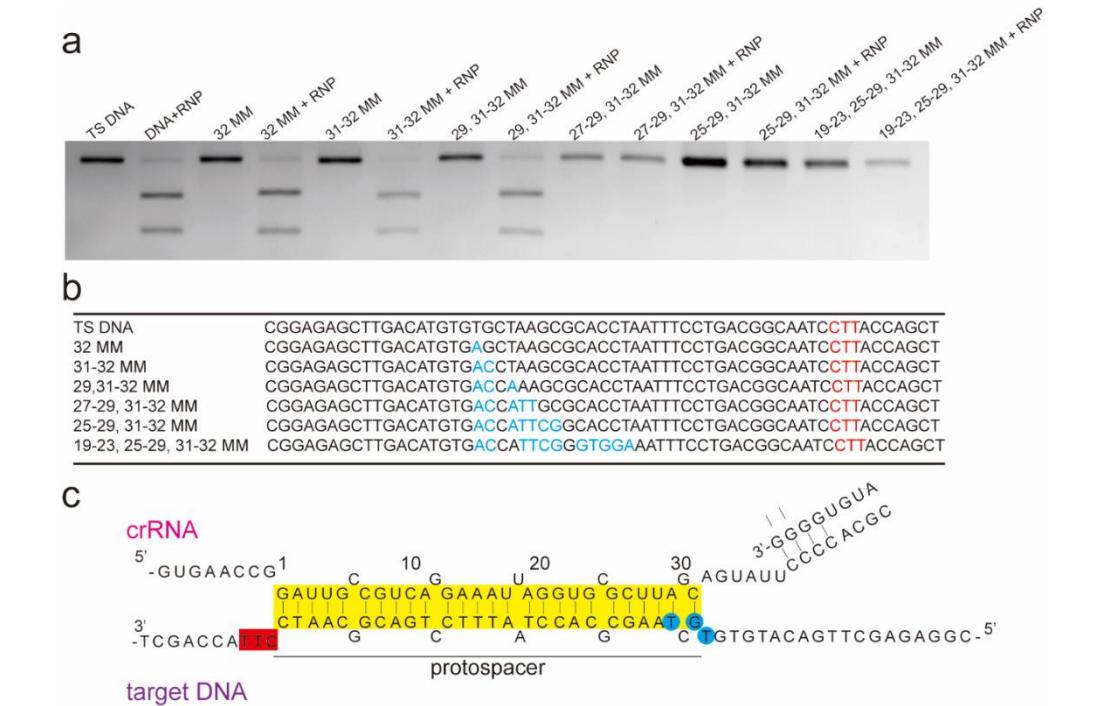


Supplementary Fig. 14 Biochemical analysis for Cas5-HNH/Cascade complex. a-d, Plasmid digestion analysis indicates that mutations in the residues of Cas5-HNH domain affected nuclease activity. **e-h**, EMSA results for the corresponding mutants in (a-d). The red labeled lane represents the mutant that disrupts DNA binding. **i**, Digestion assays shows that mutants of residues associated

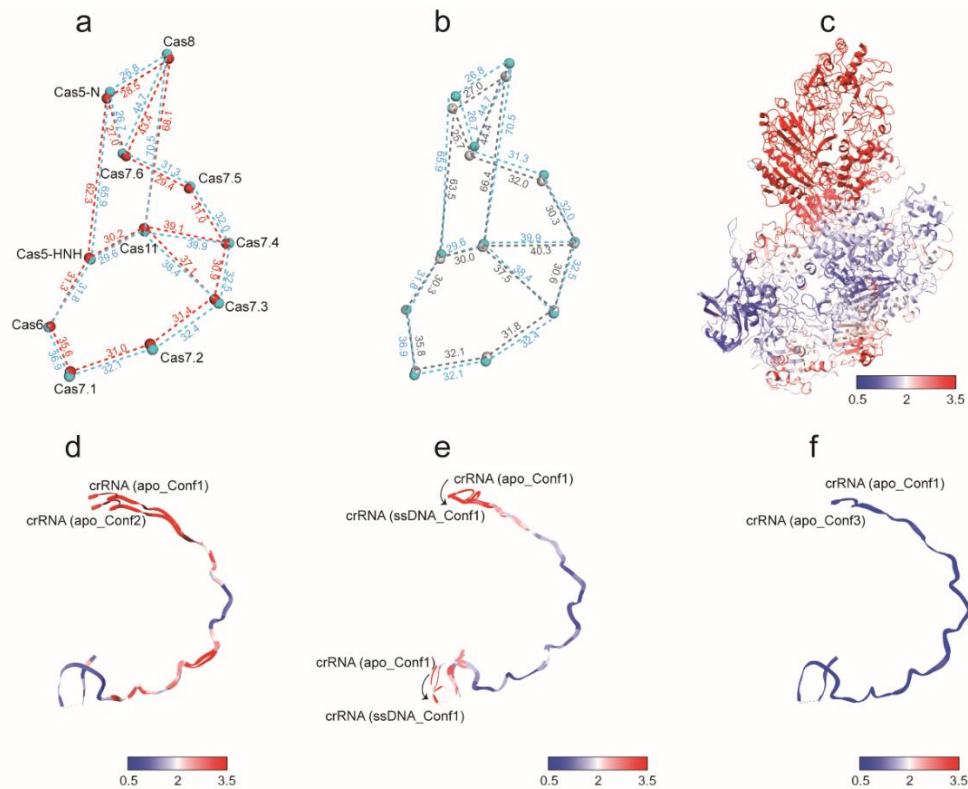
with DNA-binding abolish or reduce the enzyme's function. **j**, EMSA results for the related mutants in **(i)**. Two mutants with increased DNA affinity (K284A and Q309A) are highlighted in green.



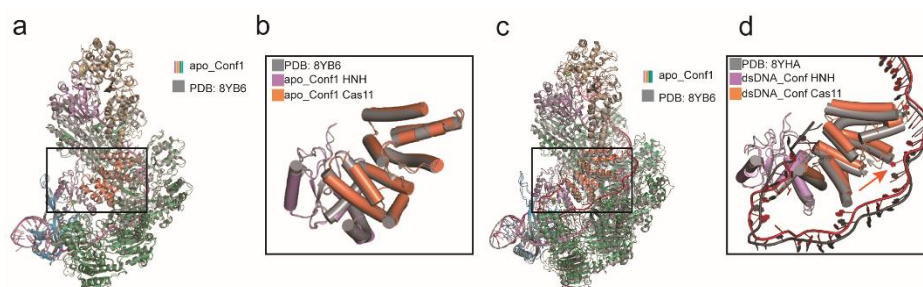
Supplementary Fig. 15 Cleavage order analysis of Cas5-HNH/Cascade complex. **a-b**, EMSA was used to track the cleavage progression over 30 minutes. The target strand DNA (TD) was labeled with Cy5 **(a)** and the non-target strand DNA (NTD) was labeled with Cy3 **(b)** at both 5'-end. **c**, The complex cleaves the TD first, followed by the NTD. Source data are provided as a Source Data file.



Supplementary Fig. 16 Mismatch tolerance of Cas5-HNH/Cascade complex. **a**, In vitro DNA cleavage assay with mismatched substrates (MM: mismatch). **b**, Sequence list of DNA substrates used in **a**, with mismatched nucleotides highlighted in cyan and the PAM sequence noted in red. **c**, Schematic representation of crRNA and target DNA. The spacer region is highlighted by a yellow rectangle, with the PAM sequence shown in red. Mismatches tolerated by the Cas5-HNH/Cascade complex at PAM-distal positions are marked with blue circles.



Supplementary Fig. 17 Comparison of Cas5-HNH/Cascade complexes. **a**, Comparison of the Cas5-HNH/Cascade complex bound to ssDNA (red) and apo_Conf1(cyan) based on their center of mass (COM). Distances between subunits (in Å) are labeled. **b**, Comparison of apo_Conf1 (cyan) and apo_Conf2 (grey), with labeled distances between subunits' COM (in Å). **c**, apo_Conf1 is color-coded by the RMSD values when aligned with apo_Conf2, using crRNA as a reference. **d-f**, Comparison of the crRNA, color-coded by RMSD values: between apo_Conf1 and apo_Conf2 (**d**), between apo_Conf1 and ssDNA_Conf1 (**e**), and between apo_Conf1 and apo_Conf3 (**f**).



Supplementary Fig. 18 Comparison of Cas5-HNH/Cascade complex with published structures. **a-d**, Structural comparison of the target-free Cas5-HNH/Cascade complex (apo_Conf1) and the dsDNA-bound complex (dsDNA_Conf) with published structures available in the PDB under accession numbers 8YB6 and 8YHA, respectively.

Supplementary Table 1

Cryo-EM data collection, refinement and validation statistics.

States	Apo_Conf1	Apo_Conf2	Apo_Conf3	ssDNA_Conf1	ssDNA_Conf2	dsDNA_Conf
Codes	PDB 8ZLU	PDB 8ZM3	PDB 8ZOL	PDB 8ZP7	PDB 8ZP9	PDB 9JXS
	EMD-60233	EMD-60235	EMD-60297	EMD-60328	EMD-60330	EMD-61880
Data collection and processing						
Magnification		165,000			96,000	96000
Voltage (kV)		300			300	300
Electron exposure (e-/Å ²)		60			60	48.62
Defocus range (μm)		-1.8 - -2.5			-1.2 - -2.2	-1.0 - -1.2
Pixel size (Å)		0.83			0.808	0.81
Micrographs (no.)		7,382			4,040	10,379
Symmetry imposed		C1			C1	C1
Total exposure time (s)		3.72			7.82	3.72
Detector		Falcon4			Falcon4	Falcon4
Initial particle images (no.)		1,989,579			1,705,634	4,187,859
Final particle images (no.)	181,932	20,320	127,239	57,434	53,050	73,951
Map resolution (Å)	2.47	3.10	2.55	3.00	2.81	2.93
FSC threshold	0.143	0.143	0.143	0.143	0.143	0.143
Map resolution range (Å)	2.0-4.0	2.5-6.5	2.0-6.0	2.0-6.0	2.0-6.0	2.0-6.0
B factor range (Å ² ;	16.34 -	22.26 -	6.79 -	45.6 -	20.34 -	35.42 -
	113.94	107.09	293.26	263.6	195.57	304.25
Refinement						
Initial model used	N/A	N/A	N/A	N/A	N/A	N/A
Map correlation coefficient	0.81	0.75	0.70	0.85	0.80	0.89
Model composition						
Non-hydrogen atoms	27,059	26,952	19,552	27,640	19,299	28,057
Protein residues	25,799	25,758	18,292	25,711	17,749	25,656
Ligands	2	2	2	2	2	2
RNA	1,258	1,192	1,258	1,258	879	1258
DNA	0	0	0	669	669	1141
R.m.s. deviations						
Bond lengths (Å)	0.002	0.011	0.006	0.006	0.004	0.007
Bond angles (°)	0.558	1.264	0.712	1.051	0.803	1.179
Validation						
MolProbity score	2.26	1.96	1.97	2.47	2.28	2.64
Clash score	5.89	8.34	9.20	9.85	7.30	11.13
Poor rotamers (%)	4.29	0.56	0.21	3.91	3.35	5.13
Ramachandran plot						
Disallowed (%)	0.21	0.12	0.26	0.31	0.13	0.21
Allowed (%)	6.99	8.37	7.54	8.27	6.95	9.50
Favored (%)	92.79	91.51	92.20	91.43	92.92	90.29