

Structural insights into C-terminus-mediated RNA target cleavage by a mesophilic prokaryotic Argonaute

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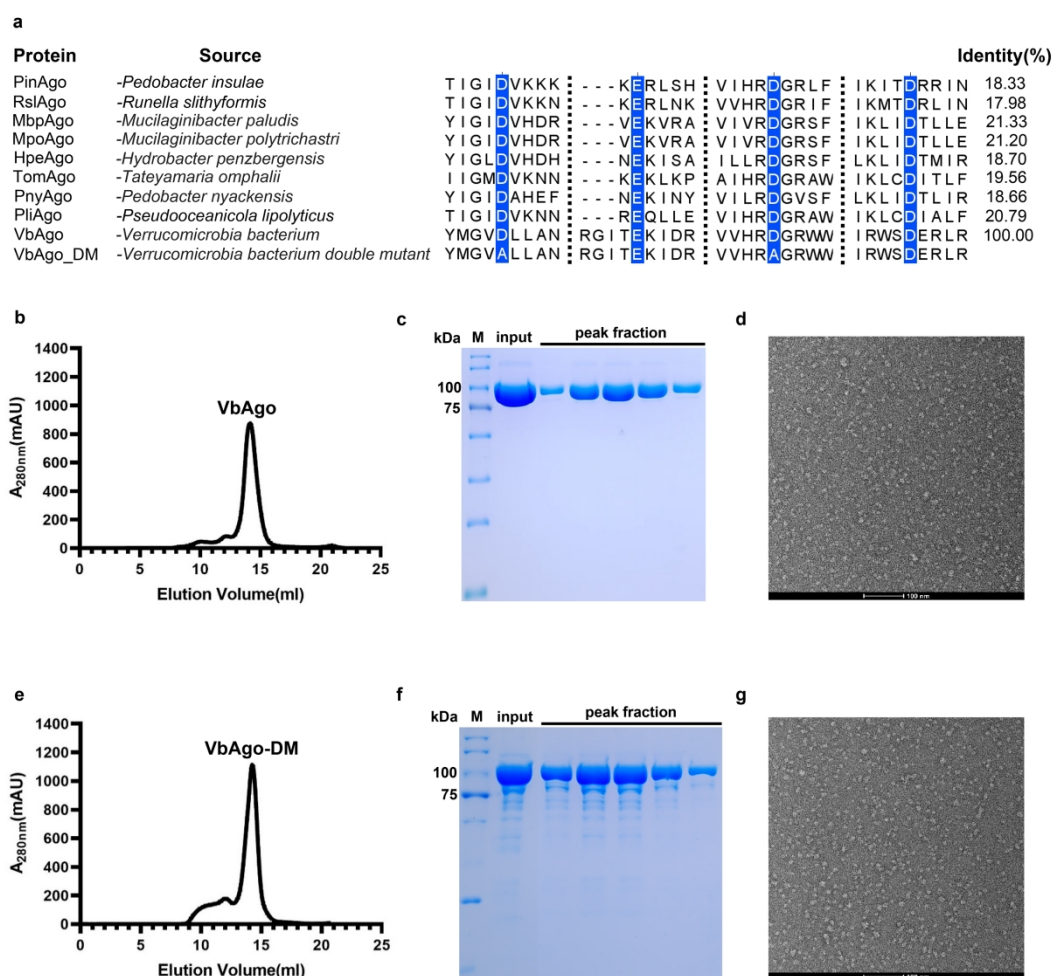
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The supplementary information of this article includes totally 19 supplementary
figures and 2 supplementary tables.

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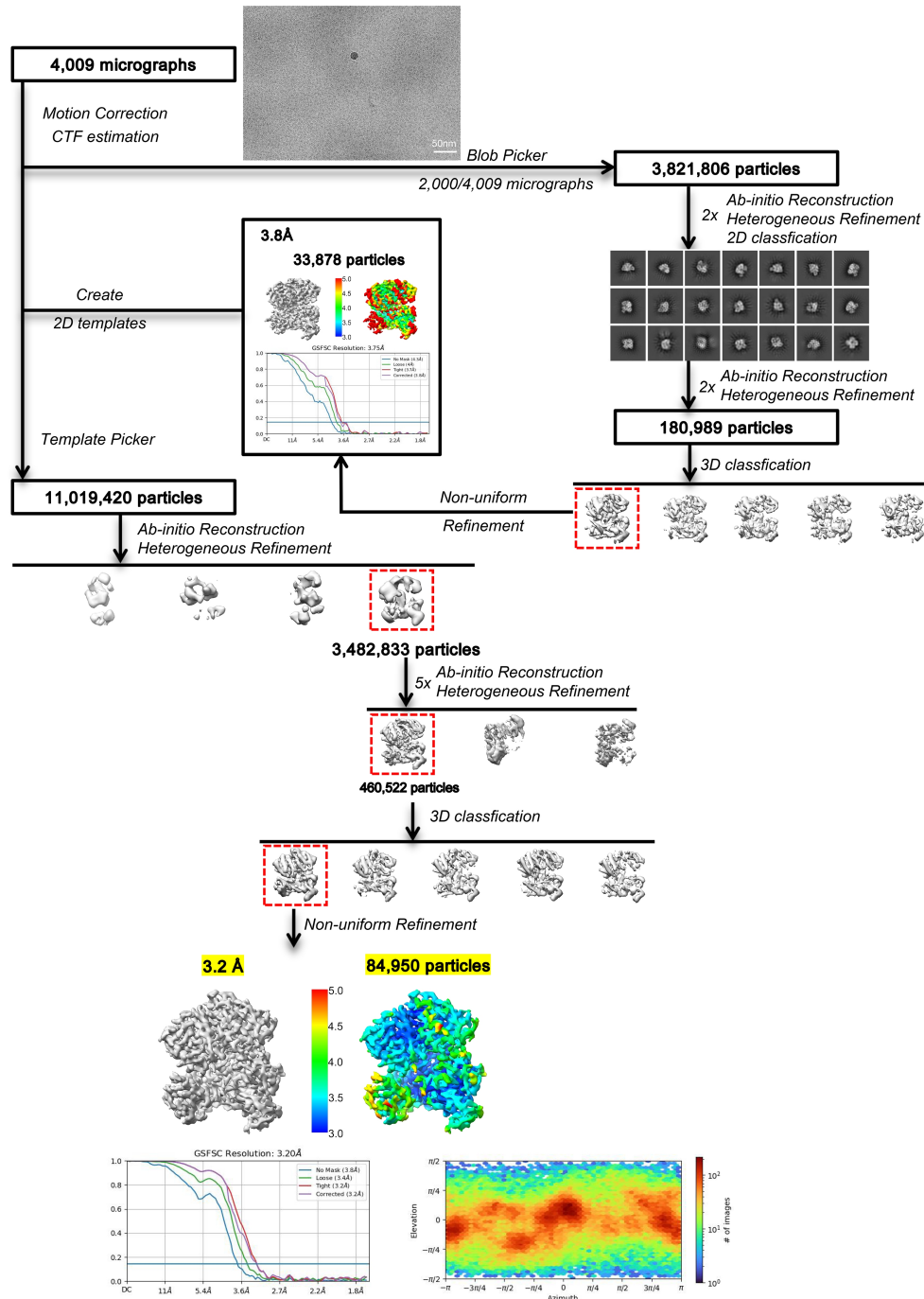
Supplementary Table 1 in page 26

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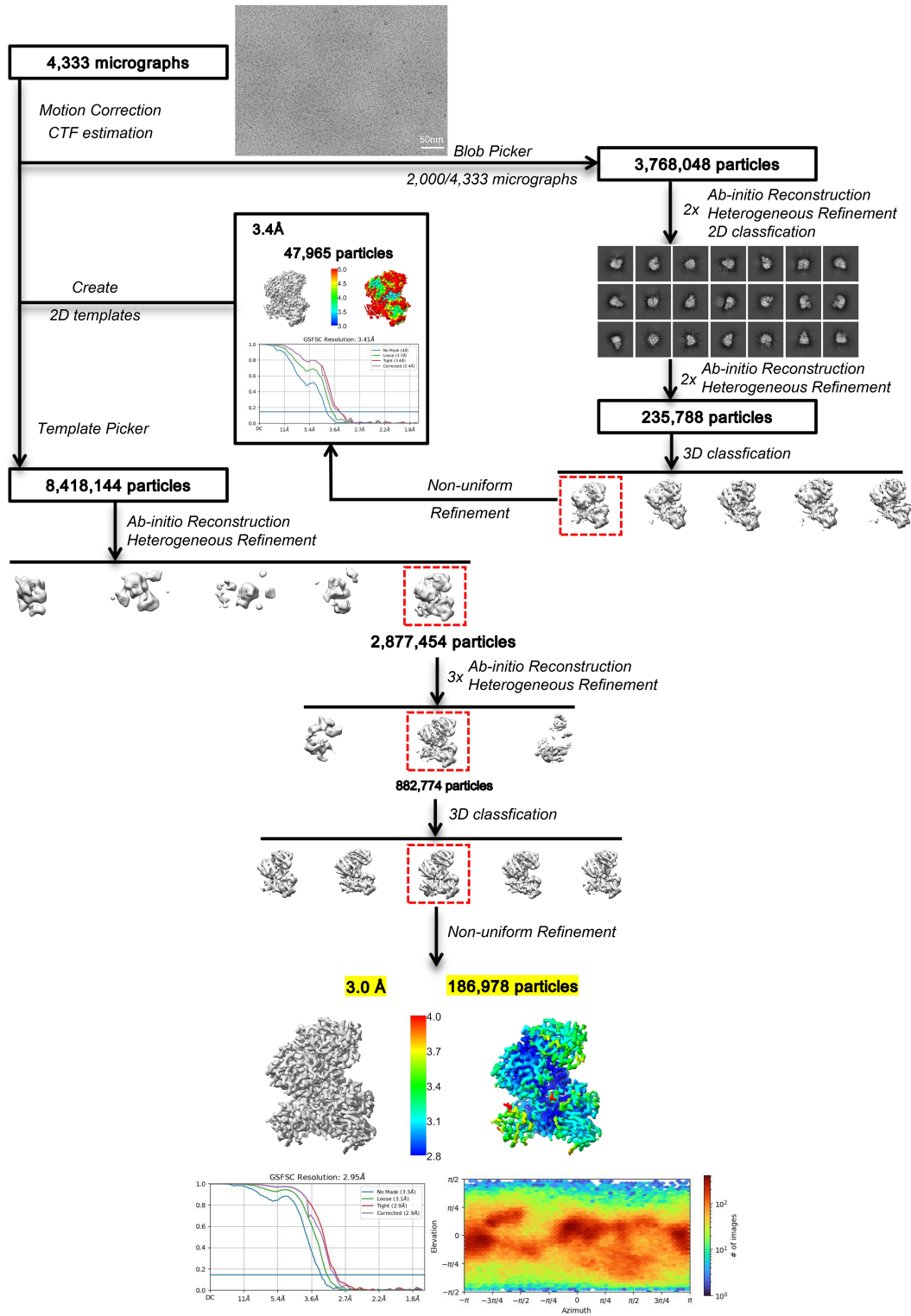
Supplementary Figure 1. Sequence analysis and cryo-EM sample preparation for VbAgo and VbAgo-DM.

(a) Multiple sequence alignment of VbAgo and other gDtr-pAgos, with conserved catalytic residues highlighted in a blue background. VbAgo-DM, the catalytically inactive mutant, D550A and D617A. The information is provided in Supplementary Datasets 1 and 2. (b-g) Purification of VbAgo and VbAgo-DM electron microscopy samples. Size-exclusion chromatography of VbAgo on Superdex 200 increase 10/300 GL (b), SDS-PAGE analysis (c) and negative staining of the final sample (d). Size-exclusion chromatography of VbAgo-DM on Superdex 200 increase 10/300 GL (e), SDS-PAGE analysis (f) and negative staining of the final sample (g). Source data are provided as a Source Data file.



Supplementary Figure 2. Structure determination of VbAgo_{apo}.

The VbAgo_{apo} image processing flowchart includes a representative raw cryo-EM micrograph and corresponding 2D class averages. The local resolution estimation of the final cryo-EM density, along with the associated resolution and the number of particles, is highlighted with a yellow background. Additionally, the angular distribution of the reconstruction and the Fourier shell correlation (FSC) plots surrounding the final cryo-EM density are presented.

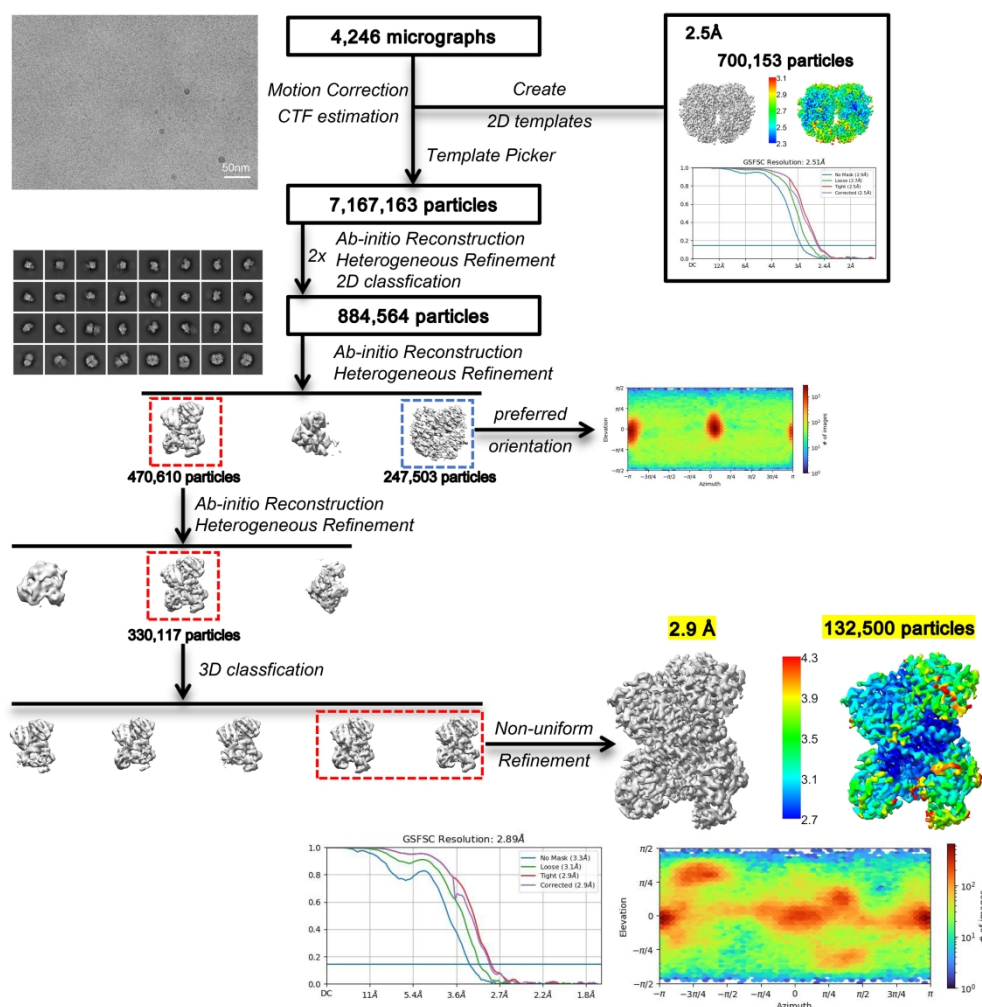


Supplementary Figure 3. Structure determination of VbAgo_{gDNA}.

The VbAgo_{gDNA} image processing flowchart includes a representative raw cryo-EM micrograph and corresponding 2D class averages. The local resolution estimation of the final cryo-EM density, along with the associated resolution and the number of

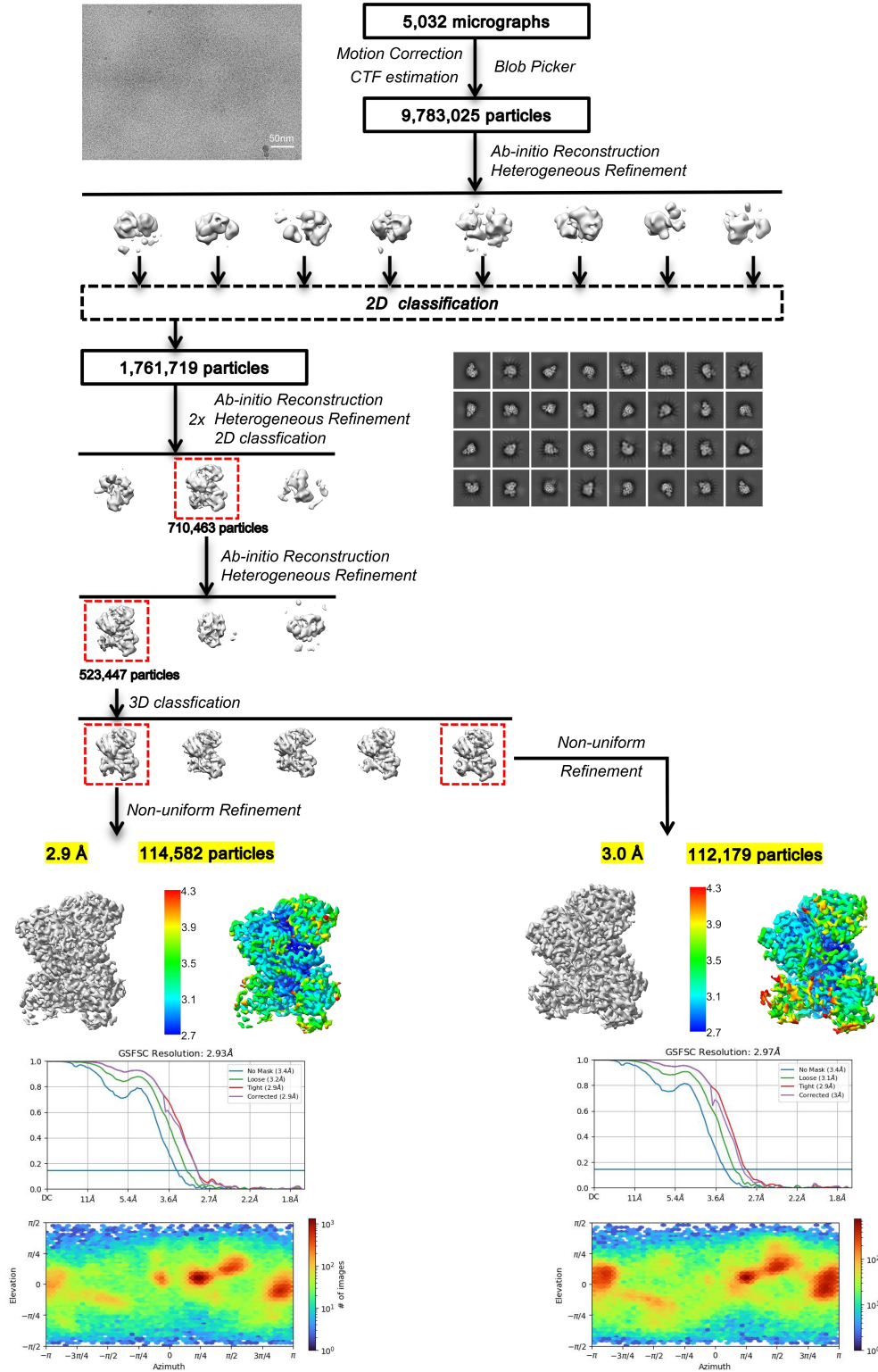
particles, is highlighted with a yellow background. Additionally, the angular distribution of the reconstruction and the Fourier shell correlation (FSC) plots surrounding the final cryo-EM density are presented.

surrounding the final cryo-EM density are presented.



Supplementary Figure 5. Structure determination of the VbAgo-gDtR formed after 10-minute incubation with target RNA.

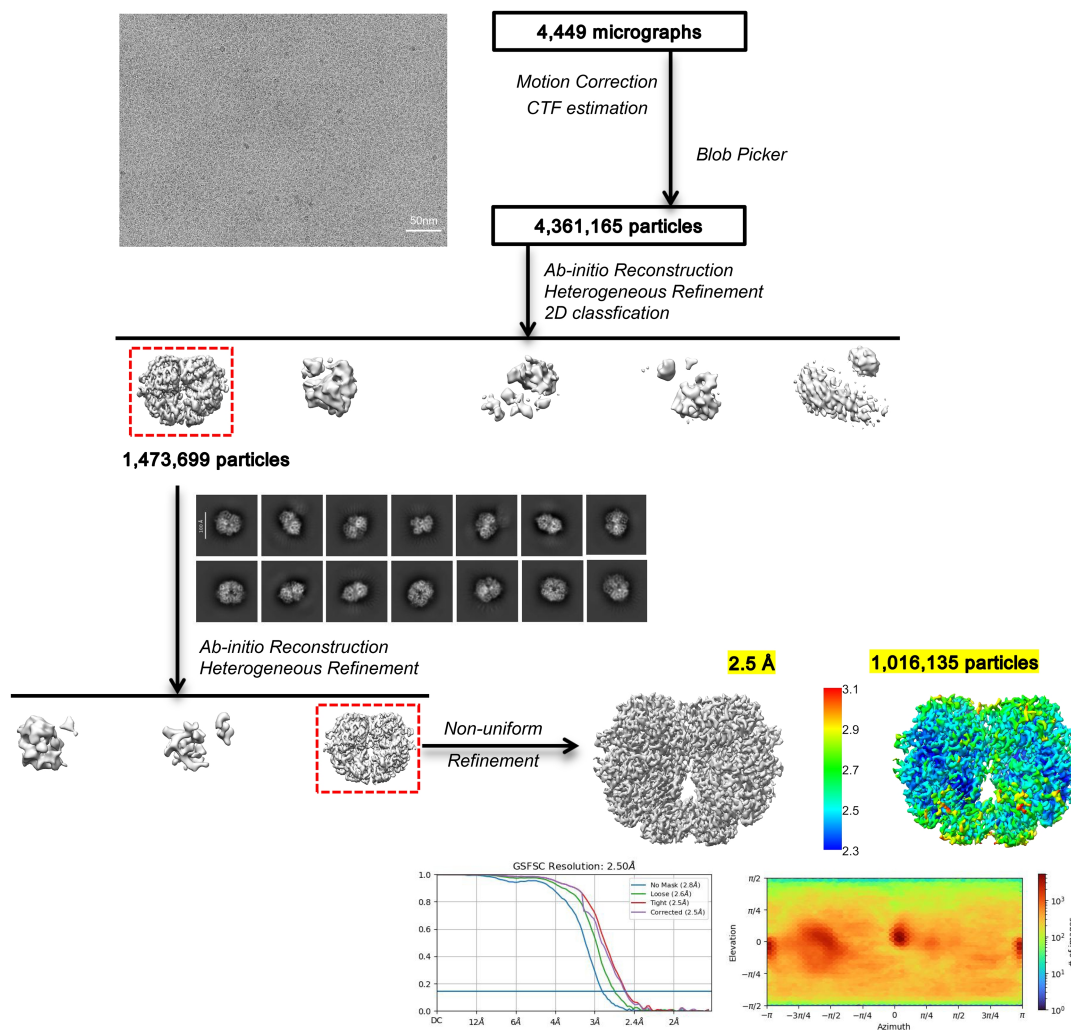
The 10-minute dataset image processing flowchart includes a representative raw cryo-EM micrograph and corresponding 2D class averages. The local resolution estimation of the final cryo-EM density, along with the associated resolution and the number of particles, is highlighted with a yellow background. Additionally, the angular distribution of the reconstruction and the Fourier shell correlation (FSC) plots surrounding the final cryo-EM density are presented.



Supplementary Figure 6. Structure determination of the VbAgo-gDtr formed after 40-minute incubation with target RNA.

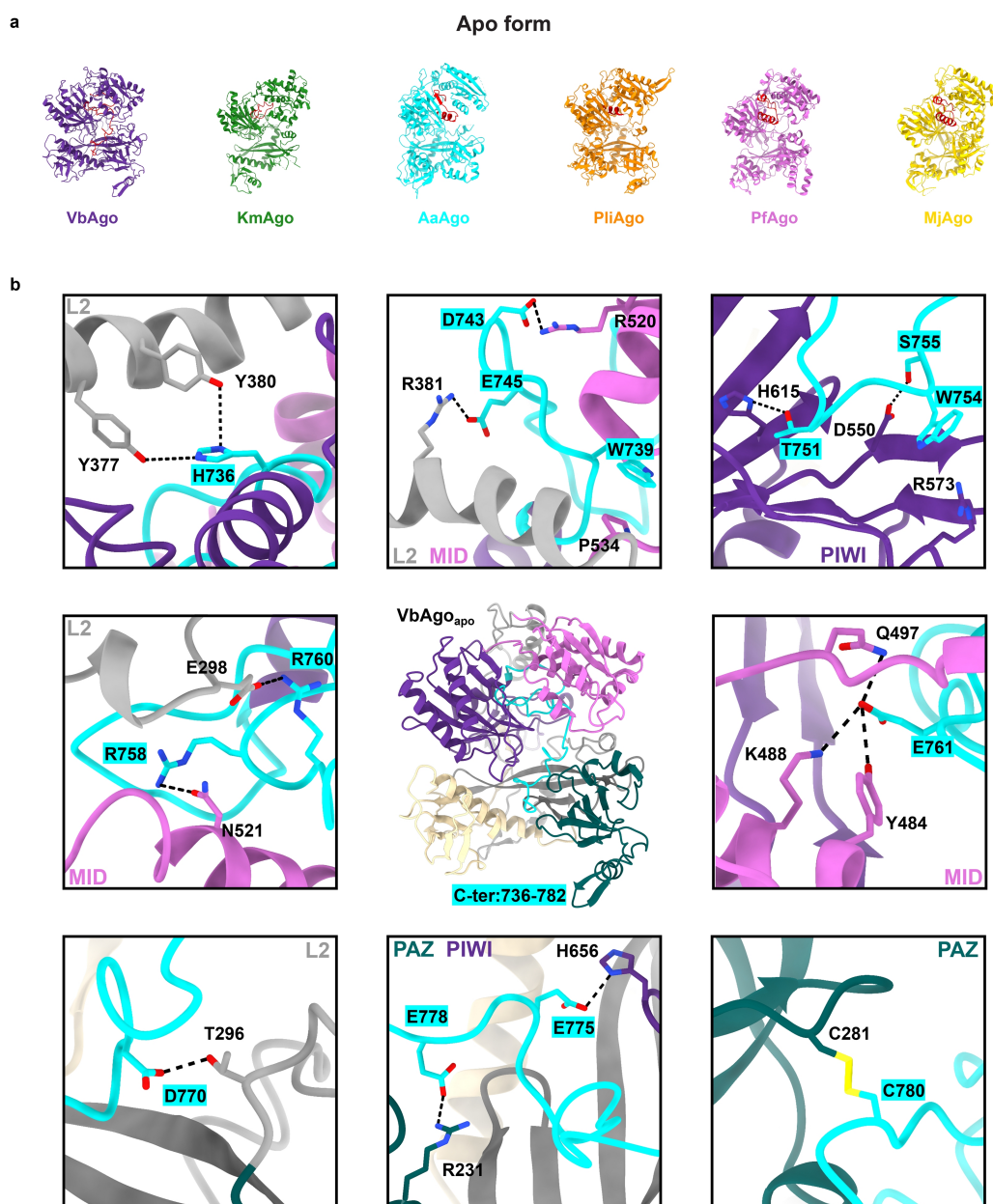
The 40-minute dataset image processing flowchart includes a representative raw cryo-EM micrograph and corresponding 2D class averages. The local resolution

estimation of the final cryo-EM density, along with the associated resolution and the number of particles, is highlighted with a yellow background. Additionally, the angular distribution of the reconstruction and the Fourier shell correlation (FSC) plots surrounding the final cryo-EM density are presented.



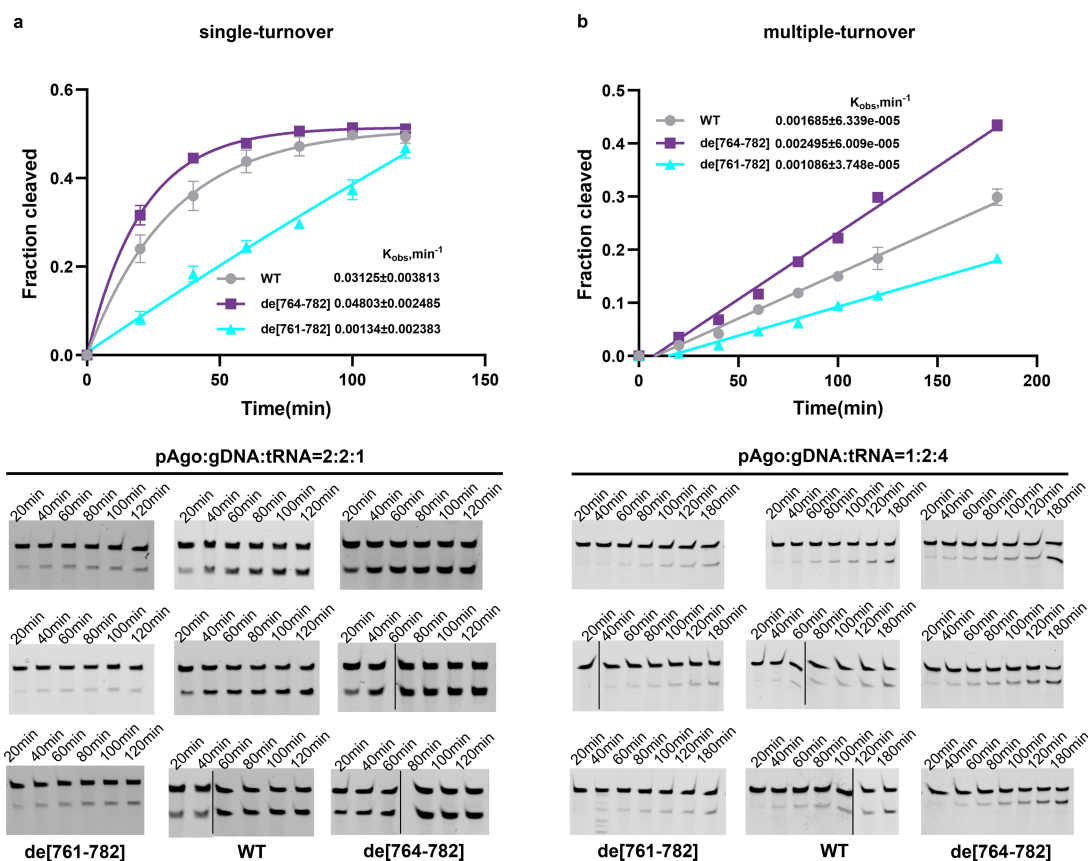
Supplementary Figure 7. Structure determination of dimer-VbAgo-DM_{gDfR}.

The dimer-VbAgo-DM_{gDfR} image processing flowchart includes a representative raw cryo-EM micrograph and corresponding 2D class averages. The local resolution estimation of the final cryo-EM density, along with the associated resolution and the number of particles, is highlighted with a yellow background. Additionally, the angular distribution of the reconstruction and the Fourier shell correlation (FSC) plots surrounding the final cryo-EM density are presented.



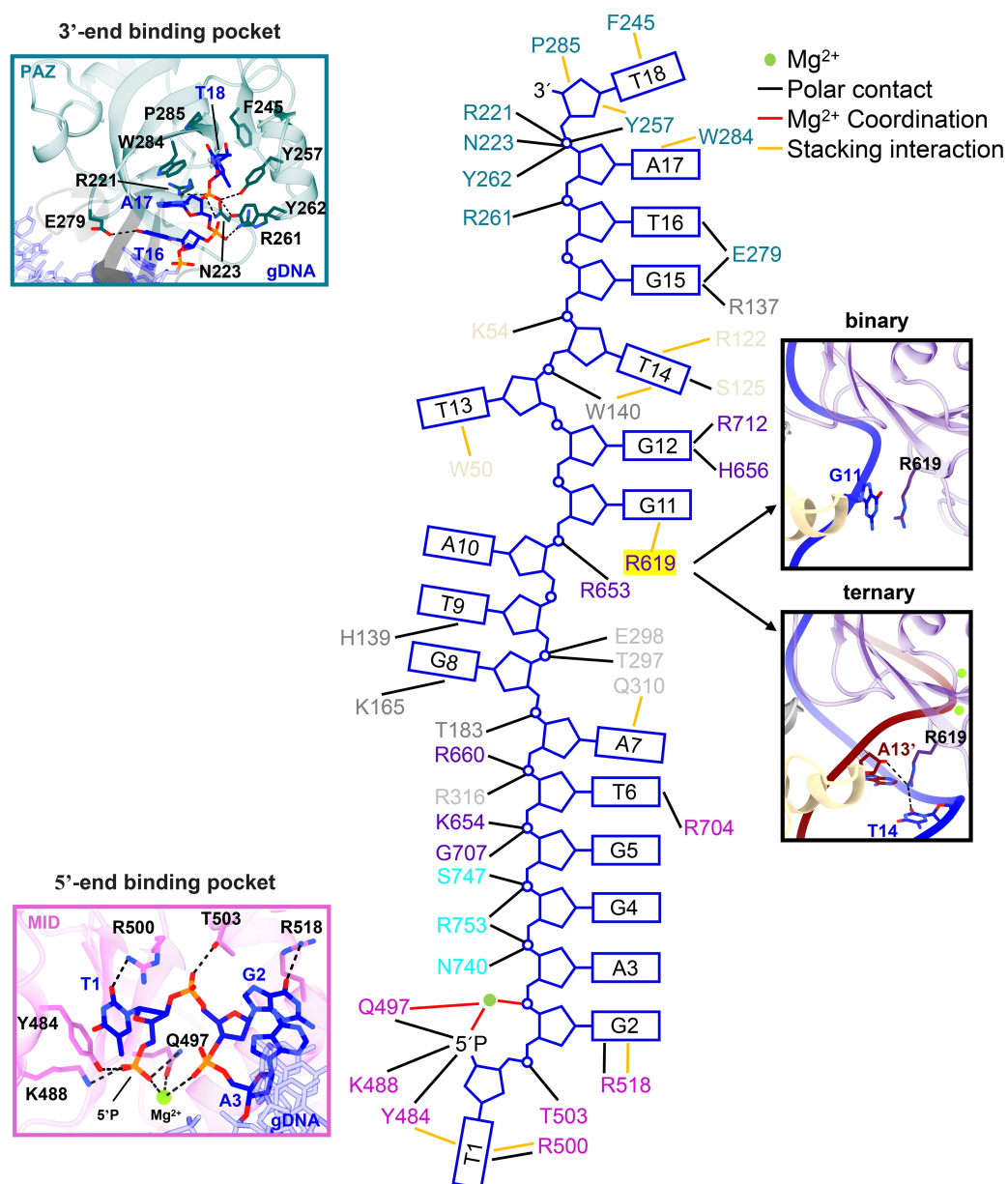
Supplementary Figure 8. Unique C-terminus in the VbAgo_{apo} state.

(a) Structural comparison of the apo forms of VbAgo, KmAgo (PDB: 8HXS), AaAgo (PDB: 1YVU), PliAgo (PDB: 7R8F), PfAgo (PDB: 1U04), and MjAgo (PDB: 5G5S). The structures were superimposed, focusing on their C-terminal regions, which are highlighted in red. (b) Schematic representation of the interactions between the C-terminus and other domains in the VbAgo_{apo} state; The atomic model is located at the center, and the color-coding corresponds to Fig.1a.

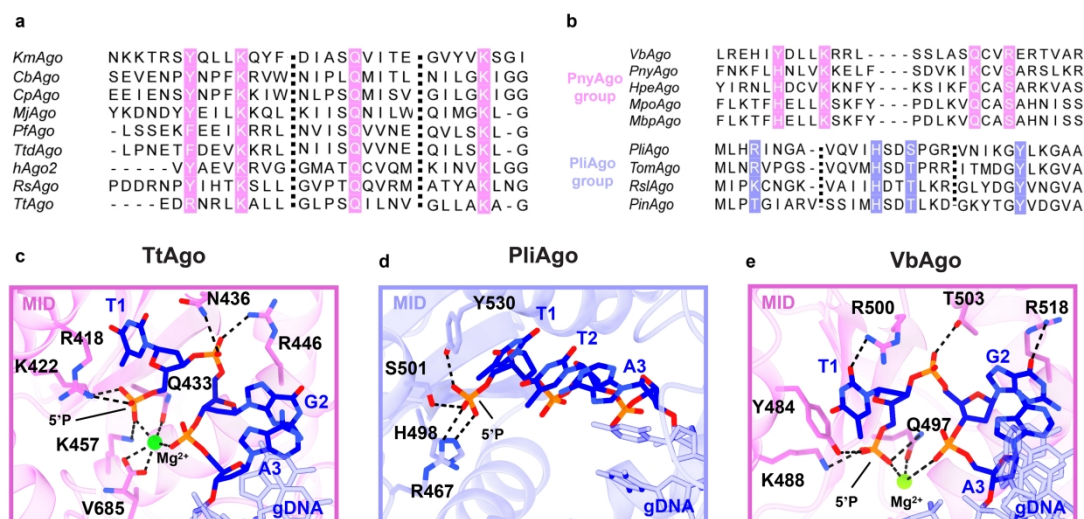


Supplementary Figure 9. RNA cleavage kinetics of VbAgo and its mutants.

(a) Single-turnover RNA cleavage kinetics of VbAgo and its mutants are shown. The fraction of cleaved product (mean $\pm$ SEM) is plotted on the Y-axis against time on the X-axis. Data were fitted using a single exponential equation (see Materials and Methods), and the corresponding observed rate constant (k_{obs}) is indicated. All experiments were performed in triplicate. (b) Multiple-turnover RNA cleavage kinetics of VbAgo and its mutants are also presented. The fraction of cleaved product (mean $\pm$ SEM) is plotted on the Y-axis against time on the X-axis. Data were fitted using a linear equation (see Materials and Methods), and the corresponding observed rate constant (k_{obs}) is indicated. All experiments were performed in triplicate. Source data are provided as a Source Data file.

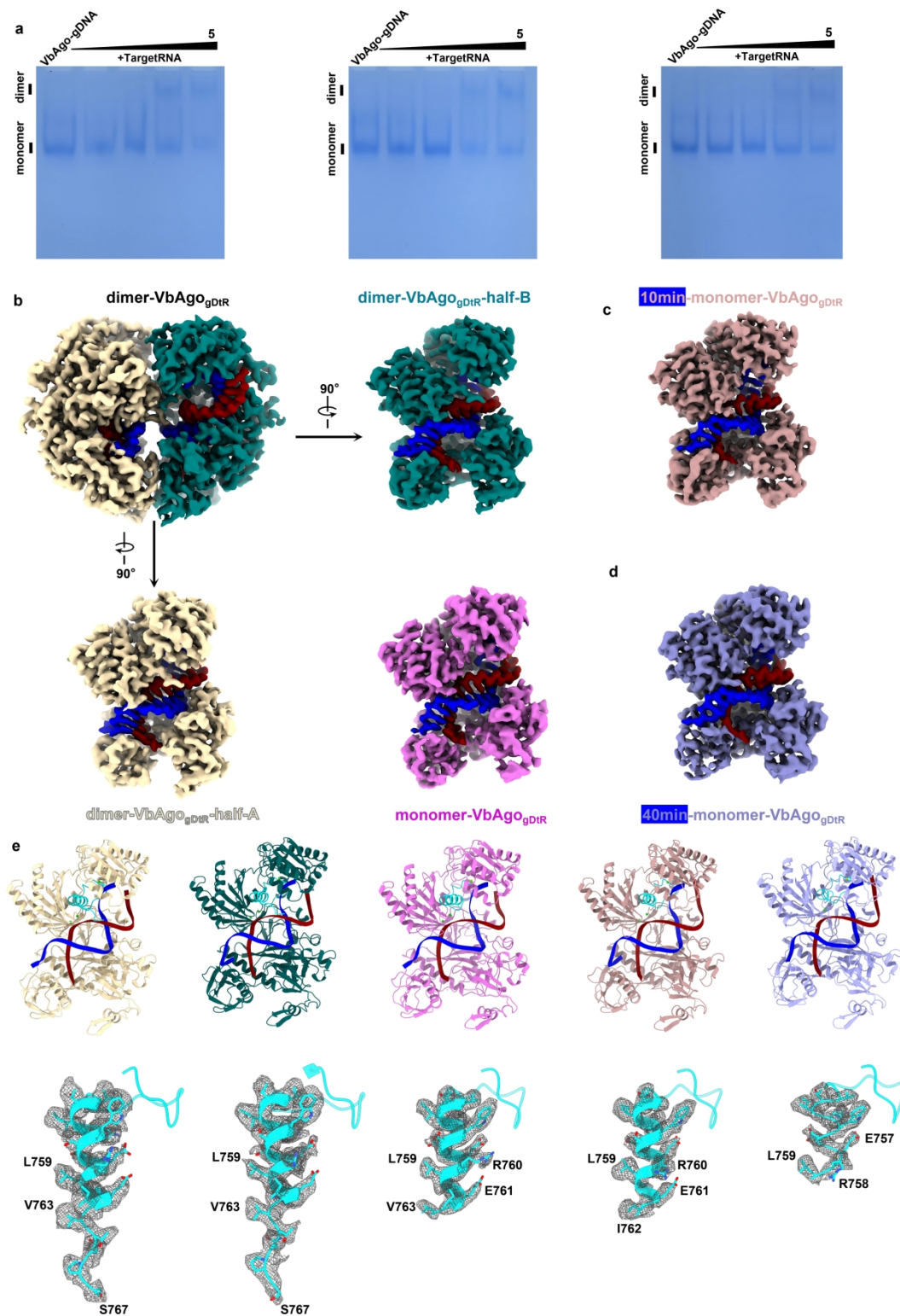


Supplementary Figure 10. 5'P-gDNA in the VbAgo_{gDNA} binary complex. Schematic of major contacts between VbAgo and the 5'P-gDNA (blue). Residues colored by domain, as in Figure 2A. In addition, the figure illustrates the interaction details of the binding pockets at the 3'-and 5'-ends, respectively.



Supplementary Figure 11. Comparison of the MID pocket of VbAgo with other pAgos.

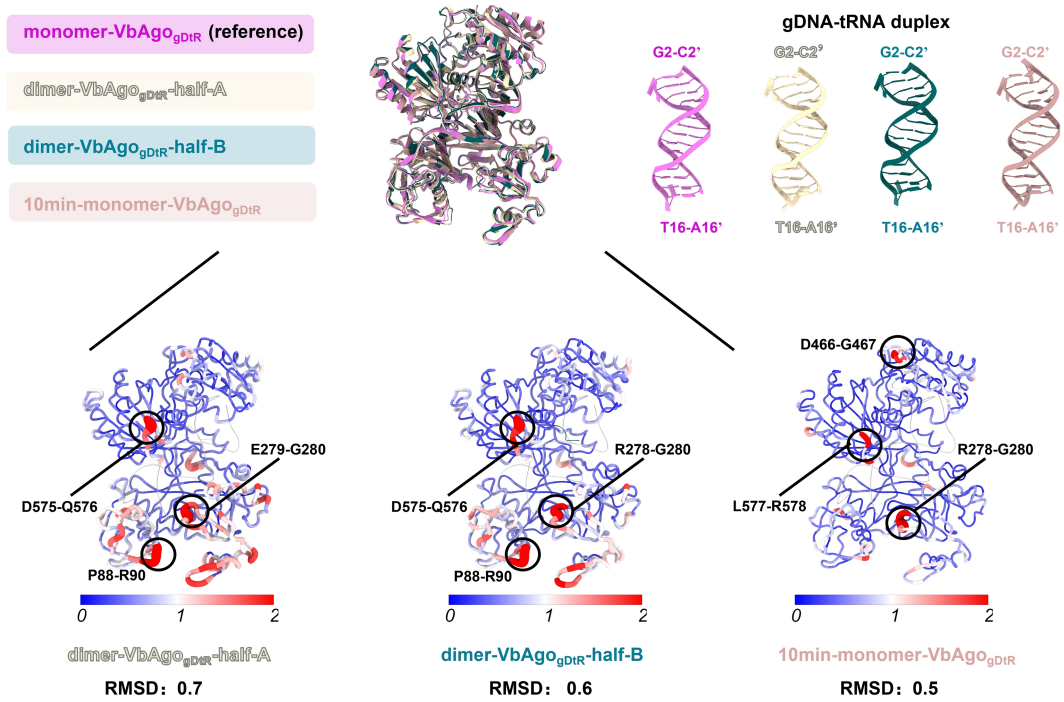
(a) Multiple sequence alignment of some representative prokaryotic and eukaryotic Argonaute proteins. Conserved residues are shaded and highlighted in orchid. The information is provided in Supplementary Dataset 1. (b) Multiple sequence alignment of the PnyAgo group (upper) and the PliAgo group (lower). Conserved residues are shaded and highlighted in orchid for the upper panel and in light purple for the lower panel. The information is provided in Supplementary Dataset 3. (c-e) Structural overview of guide 5'-end binding in Ago-DNA complexes. Panels c, d, and e show the TtAgo-P-gDNA (PDB: 3DLH), PliAgo-P-gDNA (PDB: 7R8H), and VbAgo-P-gDNA complexes, respectively. PliAgo, TtAgo, and VbAgo are shown as semi-transparent ribbon models. Amino acid residues involved in 5'-end binding are depicted as sticks and labeled. Salt bridges between residues and phosphate groups, along with Mg^{2+} coordination bonds, are indicated by black dashed lines.



Supplementary Figure 12. The overall structures of the VbAgo_{gDIR} ternary complexes in the pre-cleavage state.

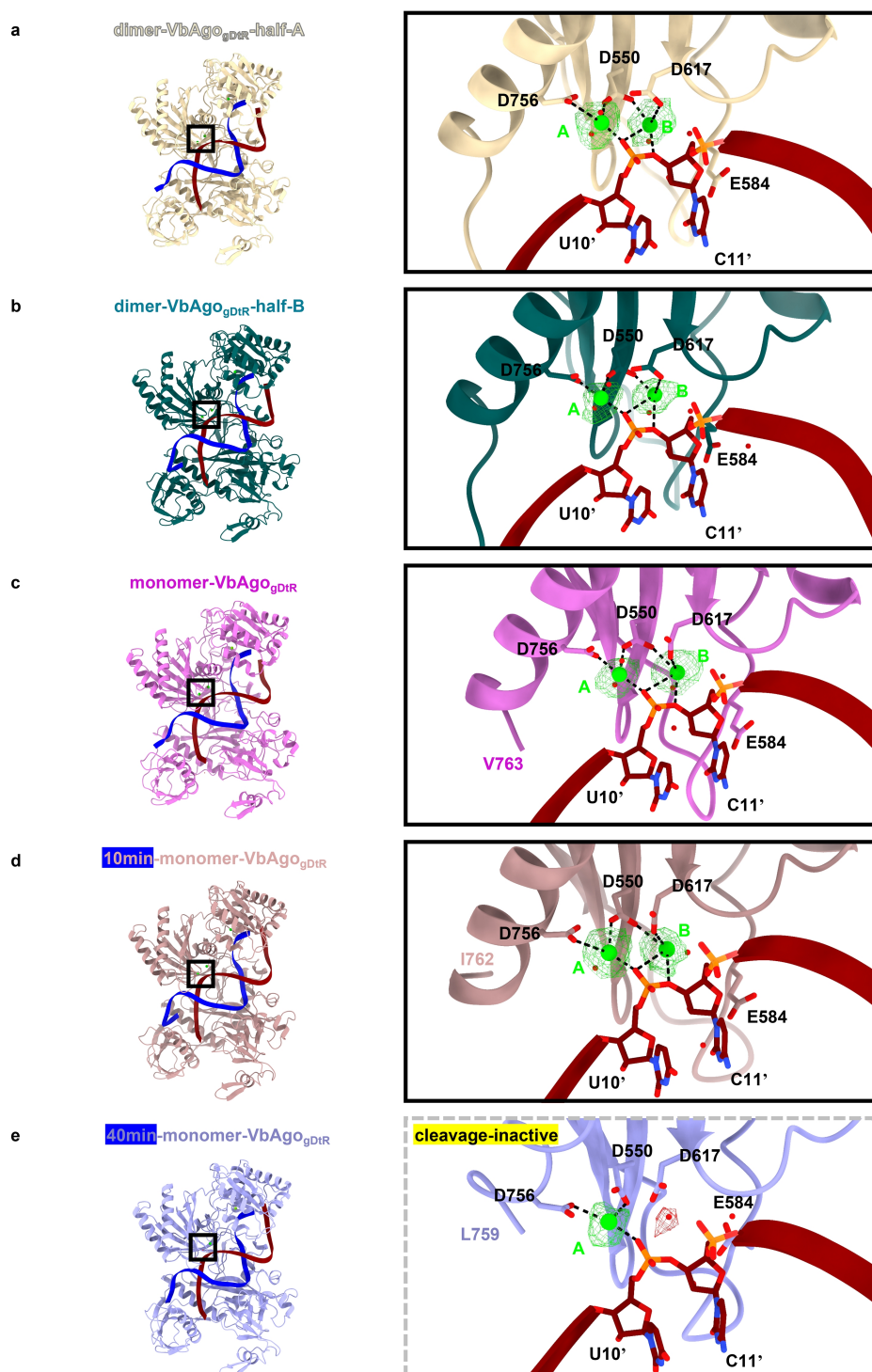
(a) Analysis of VbAgo-gDNA binary complexes with increasing concentrations of target RNA. Complexes were pre-assembled at a 1:1 molar ratio of protein to gDNA.

Consistent migration patterns were observed across three independent experiments. **(b-d)** Cryo-EM density maps of dimer VbAgo_{gDtr} with its two promoters, monomer VbAgo_{gDtr} **(b)**, 10-minute monomer VbAgo_{gDtr} **(c)**, and 40-minute monomer VbAgo_{gDtr} **(d)**. **(e)** The atomic models corresponding to panel **(b-d)** are displayed with the C-terminus in cyan and the cryo-EM density maps rendered as mesh.



Supplementary Figure 13. Pairwise structural alignments of the VbAgo_{gDtr} ternary complexes in the pre-cleavage state.

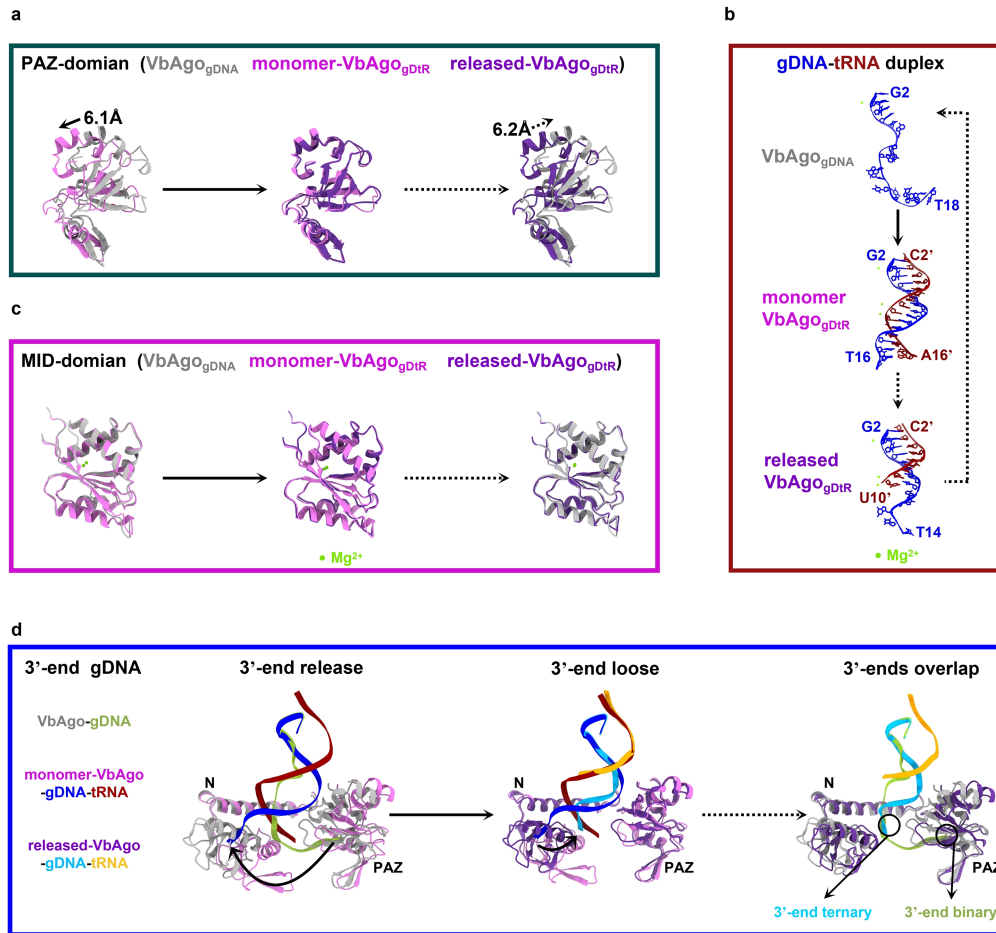
Superimposition of the dimeric VbAgo-gDtr complex with its two promoter-bound states and the 10-minute monomeric VbAgo-gDtr structure, all aligned to the monomeric VbAgo-gDtr reference. C α root-mean-square deviations (RMSDs) between corresponding residues increase along a blue-to-red color gradient.



Supplementary Figure 14. The catalytic pockets of the VbAgo_{gDIR} ternary complexes in the pre-cleavage state.

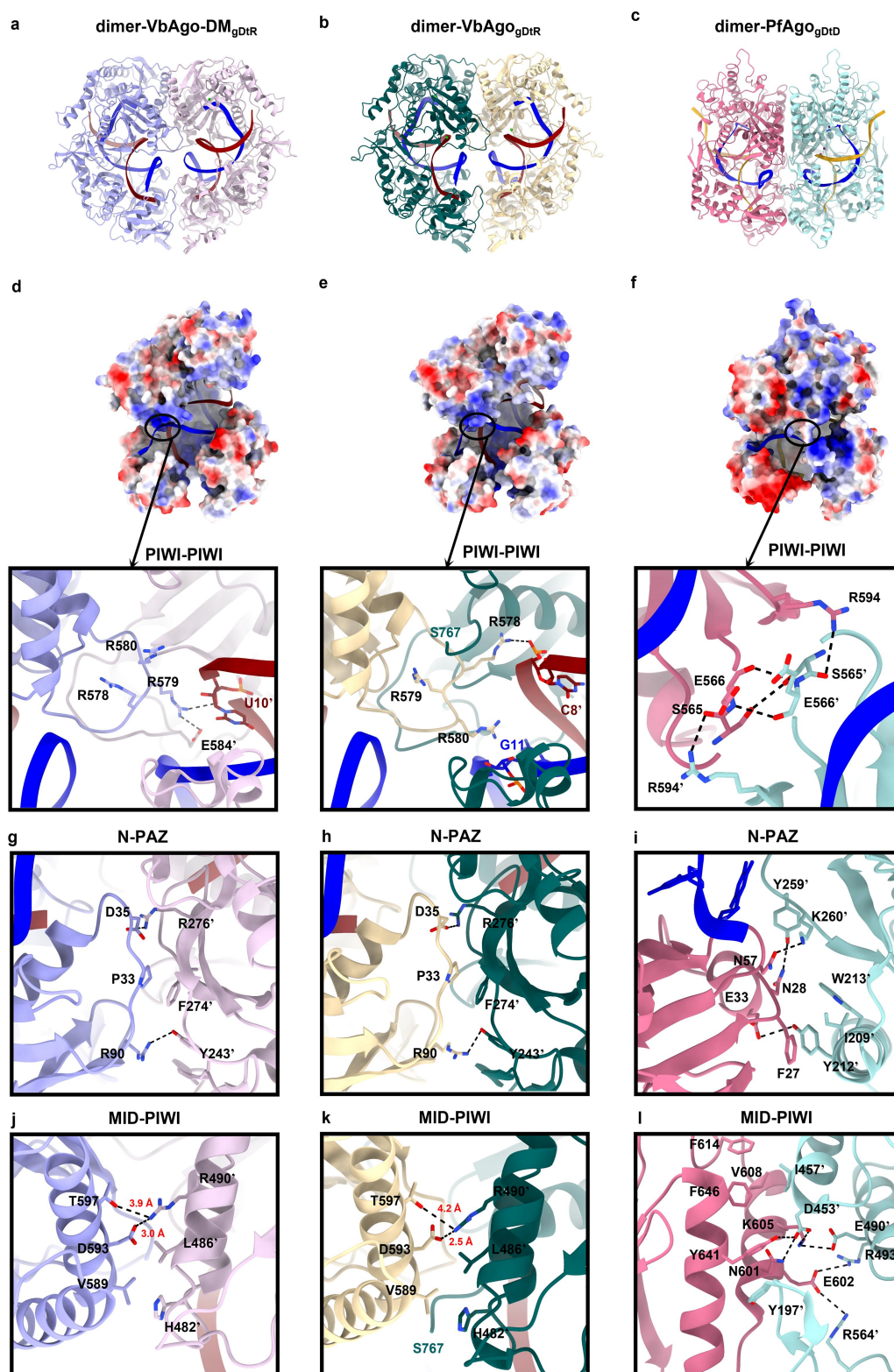
The atomic model is depicted on the left, while the detailed interactions within the catalytic pocket are shown on the right. There are always two magnesium ions and several water molecules present. The side chain of the glutamate finger residue E584 is oriented outward from the pocket across all conformations (**a-d**). In the 40-minute

monomer VbAgo_{gDiR}, the position typically occupied by the second magnesium ion (Mg^{2+} B) is instead occupied by a water molecule (e), which we designated as the cleavage-inactive state. The pair of Mg^{2+} cations are labeled and depicted as green spheres. Water molecules are represented as red spheres.



Supplementary Figure 15. Comparative structural analysis of the binary complex and monomeric ternary complexes related to Figure 3.

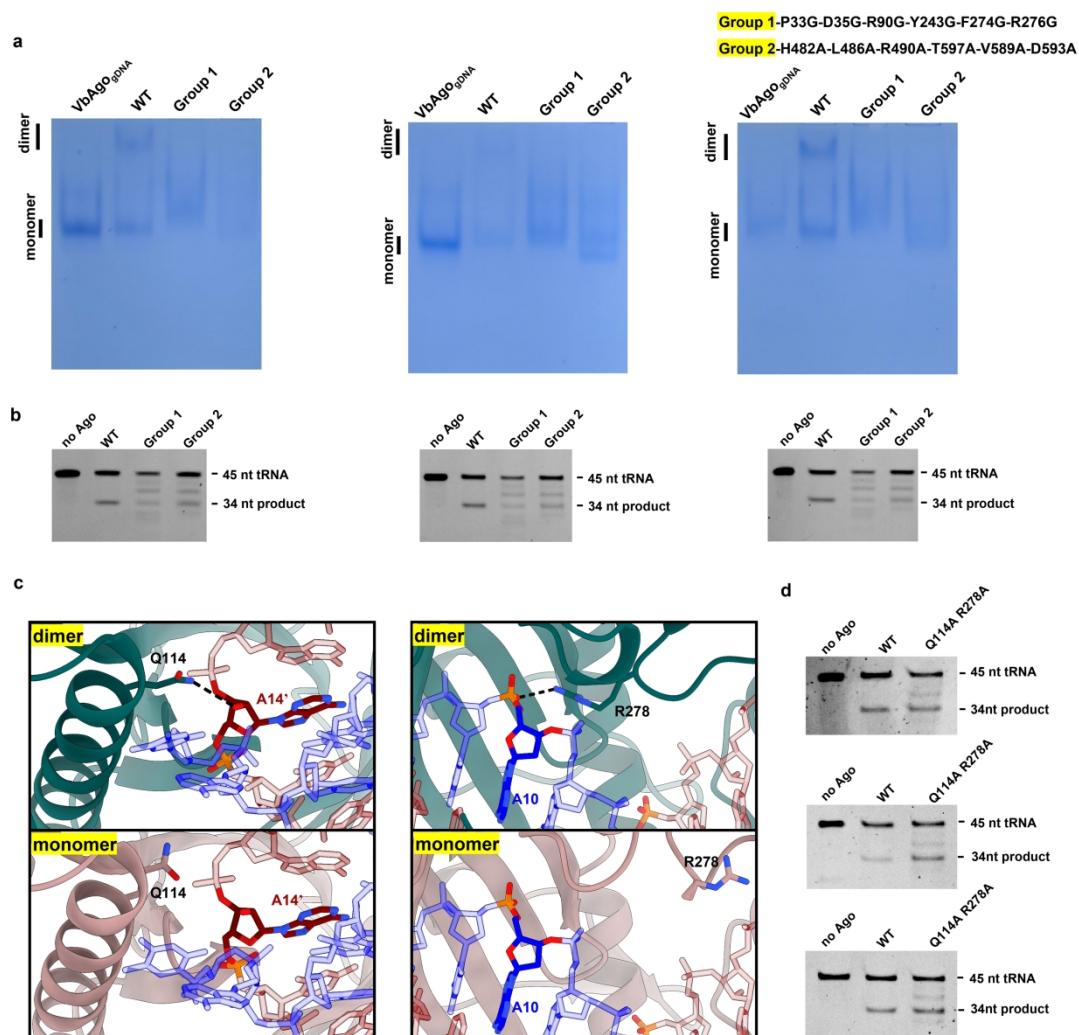
Conformational changes are shown for the PAZ domain (a), MID domain (b), gDNA-tRNA duplex (c), and 3'-end gDNA (d). Panels a-c: Color coding corresponds to Fig. 3b. The color coding for the newly incorporated gDNA and tRNA in panel d is indicated in the left margin of the panel.



Supplementary Figure 16. Comparative analysis of the dimerization interfaces in dimeric VbAgo_{gDfR} ternary complexes and dimer-PfAgo_{gDfD} (PDB: 8JPX).

(a) Atomic model of the dimeric VbAgo-DM_{gDfR}; (b) atomic model of the dimeric VbAgo_{gDfR}; (c) atomic model of the dimeric PfAgo_{gDfD}. The dimerization interaction

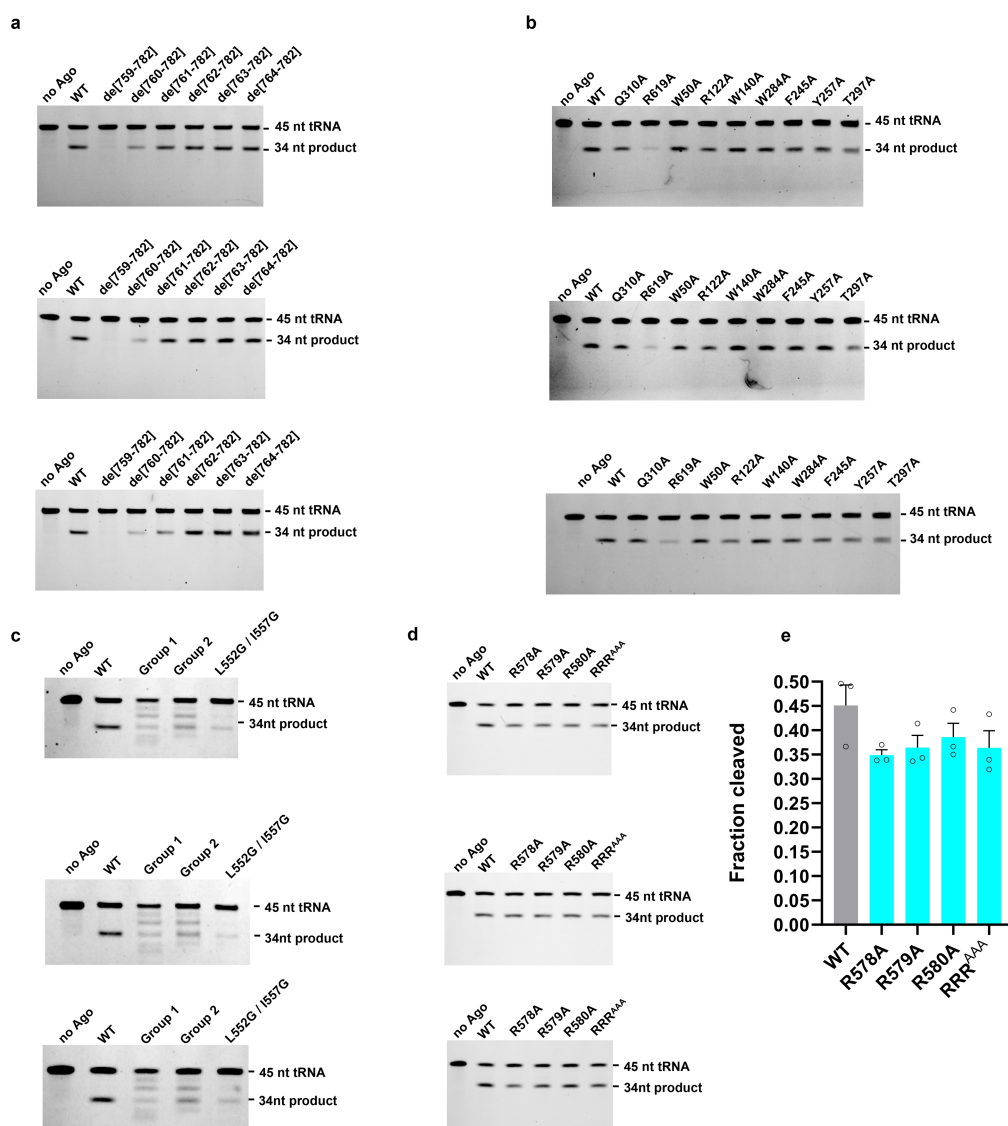
interfaces correspond to **d, g, j** for VbAgo-DM_{gDIR}; **e, h, k** for VbAgo_{gDIR}; and **f, i, l** for PfAgo_{gDIR}.



Supplementary Figure 17. Dimeric VbAgo serves as a precise cleavage surveillance state.

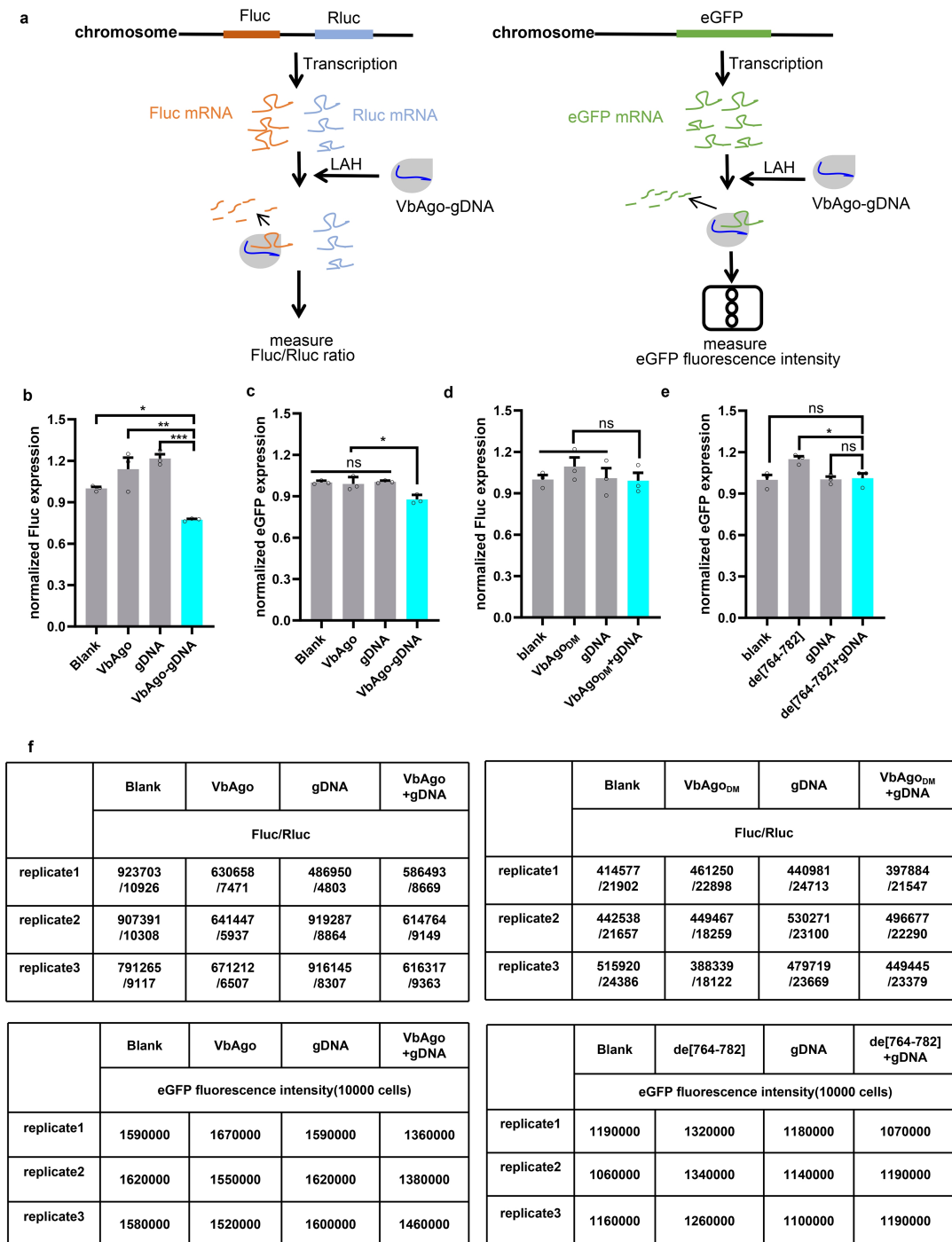
(a) Native-PAGE analysis of VbAgo-WT, VbAgo-gDNA, and Group 1/2 variants, showing reproducible migration patterns across three experiments. (b) Cleavage activity of VbAgo-WT, Group 1, and Group 2 variants, demonstrating consistent results across three independent replicates. (c) Additional interactions between VbAgo and the guide-target duplex are depicted within the dimer structure (top), with their

corresponding positions mapped onto the monomer (bottom). **(d)** Cleavage activity of Q114A and R278A variants, demonstrating consistent results across three independent replicates. Source data are provided as a Source Data file.



Supplementary Figure 18. Urea-PAGE analysis of the cleavage activity of its mutants.

(a-c) The urea-PAGE results corresponding to Fig.1c, 2f, 3e. **(d-e)** Cleavage activity of VbAgo and its three consecutive arginine residues within the PIWI-PIWI dimerization interface. Source data are provided as a Source Data file.



Supplementary Figure 19. Analysis of RNA Cleavage Activity of VbAgo in HEK293T Cells.

(a) Schematic of the intracellular RNA cleavage assay for VbAgo. Dual-luciferase analysis is shown on the left, and EGFP fluorescence analysis is shown on the right.

(b-c) Analysis of VbAgo RNA cleavage activity *in vivo*. The effects of VbAgo alone,

guide DNA (gDNA) alone, and pre-assembled VbAgo-gDNA complexes (VbAgo:gDNA = 1:1) on the dual-luciferase ratio (Fluc/Rluc) are presented. Corresponding effects on GFP fluorescence intensity in HEK293T cells are shown in panel c. **(d-e)** Analysis of in vivo RNA cleavage activity for VbAgo-DM and de[764 - 782] mutants. Data represent the mean $\pm$ SD from n = 3 biological replicates per group; statistical significance was analyzed by Student's t-test (ns = not significant, *p < 0.05, **p < 0.01, ***p < 0.001). **(f)** Original data corresponding to the analyses presented in panels b-e. Source data are provided as a Source Data file.

Supplementary Table 1. Oligonucleotides used in the single-stranded nucleic acid cleavage assays.

Name	Sequence (5'-3')
5'P_18nt-gDNA	5'P-TGAGGTAGTAGGTTGTAT
19nt-U-tRNA	AUACAACCUACUACCUCAU
45nt-FAM-U-tRNA	FAM-AAACGACGGCCAGUGCCAAGCUUACUAUAC
	AACCUACUACCUCAU
3'-Cy3_18nt-gDNA	5'P-TGAGGTAGTAGGTTGTAT-Cy3-3'
3'-BHQ2_18nt-gDNA	5'P-TGAGGTAGTAGGTTGTAT-BHQ2-3'
Fluc-gDNA	5'P-TATTCAGCCCATAGCG
eGFP-gDNA	5'P-TGCACGCCGTAGGTCA

Supplementary Table 2. Cryo-EM data collection, refinement and validation statistics.

	VbAgo_{apo} (9V65) (EMD-64797)	VbAgo_{gDNA} (9V66) (EMD-64798)	monomer-VbAgo_{gDtr} (9V93) (EMD-64859)	dimer-VbAgo_{gDtr} (9V8A) (EMD-64832)
Data collection and processing				
Magnification	105,000	105,000	105,000	105,000
Voltage (kV)	300	300	300	300
Electron exposure (e ⁻ /Å ²)	54	54	54	54
Defocus range (μm)	-1.0 ~ -1.5	-1.0 ~ -1.5	-1.0 ~ -1.5	-1.0 ~ -1.5
Pixel size (Å)	0.851	0.851	0.851	0.851
Symmetry imposed	C1	C1	C1	C1
Initial particle images (no.)	11,019,420	2,877,454	8,377,950	8,377,950
Final particle images (no.)	84,950	186,978	143,760	125,387
Map resolution (Å)	3.20	2.95	2.81	2.66
FSC threshold	0.143	0.143	0.143	0.143
Map resolution range (Å)	2.44 ~ 42.73	2.62 ~ 44.71	2.42 ~ 43.46	1.84 ~ 43.07
Refinement				
Initial model used	AlphaFold2	VbAgo _{apo}	VbAgo _{apo}	VbAgo _{apo}
Model resolution (Å)	3.40	3.09	2.92	2.89
FSC threshold	0.5	0.5	0.5	0.5
Map sharpening B factor (Å ²)	-119.9	-113.0	-86.6	-70.5
Model composition				
Non-hydrogen atoms	6,210	6,458	6,727	13,558
Protein residues	779	760	760	1528
Nucleotide	---	18	32	65
Ligand	---	MG: 1	MG: 3	MG: 6
B-factors (Å²)				
Protein	27.67/134.81/71.53	18.72/111.33/55.68	13.80/86.57/44.36	21.88/85.65/44.29
Nucleotide	---	44.28/88.56/62.96	25.73/56.82/33.97	24.11/68.09/33.76
Ligand	---	38.63/38.63/38.63	22.61/24.83/23.82	23.09/29.87/25.99
Water	38.51/38.51/38.51	15.66/53.90/30.69	34.03/47.78/40.53	23.15/39.98/32.85
R.m.s. deviations				
Bond lengths(Å)	0.003	0.003	0.003	0.004
Bond angles (°)	0.592	0.485	0.565	0.620
Validation				
MolProbity score	1.72	1.38	1.32	1.38
Clashscore	6.54	4.64	4.12	4.73
Poor rotamer (%)	0.00	0.00	0.00	0.00
Ramachandran plot				
Favored (%)	94.71	97.23	97.36	97.24
Allowed (%)	5.29	2.77	2.64	2.76
Disallowed (%)	0.00	0.00	0.00	0.00

Supplementary Table 2. Cryo-EM data collection, refinement and validation statistics.

	10min monomer-VbAgo_{gDfR} (9V9G) (EMD-64871)	40min monomer-VbAgo_{gDfR} (9V9I) (EMD-64873)	released- VbAgo_{gDfR} (9V7Z) (EMD-64826)	dimer- VbAgo-DM_{gDfR} (9V7S) (EMD-64822)
Data collection and processing				
Magnification	105,000	105,000	105,000	105,000
Voltage (kV)	300	300	300	300
Electron exposure (e ⁻ /Å ²)	54	54	54	54
Defocus range (μm)	-1.0 ~ -1.5	-1.0 ~ -1.5	-1.0 ~ -1.5	-1.0 ~ -1.5
Pixel size (Å)	0.851	0.851	0.851	0.851
Symmetry imposed	C1	C1	C1	C1
Initial particle images (no.)	1,965,093	1,761,719	1,761,719	4,361,165
Final particle images (no.)	132,500	114,582	112,179	1,016,135
Map resolution (Å)	2.89	2.93	2.97	2.50
FSC threshold	0.143	0.143	0.143	0.143
Map resolution range (Å)	2.47 ~ 44.73	2.56 ~ 44.27	2.61 ~ 47.11	2.20 ~ 35.58
Refinement				
Initial model used	VbAgo _{apo}	VbAgo _{apo}	VbAgo _{apo}	VbAgo _{apo}
Model resolution (Å)	3.03	3.14	3.12	2.67
FSC threshold	0.5	0.5	0.5	0.5
Map sharpening B factor (Å ²)	-92.6	-94.9	-98.3	-90.5
Model composition				
Non-hydrogen atoms	6,784	6,718	6541	13,421
Protein residues	760	756	757	1512
Nucleotide	34	33	23	65
Ligand	MG: 3	MG: 3	MG: 3	MG: 2
B-factors (Å²)				
Protein	14.29/91.10/46.67	18.83/129.80/62.78	14.07/114.52/60.53	10.05/79.33/35.87
Nucleotide	26.05/67.52/38.28	42.65/100.31/57.98	46.48/92.11/61.18	18.60/58.06/28.85
Ligand	31.30/32.41/31.83	33.06/54.63/43.44	34.47/59.58/48.46	20.13/20.17/20.15
Water	17.43/37.20/25.31	22.16/46.34/30.25	21.23/40.76/31.41	13.94/33.23/24.92
R.m.s. deviations				
Bond lengths (Å)	0.003	0.003	0.003	0.003
Bond angles (°)	0.515	0.586	0.548	0.535
Validation				
MolProbity score	1.33	1.53	1.31	1.33
Clashscore	4.48	6.66	4.58	3.49
Poor rotamer (%)	0.00	0.00	0.00	0.00
Ramachandran plot				
Favored (%)	97.49	97.08	97.62	96.88
Allowed (%)	2.51	2.92	2.38	3.12
Disallowed (%)	0.00	0.00	0.00	0.00