

Supplementary Information

Anti-phage defense mechanism involving phage-encoded DNA binding protein and bacterial reverse transcriptase DRT4

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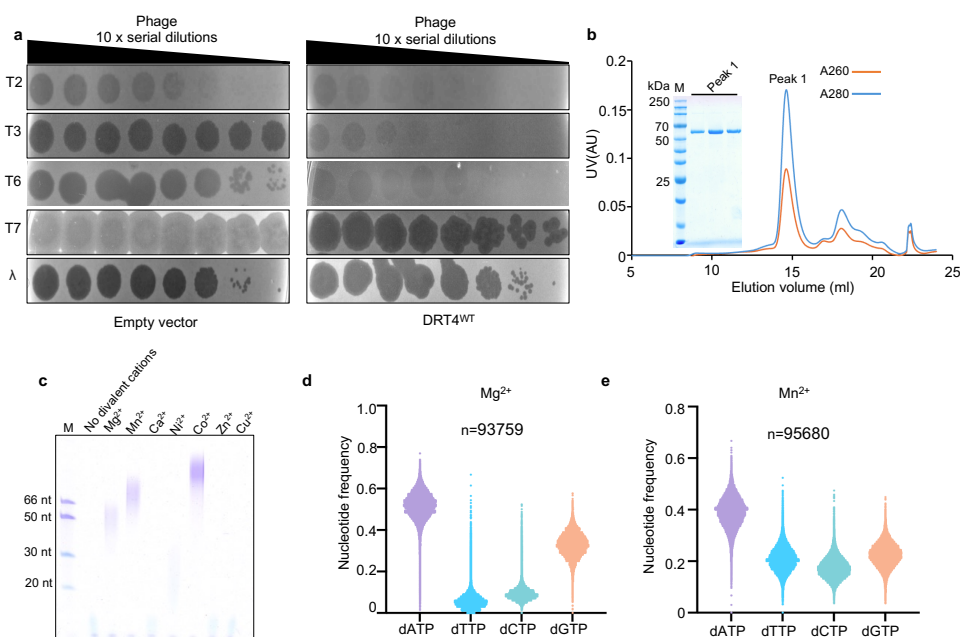
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Supplementary Figure 1. Biochemical and functional characterization of DRT4. a

Plaque formation of different dsDNA phages on cells expressing empty vector or DRT4.

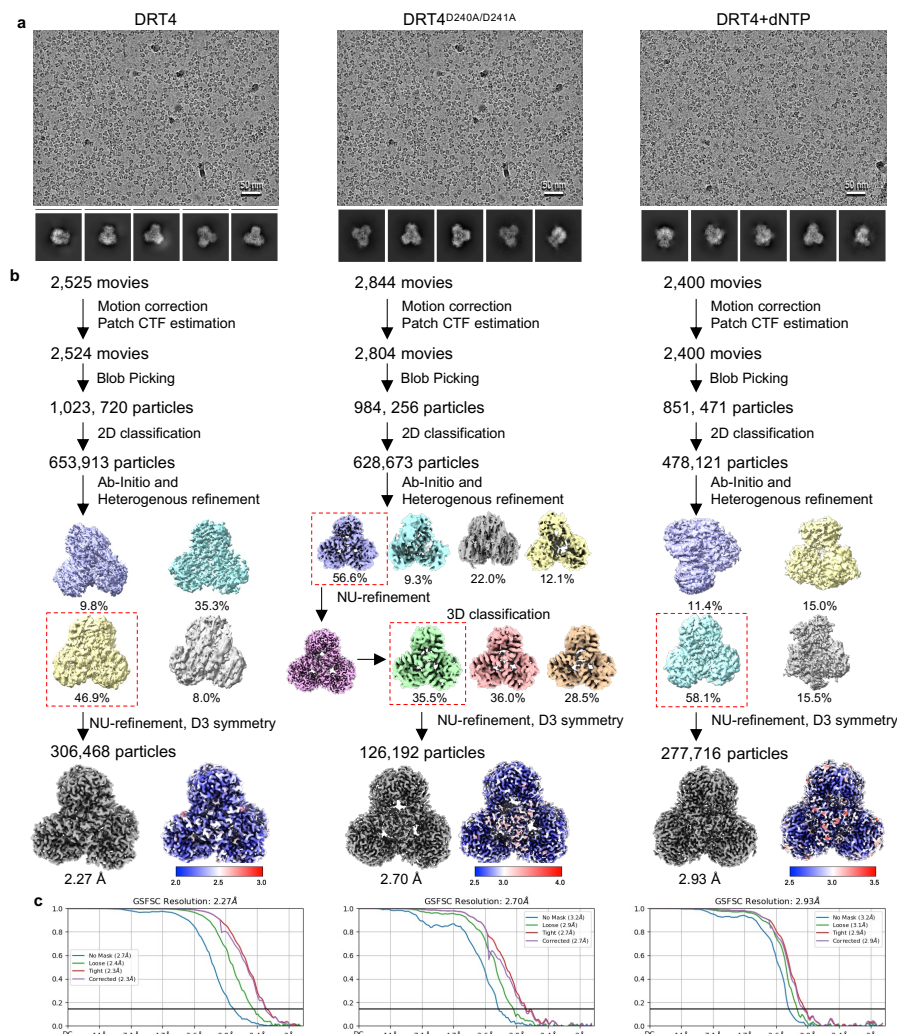
10-fold serial dilutions of the phage lysate were dropped on the plates. **b** Size-exclusion

(Superose 6 Increase 10/300 GL Cytiva) and SDS-PAGE profiles of the purified recombinant D240A/D241A mutant. **c** Divalent metal ion-dependent polymerization

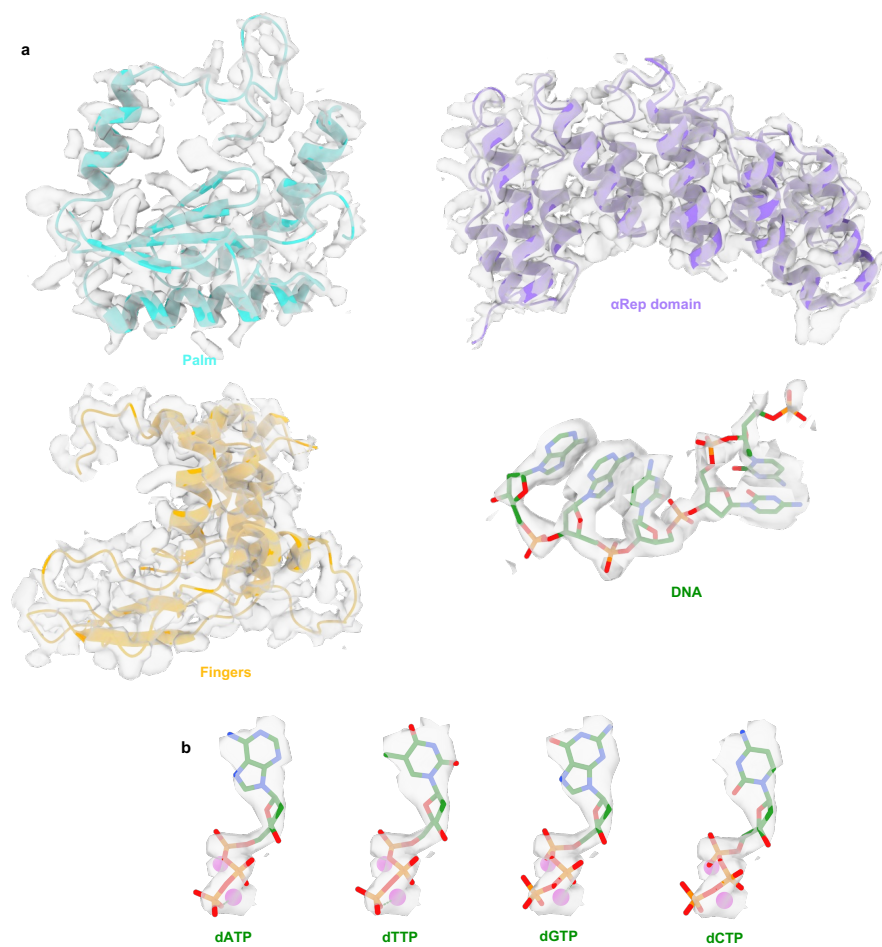
activity of DRT4. **d** Nucleotide frequency of the DRT4-synthesized ssDNA products in

the presence of MgCl₂. **e** Nucleotide frequency of the DRT4-synthesized ssDNA

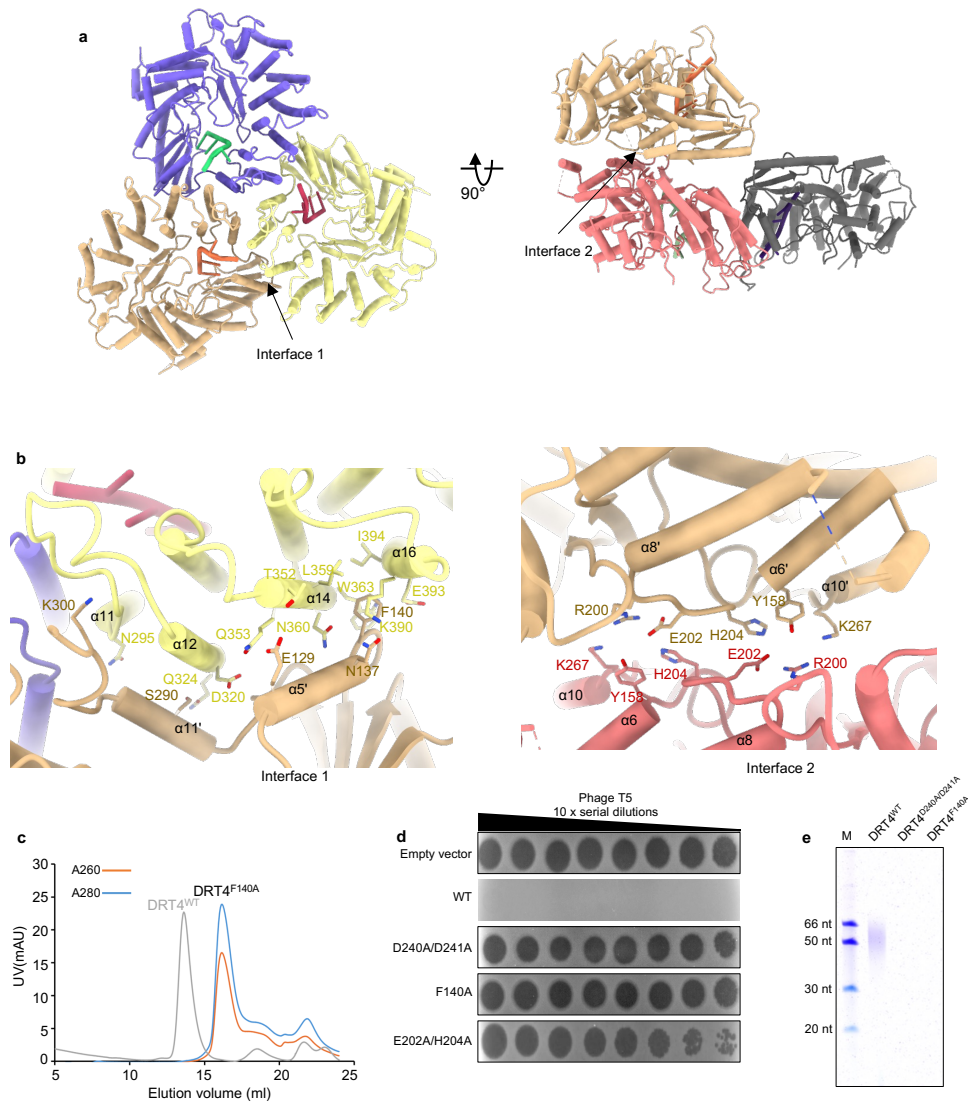
products in the presence of MnCl₂.



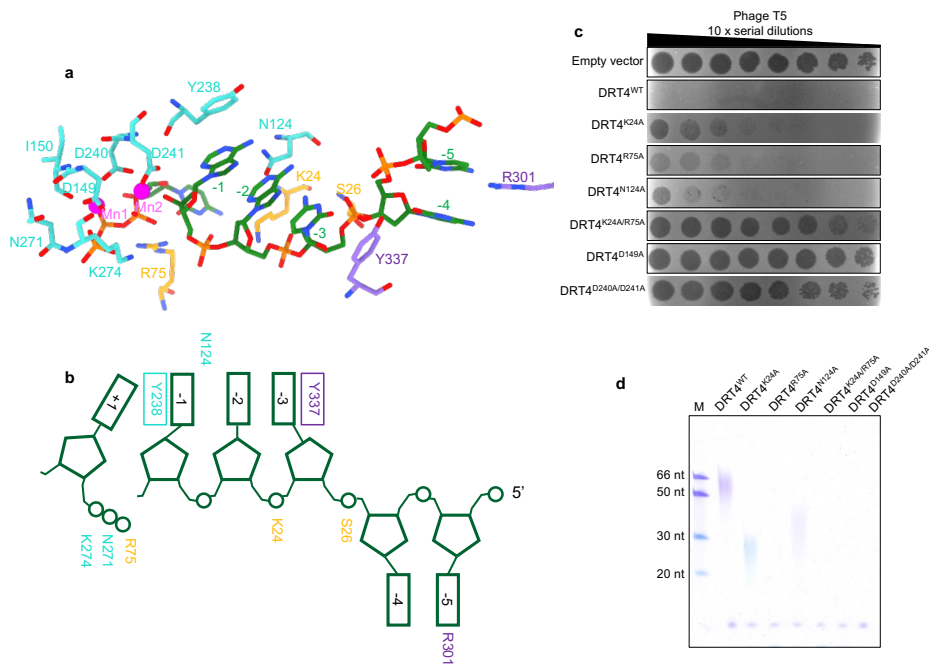
Supplementary Figure 2. Cryo-EM reconstruction of DRT4 in different functional states. **a** Representative cryo-EM images and 2D classification averages of DRT4, D240A/D241A, and DRT4 in the presence of dNTP. **b** Flowchart of cryo-EM data processing. **c** Fourier shell correlation (FSC) curves were calculated using two independent half maps, and resolution was estimated using the FSC=0.143 cutoff.



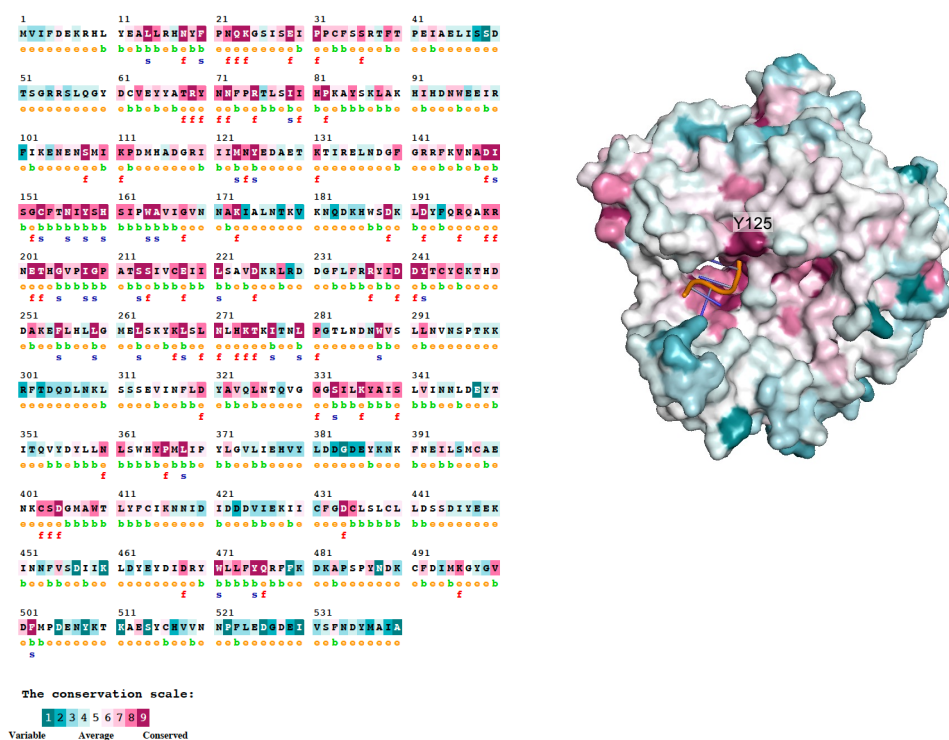
Supplementary Figure 3. Cryo-EM densities of DRT4. **a** Densities for the indicated regions are shown in the context of the atomic model of wild-type DRT4. **b** Four dNTPs were modeled into the substrate density in the substrate bound DRT4.



Supplementary Figure 4. Assembly of DRT4 hexamer. **a** Two key inter-subunit interfaces of DRT4 hexamer are indicated. Interface 1 mediates intra-trimer stabilization through extensive interactions between the $\alpha 5$ helix (palm subdomain) and $\alpha 11$ helix (α Rep domain) of one subunit and $\alpha 12$, $\alpha 14$, and $\alpha 16$ helices (α Rep domain) of an adjacent subunit. Interface 2 governs the inter-trimer interactions, forming between corresponding subunits of the upper and lower trimers. **b** Detailed interactions of two interfaces. **c** Size-exclusion (Superose 6 Increase 10/300 GL Cytiva) profiles of the purified recombinant DRT4 and F140A mutant. **d** Plaque formation of phage T5 on cells expressing empty vector, DRT4 WT, or indicated mutants. 10-fold serial dilutions of the phage T5 lysate were spotted on plates. **e** Polymerization activity of the F140A mutant in the presence of 5 mM MgCl₂.

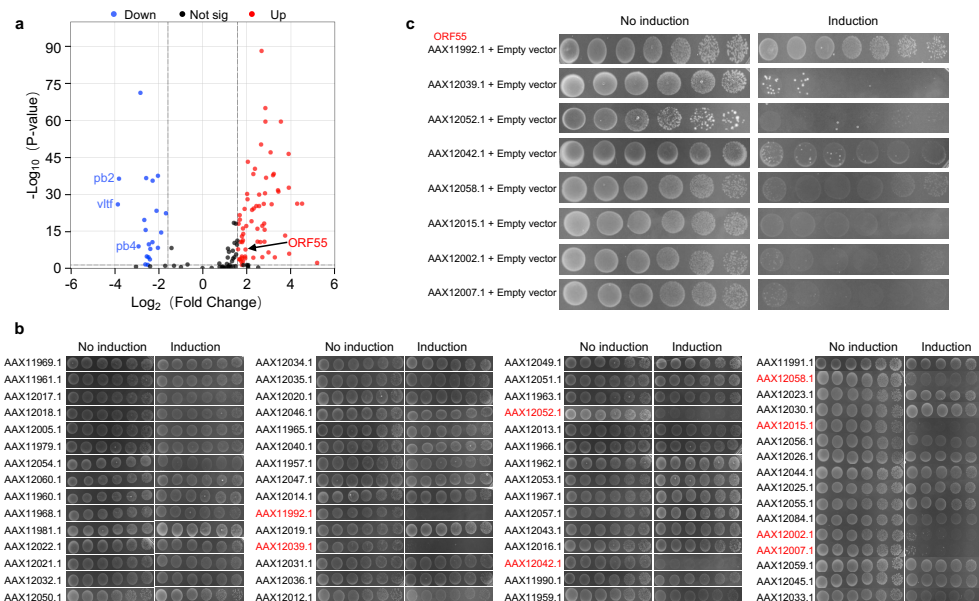


Supplementary Figure 5. Interactions of DRT4 with dNTP and DNA. **a** Detailed interactions of DRT4 with dNTP and DNA, only residues involved in polar and base-stacking interactions are shown. Residues color follow same scheme as **Fig. 2c**. **b** Schematic drawing highlighting the DRT4-DNA interactions. **c** Plaques formation of phage T5 on cells expressing empty vector, DRT4 WT, or indicated mutants. 10-fold serial dilutions of the phage T5 lysate were spotted on plates. **d** Polymerization activity of DRT4 and mutants in the presence of 5 mM MgCl_2 .

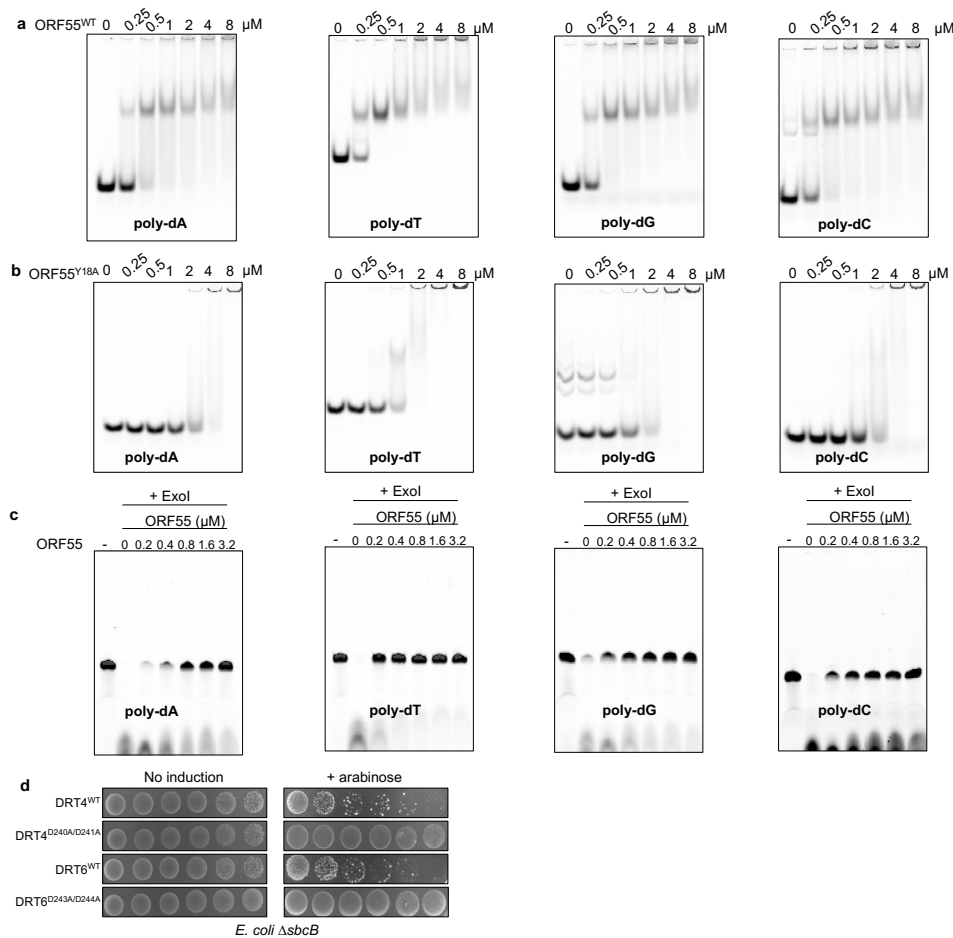


Supplementary Figure 6. Evolutionary conservation analysis of the DRT4 system.

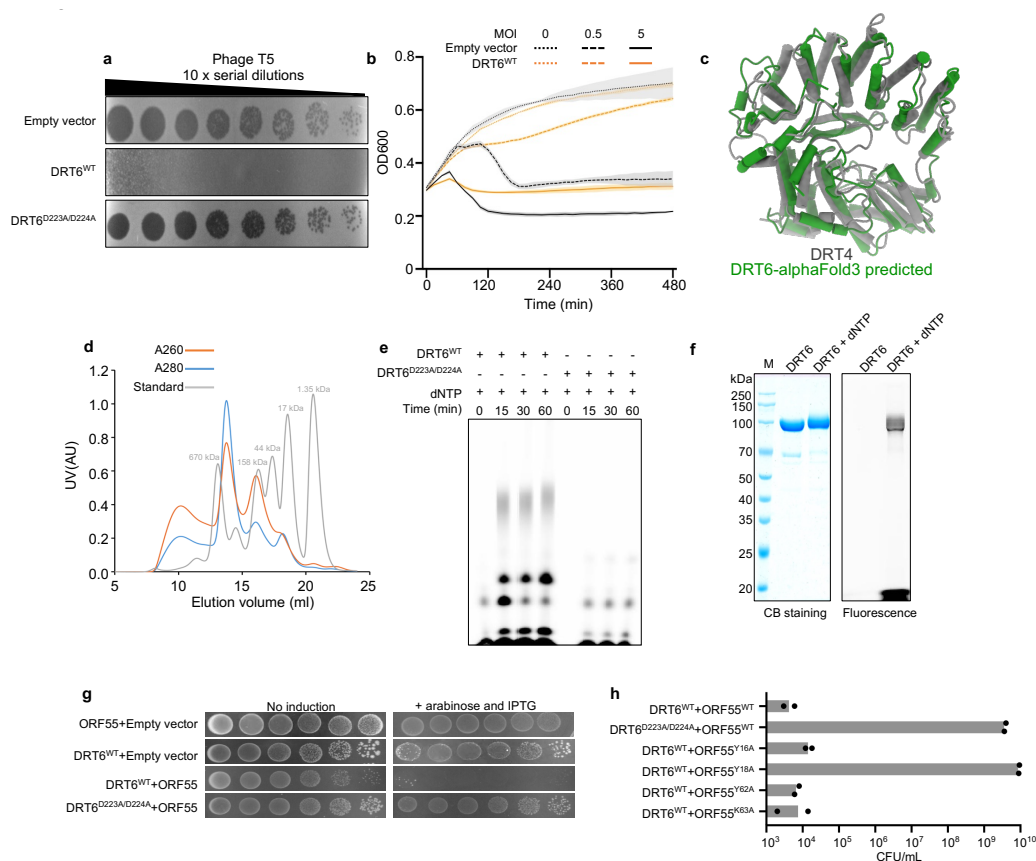
Alignment of 174 DRT4 protein sequences shows the residues forming active sites and the priming residue Tyr125 are highly conserved. Evolutionary conservation was calculated by the ConSurf Server.



Supplementary Figure 7. Identification of the phage T5 trigger of the DRT4-mediated antiviral immunity. **a** Volcano plot showing differential gene expression of *E. coli* expressing the wild-type DRT4 compared with *E. coli* expressing the mutant D240A/D241A after phage T5 infection for 60 min. Data were analyzed for differential expression between groups using DESeq2 ($|\text{FoldChange}| \geq 3$ & $\text{Padjust} \leq 0.05$). Blue, black, and red points depict downregulated, non-significant, and upregulated genes, respectively. Data represent three biological replicates, and results are shown using adjusted P-values. Three downregulated late phage genes were labelled. **b** The survival status of *E. coli* cells upon co-expression of DRT4 with indicated phage T5 protein. The protein identifiers (protein_id) of all phage T5 proteins correspond to those in GenBank accession AY587007.1. For clarity, AAX11992.1 is referred to as ORF55 in this study. **c** The survival status of *E. coli* cells upon co-expression of the pBAD empty vector with indicated phage T5 protein.



Supplementary Figure 8. ORF55 binds to single-stranded DNA. **a** EMSA of phage T5 ORF55 binding to different DNA homopolymers. Increasing concentrations of ORF55 were incubated with the 5'-FAM-labeled 30-nt ssDNA. **b** EMSA of phage T5 ORF55 Y18A mutant protein binding to different DNA homopolymers. **c** ExoI treatment of 5'-FAM-labeled 30-nt ssDNA in the presence of increasing concentrations of ORF55. **d** The survival status of *E. coli* upon expression of DRT4, DRT6 or indicated mutant in the *sbcB*-knockout *E. coli*. *sbcB* encodes ExoI.



Supplementary Figure 9. ORF55 activates DRT6-mediated antiviral immunity. **a** Plaque formation of phage T5 on cells expressing empty vector, DRT6 WT, or catalytically inactive mutant D223A/D224A. 10-fold serial dilutions of the phage T5 lysate were spotted on plates. **b** Bacterial growth curves in the presence of either DRT6 or empty vector upon infection by phage T5 at indicated MOI. **c** Superimposition of AlphaFold3-predicted DRT6 structure with the cryo-EM structure of DRT4. **d** Size-exclusion (Superose 6 Increase 10/300 GL Cytiva) and SDS-PAGE profiles of the purified MBP-DRT6. **e** DNA polymerization activity of DRT6. **f** Analysis of labeled nucleotides covalently attached to DRT6. Protein samples were analyzed on an SDS-PAGE gel and visualized using fluorescent readout (right panel), then stained with Coomassie Blue (left panel). **g** The survival status of *E. coli* cells upon co-expressing of DRT6 with ORF55 or indicated mutants. **h** Bar graphs quantifying cell viability in colony forming units (CFUs) upon co-expression of ORF55 or indicated mutant with DRT6 or catalytically inactive mutant.

conserved residues involved in metal ion and dNTP binding are colored cyan.

Supplementary Table 1. Cryo-EM data collection, refinement and validation statistics

	DRT4 (PDB 9VDP) (EMD-64991)	DRT4 ^{D240A/D241A} (PDB 9VDV) (EMD-64992)	Substrate bound DRT4 (PDB 9VDO) (EMD-64990)
Data collection and processing			
Magnification	50,000	50,000	50,000
Voltage (kV)	300	300	300
Electron exposure (e-/Å ²)	40	40	40
Defocus range (μm)	-0.5 to -2.5	-0.5 to -2.5	-0.5 to -2.5
Pixel size (Å)	0.95	0.95	0.95
Symmetry imposed	D3	D3	D3
Initial particle images (no.)	653,913	628,673	478,121
Final particle images (no.)	306,468	126,129	277,716
Map resolution (Å)	2.27	2.70	2.93
FSC threshold	0.143	0.143	0.143
Map resolution range (Å)	2.0-3.0	2.5-4.0	2.5-3.5
Refinement			
Initial model used	AlphaFold3	This study	This study
Model resolution (Å)	2.3	2.7	2.9
FSC threshold	0.143	0.143	0.143
Map sharpening <i>B</i> factor (Å ²)	-82.8	-94.3	-123.9
Model composition			
Non-hydrogen atoms	24,888	24,258	25,026
Protein residues	2,964	2,964	2,964
Nucleotides	30	0	36
Ligands	0	0	12
<i>B</i> factors (Å ²)			
Protein	85.28	34.05	14.95
Nucleotide	61.67		21.07
Ligand			7.75
R.m.s. deviations			
Bond lengths (Å)	0.004	0.007	0.005
Bond angles (°)	0.714	0.997	0.812
Validation			
MolProbity score	1.87	2.28	1.56
Clash score	4.69	8.04	7.29
Ramachandran plot			
Favored (%)	96.33	96.94	97.11
Allowed (%)	3.47	2.86	2.69
Disallowed (%)	0.2	0.2	0.2

Supplementary Table 2. Information of phage T5 proteins in Supplementary Figure 7b

Number	Protein ID	Product
1	AAX12046.1	ORF109
2	AAX12002.1	ORF085
3	AAX12045.1	DNA ligase
4	AAX12042.1	ORF105
5	AAX12059.1	deoxyuridine 5'-triphosphate nucleotide hydrolase
6	AAX12040.1	ORF103
7	AAX11957.1	ORF020
8	AAX12007.1	ORF070
9	AAX12047.1	ORF110
10	AAX11990.1	ORF053
11	AAX11991.1	putative formate acetyltransferase
12	AAX12060.1	ORF123
13	AAX11960.1	ORF023
14	AAX11968.1	Ser/Thr protein phosphatase
15	AAX11981.1	ORF044
16	AAX11969.1	ORF032
17	AAX11992.1	ORF055
18	AAX11965.1	ORF028
19	AAX11961.1	ORF024
20	AAX12017.1	ORF080
21	AAX12018.1	ORF081
22	AAX12005.1	ORF068
23	AAX11979.1	putative endonuclease
24	AAX11962.1	ORF025
25	AAX12014.1	ORF077
26	AAX12053.1	hypothetical protein (D10 3' region)
27	AAX12058.1	exodeoxyribonuclease
28	AAX11967.1	probable phosphoesterase
29	AAX12023.1	ribonuclease HI
30	AAX12030.1	anaerobic ribonucleoside-triphosphate reductase
31	AAX12015.1	spore cortex-lytic enzyme precursor
32	AAX12057.1	D14 protein
33	AAX12019.1	ORF082
34	AAX12054.1	D11 protein
35	AAX12039.1	ORF102
36	AAX12043.1	ORF106
37	AAX12016.1	ORF079
38	AAX12049.1	DNA primase
39	AAX12051.1	hypothetical protein (D10 5' region)
40	AAX11963.1	ORF026

41	AAX12052.1	probable helicase
42	AAX12013.1	ORF076
43	AAX11966.1	ORF029
44	AAX12022.1	ORF085
45	AAX12056.1	probable exonuclease subunit 2
46	AAX12026.1	ribonucleoside diphosphate reductase I beta chain
47	AAX12021.1	ORF084
48	AAX12044.1	DNA ligase
49	AAX12025.1	dihydrofolate reductase
50	AAX12031.1	ORF094
51	AAX12032.1	ORF095
52	AAX12055.1	possible exonuclease subunit 1
53	AAX12034.1	hypothetical protein Aeh1ORF302c-like protein
54	AAX12084.1	lytic conversion lipoprotein precursor
55	AAX12035.1	ORF098
56	AAX12036.1	hypothetical protein Aeh1ORF298c-like protein
57	AAX12020.1	ORF083
58	AAX12050.1	DNA polymerase
59	AAX12012.1	putative transmembrane protein
60	AAX11959.1	ORF022
61	AAX12033.1	putative transferase