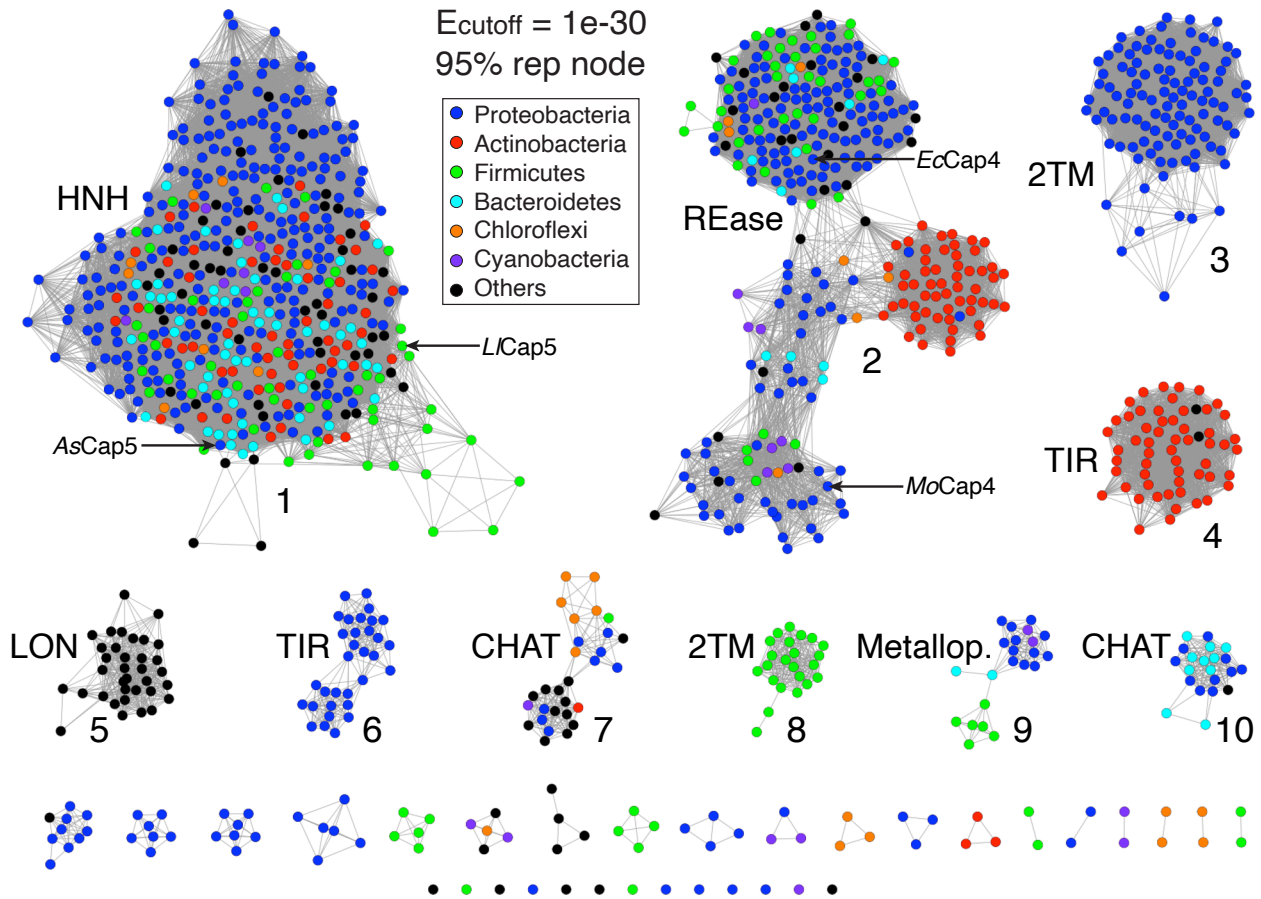
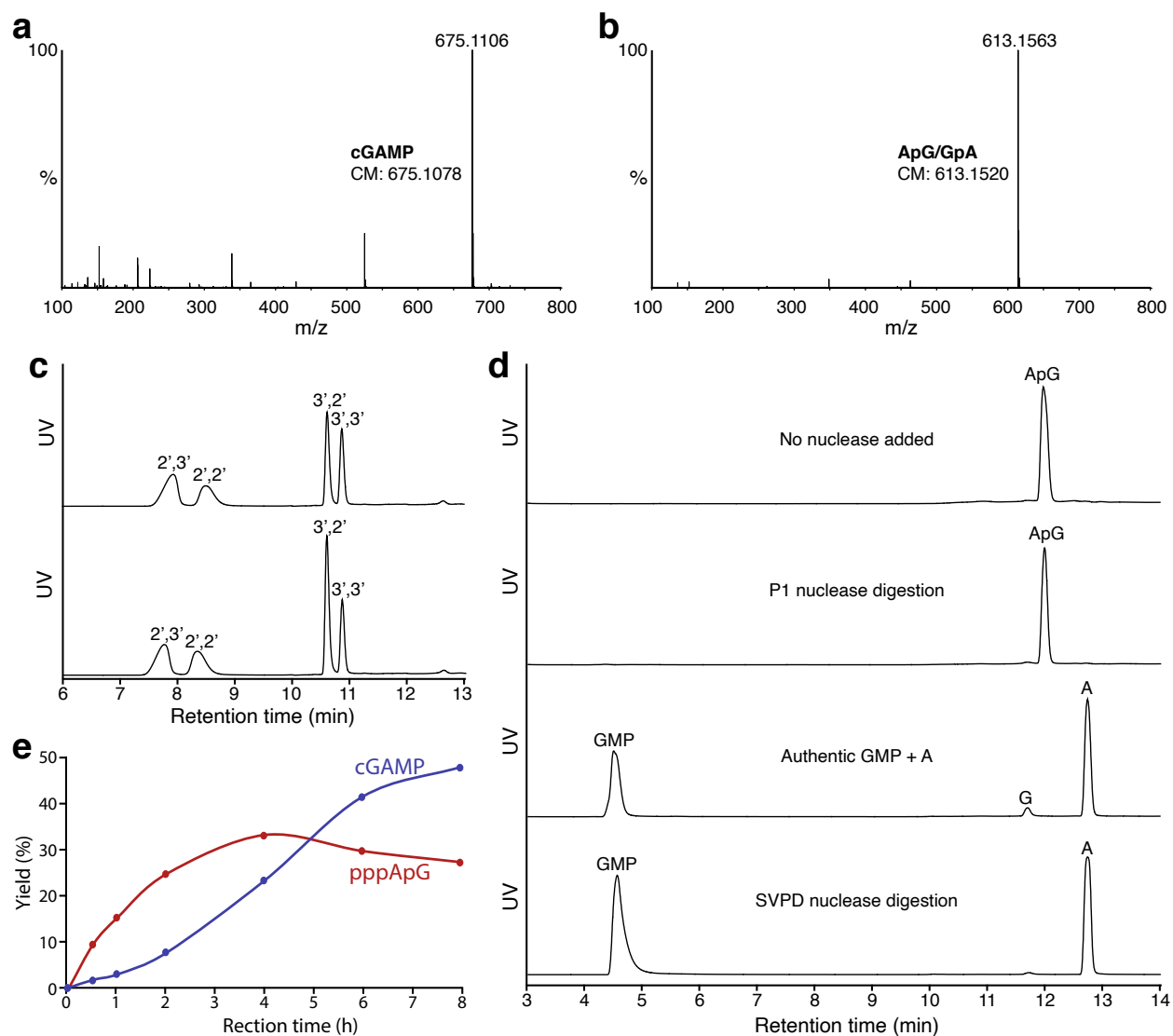


Molecular mechanisms of the CdnG-Cap5 antiphage defense system employing 3',2'-cGAMP as the second messenger

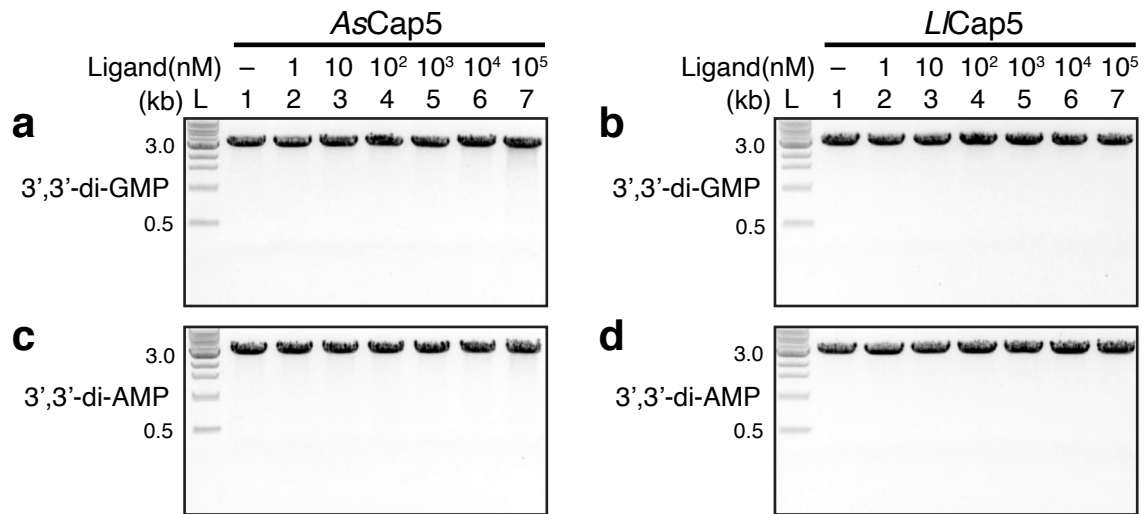
Supplementary Information



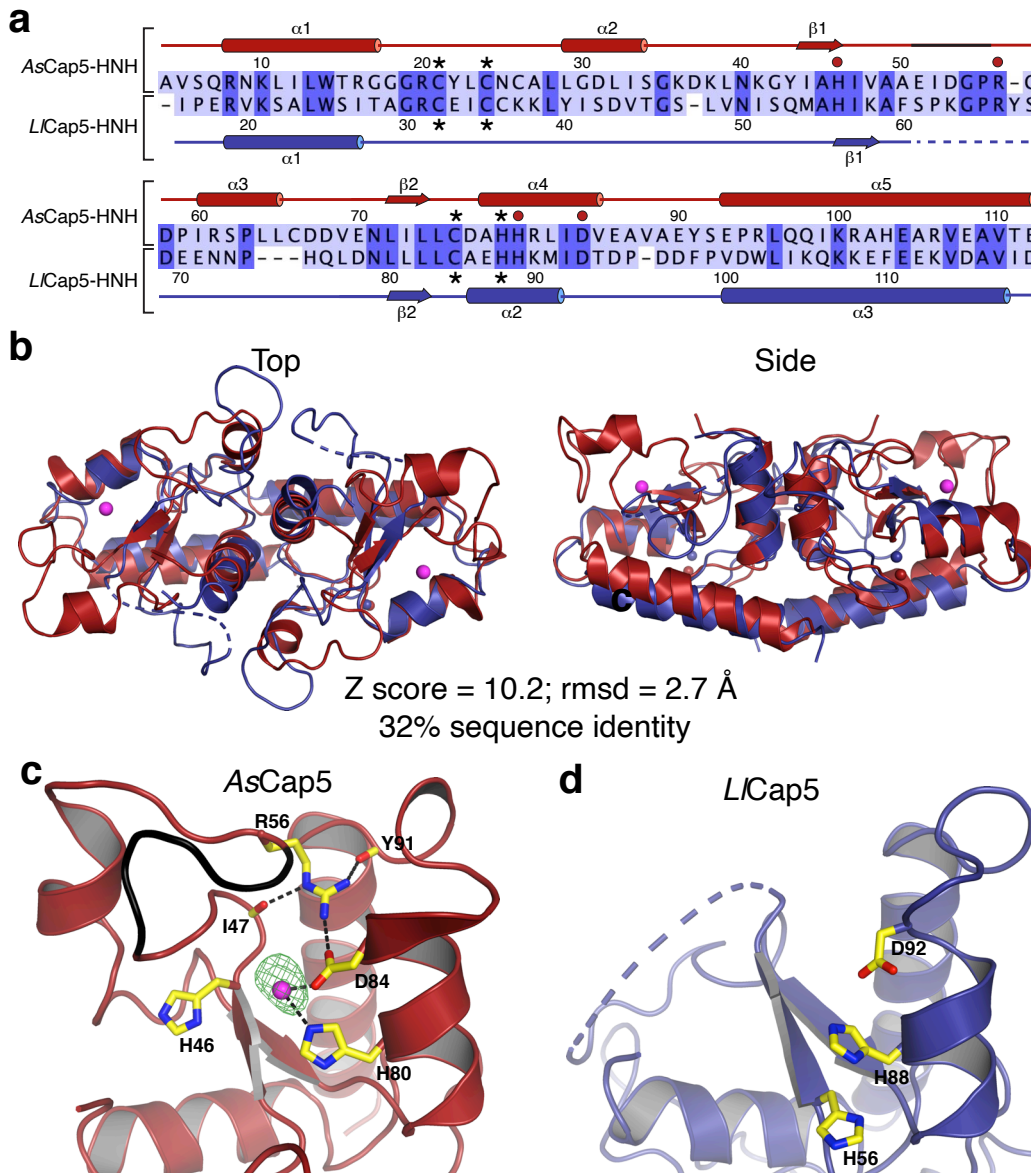
Supplementary Figure 1. Bioinformatic analysis of SAVED domain-containing proteins via Sequence Similarity Network (SSN). The analysis was based on sequences of PF18145 on the database of UniProt 2020_04 and InterPro 81. Each node (colored circle) represents a collection of SAVED proteins sharing >95% sequence identity (95% rep node). An edge (gray line) connects two nodes if the E-value measuring their sequence similarities is smaller than the cutoff value (1e-30 for this network). Only top 10 most abundant clusters are labeled, including abbreviations of effector domains associated with SAVED domain. The nodes representing two Cap5 of current study are marked with arrows. Also marked are two Cap4 proteins recently studied by Kranzusch and coworkers.



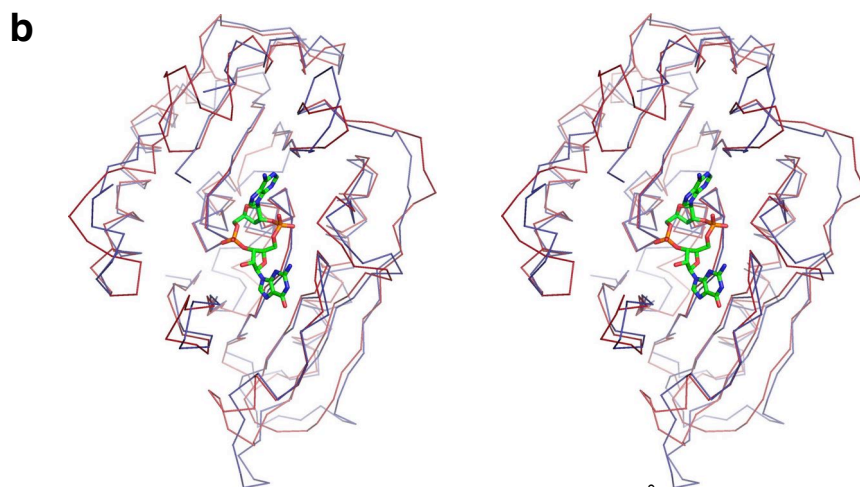
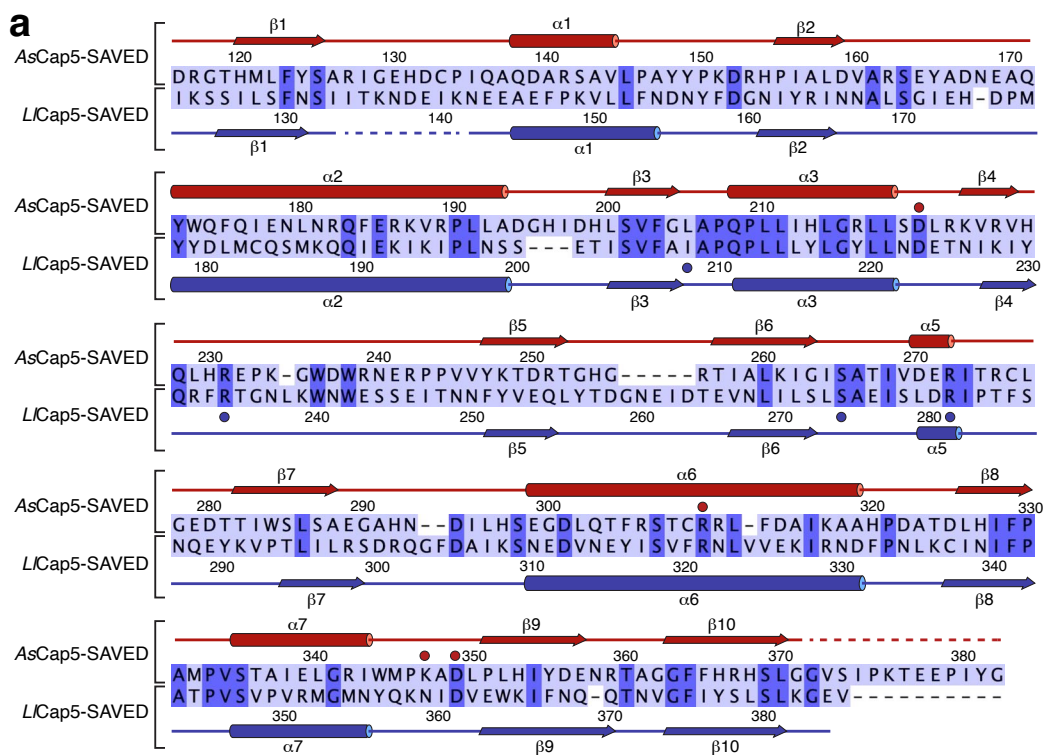
Supplementary Figure 2. Characterization of chemical structures of two compounds synthesized by AsCdnG. **a, b** High-resolution MS of the two compounds produced by AsCdnG shown in Fig. 1b. CM, calculated m/z. **c** UPLC analysis of four commercially purchased cGAMP (top panel) as well as their co-injection with cGAMP synthesized by AsCdnG (bottom panel). **d** UPLC analysis of the digested products of ApG/GpA by P1 nuclease (top panel) and snake venom phosphodiesterase (SVPD) nuclease (bottom panel). P1 nuclease only cleaves 3',5'-phosphate linkage, whereas SVPD is able to cleave both 3',5'- and 2',5'-phosphate linkages. **e** Time course of the reaction carried out by AsCdnG.



Supplementary Figure 3. In vitro DNA degradation by *AsCap5* and *L/Cap5* in the presence of 3',3'-c-di-GMP and 3',3'-c-di-AMP. The experimental conditions were the same as the ones shown in Fig. 2. Results are representative of three independent experiments.

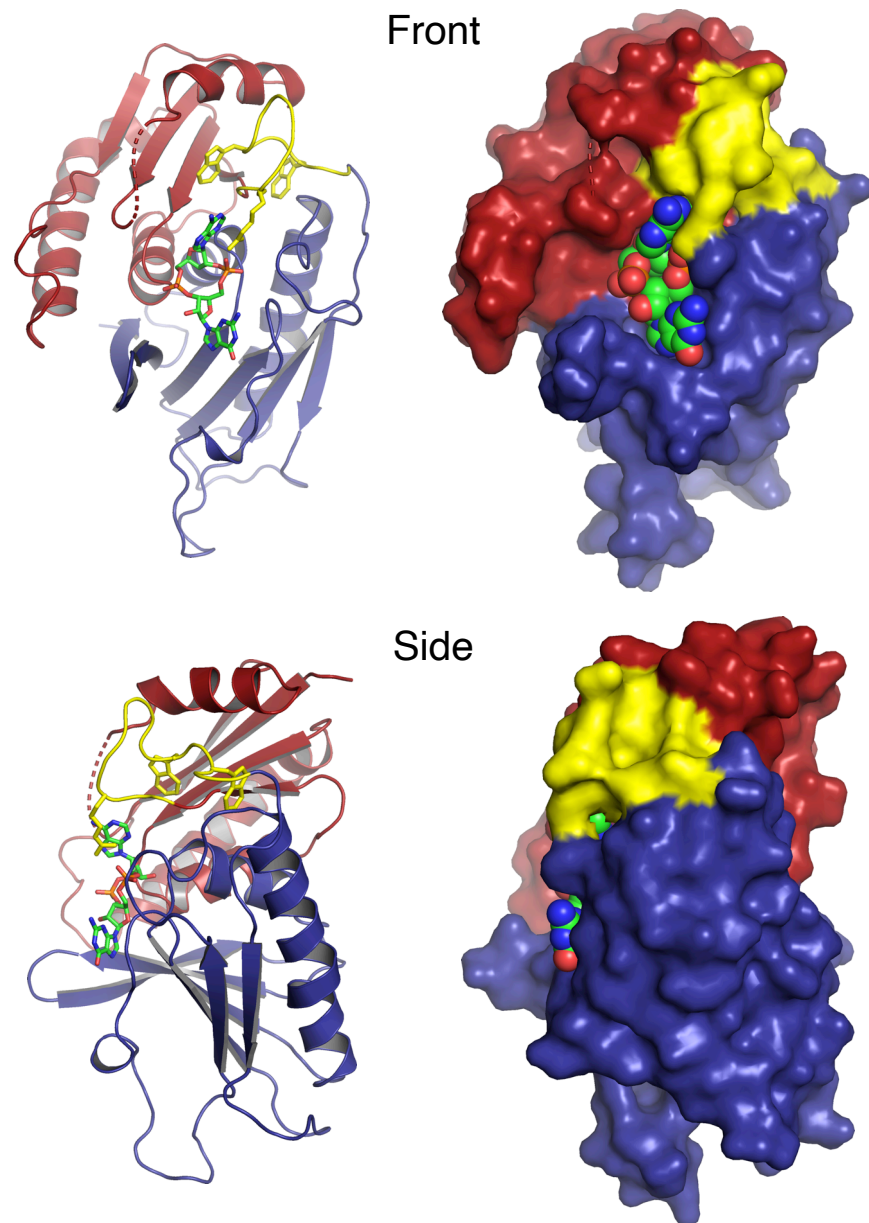


Supplementary Figure 4. Additional structural analysis of Cap5-HNH. **a** Mapping secondary structures of AsCap5-HNH (top) and L/Cap5-HNH (bottom) onto their aligned sequences. Part of the loop between $\beta 1$ and $\alpha 3$ in AsCap5-HNH having steric clash with the modeled DNA•RNA hybrid is colored black. The disordered residues in L/Cap5-HNH are depicted with dashed lines. Zinc finger residues are marked with asterisks. Important residues near the active site of AsCap5-HNH are marked with red circles. **b** Structural alignments of AsCap5-HNH and L/Cap5-HNH homodimers. The structures are depicted in cartoon, with AsCap5-HNH and L/Cap5-HNH colored red and blue, respectively. **c** Structure of AsCap5-HNH near the active site. The Mg^{2+} ion is covered with simulated-annealing F_o-F_c omit map contoured at 3σ . H46 activates a water molecule to cleave DNA. Hydrogen bonding network of the side chain of R56 is likely to be responsible for the conformation of the loop that has steric clash with the modeled DNA•RNA hybrid. **d** Structure of L/Cap5-HNH with the same region as AsCap5-HNH shown in **c**, indicating that L/Cap5-HNH is significantly less structured near the active site.

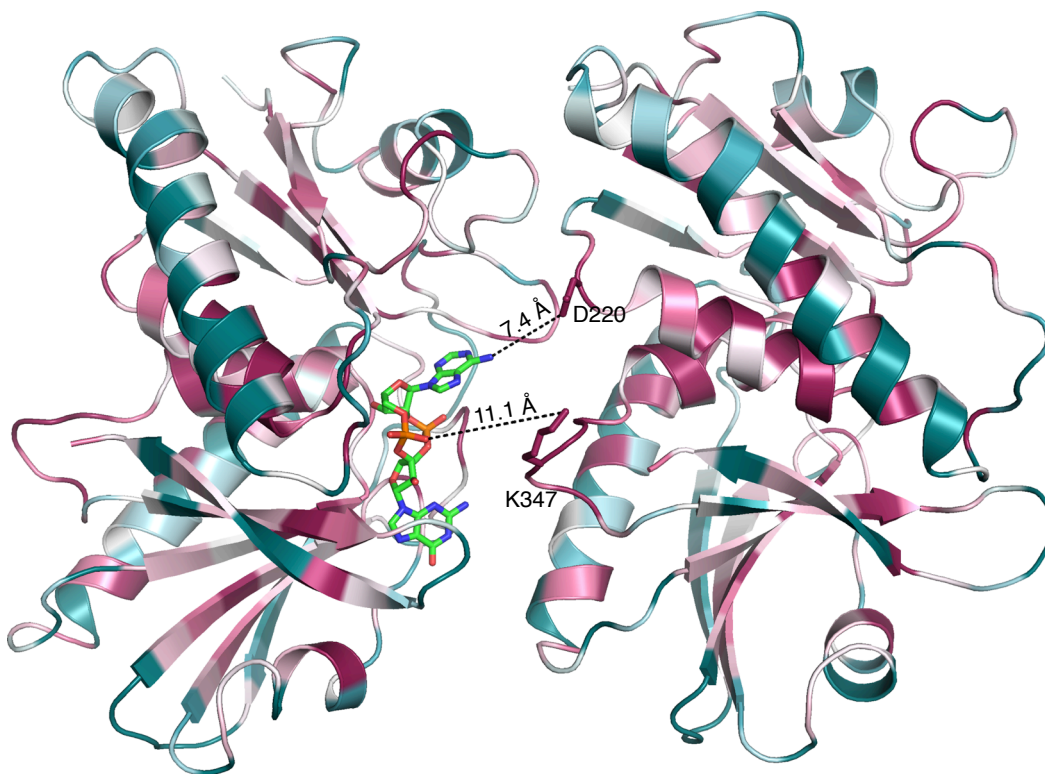


Z score = 30.0; rmsd = 1.9 Å
 23% sequence identity

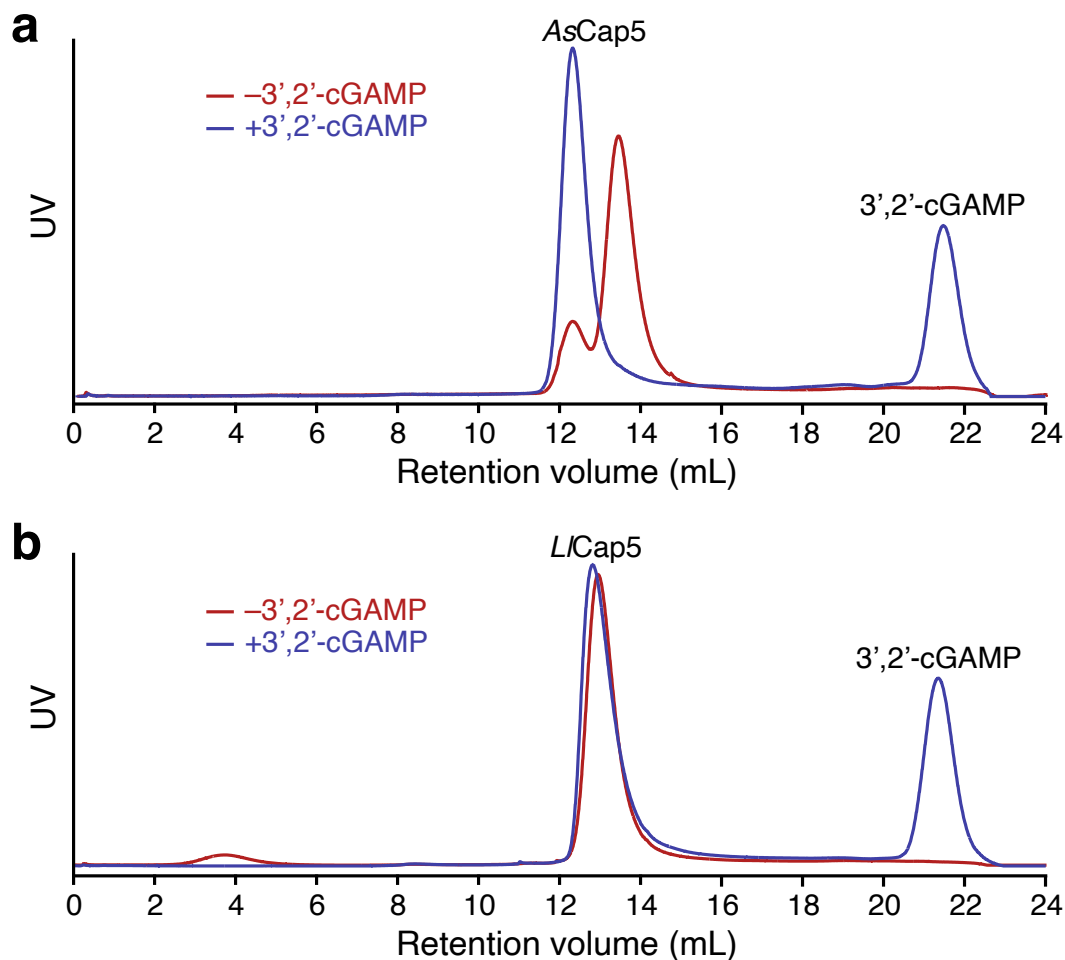
Supplementary Figure 5. Additional structural analysis of the SAVED domains of Cap5. **a** Mapping secondary structures of *AsCap5*-SAVED (top) and *L/Cap5*-SAVED (bottom) onto their aligned sequences. The two SAVED domains only share 23% sequence identity. Residues of *L/Cap5*-SAVED involved in recognition of 3',2'-cGAMP are marked with blue circles. Residues of *AsCap5*-SAVED selected for mutagenesis are marked with red circles. **b** Stereoview of structural superposition of *AsCap5*-SAVED (red) and *L/Cap5*-SAVED (blue). The structures are depicted in ribbon, and the bound 3',2'-cGAMP is in stick.



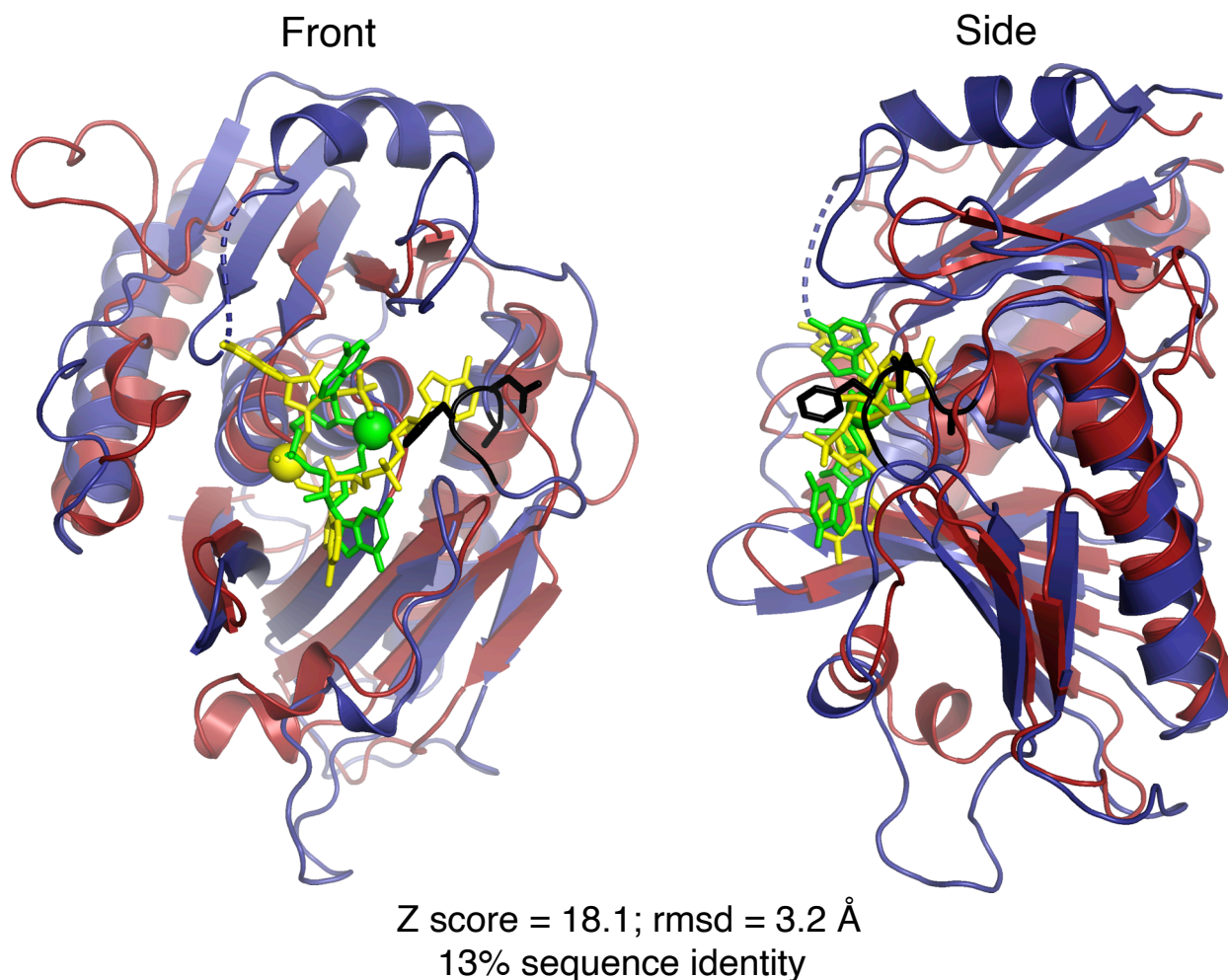
Supplementary Figure 6. Structural analysis of *LICap5*-SAVED-3',2'-cGAMP complex in the context of possible evolutionary origin of SAVED domain. The structures are depicted in Cartoon (left) and Surface (right), and oriented the same as the ones shown in Fig. 4a, b. The first CARF-like motif is colored red, and the second one is colored blue. The linker connecting these two CARF-like motifs is colored yellow. The critical residue R234, as well as the conserved WxW motif, are in the linker region and highlighted in stick. Interactions of WxW motif with its surroundings are likely to play a role of orienting R234 for its interaction with 3',2'-cGAMP.



Supplementary Figure 7. Cartoon depiction of the structure of AsCap5 SAVED dimer. The structure is the same as the one shown in Fig. 5a with the addition of the modeled 3',2'-cGAMP. Two conserved residues, D220 and K347, located at the tip of two extruding loops, are in stick. These two residues are proposed to interact with 3',2'-cGAMP. We speculate that the side chain of D220 might interact with N6 of the AMP moiety, which is 7.4 Å away. We further speculate that the side chain of K347 might interact with the 3',5'-phosphate group of the AMP moiety, which is 11.1 Å away based on the current structure. An optimal rotamer of K347 would reduce the distance to 7.2 Å. Therefore, if 3',2'-cGAMP indeed induce two SAVED domains to form closed homodimer, the relative movement would be approximately 5 Å from the structure shown here.



Supplementary Figure 8. AsCap5 and L/Cap5 do not form oligomers regardless of whether 3',2'-cGAMP is present. Purified recombinant AsCap5 and L/Cap5 were analyzed by analytical size exclusion chromatography in the absence (red curve) and presence (blue curve) of 3',2'-cGAMP. The peaks corresponding to the excess 3',2'-cGAMP were only observed in the samples with 3',2'-cGAMP (blue curves). 3',2'-cGAMP induced the shift of the retention time of AsCap5 (a). On the other hand, 3',2'-cGAMP had no effect on L/Cap5 (b). Because both the size and the shape of a protein affect its retention volume of a gel filtration column, the results shown here are difficult to interpret. It is possible that the two peaks shown in a represent two different conformers of AsCap5 homodimer, and addition of 3',2'-cGAMP shifts the relative populations of these two conformers. One possible interpretation of the results shown in b is that the exchange of conformers of L/Cap5 is too fast to be resolved by size exclusion chromatography. However, one conclusion is certain with these results: both AsCap5 and L/Cap5 do not form oligomers in the presence of 3',2'-cGAMP under the conditions these analyses were carried out. In addition, the fractions collected from both AsCap5 and L/Cap5 samples with 3',2'-cGAMP were active based on DNA degradation assays, demonstrating that 3',2'-cGAMP did not dissociate from the proteins over the course of size exclusion chromatography.



Supplementary Figure 9. Structural comparison of *L/Cap5-SAVED* to *AbCap4-SAVED*. Two superpositioned structures are depicted in cartoon, with *L/Cap5-SAVED* colored blue and *AbCap4-SAVED* colored red. The structures are oriented the same as the ones shown in Fig. 4a and Supplementary Figure 6. The bound ligands are depicted in stick, with 3',2'-cGAMP colored green and 2',3',3'-cAAA colored yellow. The phosphate atoms of the 2',5'-phosphate linkages are highlighted in spheres. Part of the loop of *L/Cap5-SAVED* having steric clash with the third nucleotide of 2',3',3'-cAAA is colored black and depicted in stick.

Supplementary Table 1. Data collection and model statistics

	AsCap5	L/Cap5	L/Cap5- + 3',2'-cGAMP
PDB ID	7RWK	7RWM	7RWS
Data Collection			
Space Group	<i>P2₁2₁2₁</i>	<i>P4₃</i>	<i>C222₁</i>
Cell dimensions a, b, c (Å) α , β , γ (°)	69.3 96.9 131.8 90.0, 90.0, 90.0	180.8 180.8 93.5 90.0, 90.0, 90.0	68.7 126.7 83.4 90.0, 90.0, 90.0
Resolution (Å)	50.0-2.4	50.0-3.4	50.0-1.8
R _{merge} (%)	6.9	6.1	5.2
<i>I</i> / σ	10.3	9.2	16.4
Completeness (%)	99.8	100	99.7
Redundancy	3.8	7.6	5.7
Refinement			
Resolution (Å)	39.7-2.4 (2.48-2.4)	43.8-3.4 (3.52-3.4)	33.0-1.8 (1.86-1.8)
No. reflections	35,452 (3,305)	41,617 (4,116)	33,873 (3,297)
R _{work} /R _{free} (%)	19.3/23.5 (24.4/27.2)	24.9/29.5 (27.5/36.6)	16.6/19.3 (16.8/21.5)
No. atoms Protein Ligand/ion Solvent	6,223 5,858 4 361	11,431 11,427 4 0	2,451 2,073 45 333
Average B factors (Å ²) Protein Ligand/ion Water	39.6 39.6 34.1 40.1	87.4 87.4 83.4 N/A	21.9 20.1 15.2 33.8
R.m.s. deviations Bond lengths (Å) Bond angles (°)	0.010 1.55	0.040 0.88	0.010 1.86
Ramachandran statistics (%) Favored Allowed Outliers	95.5 4.5 0.0	89.4 9.2 1.4	96.4 3.2 0.4

*Values in parenthesis are for highest resolution shell.

Supplementary Table 2. DNA sequences of primers employed to create AsCap5 mutants.

Mutation	Primer name	Primer sequence
K347A	K347A-Fwd	5'-GGT CGG ATA TGG ATG CCA gcg GCT GAT CTT CCT TTA CAC-3'
	K347A-Rev	5'-GTG TAA AGG AAG ATC AGC cgc TGG CAT CCA TAT CCG ACC-3'
K347R	K347R-Fwd	5'-GGT CGG ATA TGG ATG CCA cgg GCT GAT CTT CCT TTA CAC-3'
	K347R-Rev	5'-GTG TAA AGG AAG ATC AGC ccg TGG CAT CCA TAT CCG ACC-3'
K347Q	K347Q-Fwd	5'-GGT CGG ATA TGG ATG CCA cag GCT GAT CTT CCT TTA CAC-3'
	K347Q-Rev	5'-GTG TAA AGG AAG ATC AGC ctg TGG CAT CCA TAT CCG ACC-3'
D349A	D349A-Fwd	5'-CGG ATA TGG ATG CCA AAG GCT gct CTT CCT TTA CAC ATC TAT GAC-3'
	D349A-Rev	5'-GTC ATA GAT GTG TAA AGG AAG agc AGC CTT TGG CAT CCA TAT CCG-3'
R310A	R310A-Fwd	5'-CAG ACA TTT CGC TCA ACT TGC gct AGA TTA TTC GAC GCA ATC AAA GCC-3'
	R310A-Rev	5'-GGC TTT GAT TGC GTC GAA TAA TCT agc GCA AGT TGA GCG AAA TGT CTG-3'
R310K	R310K-Fwd	5'-CAG ACA TTT CGC TCA ACT TGC aag AGA TTA TTC GAC GCA ATC AAA GCC-3'
	R310K-Rev	5'-GGC TTT GAT TGC GTC GAA TAA TCT ctt GCA AGT TGA GCG AAA TGT CTG-3'
R310Q	R310Q-Fwd	5'-CAG ACA TTT CGC TCA ACT TGC cag AGA TTA TTC GAC GCA ATC AAA GCC-3'
	R310Q-Rev	5'-GGC TTT GAT TGC GTC GAA TAA TCT ctg GCA AGT TGA GCG AAA TGT CTG-3'
D220A	D220A-Fwd	5'-CTT GGC AGA CTT TTG AGC gcc TTG CGT AAG GTT CGT GTG-3'
	D220A-Rev	5'-CAC ACG AAC CTT ACG CAA ggc GCT CAA AAG TCT GCC AAG-3'