Supplementary Information

Nucleic Acid Mediated Activation of a Short Prokaryotic Argonaute Immune System

Jithesh Kottur^{1,2*¶}, Radhika Malik^{1*¶}, and Aneel K. Aggarwal^{1¶}

¹Department of Pharmacological Sciences, Icahn School of Medicine at Mount Sinai, New York,

NY, 10029, USA

²Department of Antiviral Drug Research, Institute of Advanced Virology, Thiruvananthapuram,

Kerala, 695317, India

^{*} These authors contributed equally to this work

[¶]Corresponding authors:

E-mail: jitheshkottur@iav.res.in

E-mail: radhika.malik@mssm.edu

E-mail: aneel.aggarwal@mssm.edu

This file includes: Supplementary Figures 1-11 Supplementary Table 1 and 2 Supplementary References



Supplementary Figure 1. Electron density maps of SPARTA domains. 2Fo-Fc maps of (a) APAZ, (b) Short pAgo and (c) TIR domains were shown at a contour level of 1.5 σ . The individual domains are represented as ribbon form. APAZ domain is colored as orange, MID, Insert57 and PIWI domain are colored cyan, yellow and slate, respectively. The TIR domain and connector helix are colored as green and magenta respectivley.



b.



С.



Supplementary Figure 2. Negative stain and cryo-electron microscopy . (a) Representative micrograph of a negatively stained sample of the SPARTA complex. Scale bar is 200 nm. The sample was stained with uranyl formate. (b) Representative 2-D classes after collect-ing data on negatively stained samples of the SPARTA oligomer (c) Representative micrograph of the tilted cryo-EM data of the SPARTA oligomer. This micrograph was imaged by tilting the stage to 30°.



Supplementary Figure 3. Cryo-EM analysis of the SPARTA complex. An overview workflow of the cryo-EM image processing for the SPARTA complex is shown. Schematic shows particle picking using template picker and Topaz. Various stages of data processing includes 2-D classification, Ab-initio clean-up and 3-D classification using cryoSPARC. The number of micrographs collected is highlighted in pink. The number of particles at the various stages of data processing are shown in blue. The map calculated after global refinement has a FSC_{0.143} of 3.35 Å. The 3D FSC plot to access the directional anisotropy is shown and the calculated sphericity is 0.90.



Supplementary Figure 4. Focused refinements of the SPARTA complex. A schematic workflow of the C2 symmetry based focused refinement of the 'wings' (APAZ-MID-PIWI-RNA/DNA) is described. Improvement in the resolution of the 'wings' is shown by the local resolution maps and their respective fourier shell correlation curves. FSC curves are shown and the nominal resolution of the focused maps for the 'wings' are 3.15 Å and 3.17 Å, respectively.



С.



d.



Supplementary Figure 5. Cryo-EM density for key regions of the SPARTA oligomer. (a) Cryo-EM density of the ligand, DNA/RNA duplex in two different orientations (90° apart) is shown. (b) Cryo-EM density of the key regions of MID (cyan) and PIWI (purple) domains of pAgo and APAZ (green) domain which are interacting with the RNA/DNA is depicted. (c) Cryo-EM density of one of the pAgo dimers and a close-up view of a section of the dimer interface is shown. (d) Cryo-EM density of all the TIR domains is shown and a section of the interface from TIR_B and TIR_D is shown in detail.



Supplementary Figure 6. Structural alignment of Apo SPARTA domains with homologous proteins. (a) SPARTA TIR domain structurally aligned to TIR domains from proteins with scaffolding roles (hTLR2^{TIR}, PDB:1FYW and TIR-SAVED, PDB:7QQK) and enzymatic roles (hSARM1, PDB:7NAK and NLR-RPP1^{TIR}, PDB:7DFV). (b) SPARTA APAZ domain structurally aligned with the N-PAZ-L1-L2 regions of *Clostridium butyricum* (Cb) long pAgo (PDB ID: 6QZK). (c) SPARTA MID and PIWI domains of Cb long pAgo (PDB ID: 6QZK).

Sequence alignment between S2A clade short pAgo proteins

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CrtSPARTA	101 TRAVLENASTHKRTYDLVT ENDKI I TANKNDER VDWFVIVEE I WYCRPNSVLPNELVQTKSLISKSKAKSERYTET FEEFNKKLKEVEKE-AKTYNYDAC	FHDQLKARLLE 216
WP_027160976.1	I 118 IERATRIFNLNEAVDKVAR LYIDRVEQHRRNDERSIDVWVLVL PDIVYER CRROSKRTGLAMELGDFKKRQKAKEN - LP - LLS AMGAIDQSDEAIFEDVPC	FHRRIKAEFLR 227
WP_067325603.1	I 118 IERATRIFNLNEAVDKVAR LYIDRVEQHRRNDERSIDVWVLVL PDIVYER CRPOSKRIGVAMELGDFKKRQKAKEN - LP - LLS AMGAIDQSDEAIFEDVPC	FHRRIKAEFLR 227
WP_075634049.1	I 118 IERASRIFNQHEAVDKVSRLYIDRVNKHLRNEERAVDIWILVLPEIVYERCRPESKRTGLATEKGDFGKKQKKRES-LP-LLAGVIDQSDETIFDDVPC	FHRRIKADFLN 225
WP_097544715.1	I 118 IDRVTRVFNQHEAVDKVSRLYIDRVNKHLRNEERTVDVWVLVLPELVYERCRPNSKRAGLATEKGDFAKKQKRRES-LP-LFAGVIDQSDETIFDDVPC	FHRRIKADFLN 225
WP_088201560.1	I 118 IDRATRVTNQHEAVDKVSRLYIDRVNKYLHNEERAVD IVMLVLPEIVYERCRPNSKRTGLPMEQGDFGKRQKRES-LP-LFAGVIDQSDET IFDDVPC	FHRRIKADFLN 225
WP_011971022.1	I 118 IDRATRVTNQHEAVDKVAKLYIERVNKHLHNEERAVDIWVLVLPE VYDRCRPNSKRTGLSTEKGEFAKKQKKRES-LP-LLAGIIDQSGETIFDDVPC	FHRRIKADFLT 225
WP_008877327.1	I 118 IDRATRI INLNEAVDKVAR LYIDRVRQHLRNDERSVDVWILVLPE VYER CRPOSKRTGLPMEKGDFTKRQRAKES - IP - LFS AMGV IDQTEEA I FEDVPC	FHRRIKAEFLK 227
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CrtSPARTA	217 HTIPTQILRESTLAWRDFKNTFGAPIRDFSKIEGHLAWTISTAAYYKAGGK - PWKLGDIRPGVCYLGLVYKKIEKSKNPQNAOCAAQMFLDNGDGTVFKGEVGPW	NPEKGEYHLKP 332
CrtSPARTA WP_027160976.1	217 HT IPTQILRESTLAARD FK NTFGAPIRDFSKIEGHLAAVT ISTAAYYKAGGK - PWKLGDIR PGVCYLGLVYKKIEKSKN PONACCAAQMFLDNGDGTVFKGEVGPWN 1228 - IAPTQLVRETTLAPGAFLNKANYPTRKT-QDAATVAAVNLATGLYYKTQPKPPWRLADVRPGVCYIGMVYKSLPNDPD-GHACSAAQMFLNEGDGVVFRGANGPW	NPEKGEYHLKP 332
CrtSPARTA WP_027160976.1 WP_067325603.1	217 HTIPTOILERESTLAVADENN TFGAPIRDFSKIECHLAWTISTAAYYKAGGK-PWKLGDIR PGVCYLGLVYKKIEKSKNPONACCAAOMFLDNBDGTVFKGEVGPW 228 - IAPTOLVRETTLAPGAFLNKANYPTRKT-ODAATVAVNLATGLYYKTOPKPPWRLADVRPGVCYIGMVYKSLPNDPD-GHACSAAOMFLNBDGVVFRGANGPW 228 - IAPTOLVRETTLAPGAFLNKANYPIRKT-ODATVAVNLATGFYYKTOPKPWRLADVRPGVCYIGLYYKSLPNDPD-GHACCAAOMFLNBDGVFRGANGPW	NPEKGEYHLKP 332 TGD - YEYHLKA 340 TGD - YEYHLKA 340
CrtSPARTA WP_027160976.1 WP_067325603.1 WP_075634049.1	217 HTIPTOILRESTLAVADEKNTFGAPIRDFSKIEGHLAWTISTAAYYKAGGK-PWKLGDIR PGVCYLGLVYKKIEKSKNPONACCAAOMFLDNEDGTVFKGEVGPW 1228 - IAPTOLVRETTLAPGAFLNKANYPTRKT-QDAATVAWNLATGLYYKTOPKPPWRLADVRPGVCYIGUVYKSLPNDPD-GHACSAAOMFLNEGDGVVFRGANGPW 1228 - IAPTOLVRETTLAPGAFLNKANYPIRKT-QDAATVAWNLATGLYYKTOPKPPWRLADVRPGVCYIGLVYKSLPNDPD-GHACCAAOMFLNEGDGVVFRGANGPW	NPEKGEYHLKP 332 TGD - YEYHLKA 340 TGD - YEYHLKA 340 TGD - YEYHLKR 338
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CrtSPARTA WP_027160976.1 WP_067325603.1 WP_075634049.1 WP_097544715.1 WP_088201560.1 WP_011971022.1 WP_008877327.1	217 HTIPTOILERESTLAVADEKNTFGAPIRDFSKIEGHLAWTISTAAYYKAGGK-PWKLGDIRPGVCYLGLVYKKLEKSKNPONACCAAOMFLDNEDGTVFKGEVGPW 228 - IAPTOLVRETTLAPGAFLNKANYPTRKT-QDAATVAWNLATGLYYKTOPKPPWRLADVRPGVCYIGMVYKSLPNDPD-GHACSAAOMFLNEGDGVVFRGANGPW 226 - IAPTOLVRETTLAPGAFLNKAGYPIRKT-QDRATVAWNLATGLYYKTOPKPWRLADVRPGVCYLGMVYKSLPNDPD-GHACCAAOMFLNEGDGVVFRGANGPW 226 - IAPTOLIRETTLAPGAFLNKAGYPIRKT-QDRATVAWNLATGLYYKTOPRPPWRLATIRPGVCYLGMVYKSLPNDPD-GHACCAAOMFLNEGDGVVFRGANGPW 226 - IAPTOLIRETTLAPGAFLNKAGYPTRKT-QDRATVAWNLATGLYYKTOPRPPWRLSTIRPGVCYLGMVYKSLPNDPD-GHACCAAOMFLNEGDGVVFRGANGPW 226 - IAPTOLIRETTLAPGAFLNKAGYPTRKT-QDRATVAWNLATGLYYKTOPRPPWRLSTIRPGVCYLGMVYKSLPNDPD-GHACCAAOMFLNEGDGVVFRGANGPW 226 - IAPTOLIRETTLAPGAFLNKAGYPTRKT-QDRATVAWNLATGLYYKTOPRPPWRLSTIRPGVCYLGMVYKSLPNDPD-GHACCAAOMFLNEGDGVVFRGANGPW 226 - IAPTOLIRETTLAPGAFLNKAGYPVRKT-QDRATVAWNLATGLYYKTOPRPPWRLSTIRPGVCYLGMVYKSLPNDPD-GHACCAAOMFLNEGDGVVFRGANGPW	NPEKGEYHLKP 332 TGD - YEYHLKA 340 TGD - YEYHLKA 340 TGD - YEYHLKR 338 TGD - YEYHLKR 338 TGD - YEYHLKR 338 TGD - YDYHLKA 338 TGD - YDYHLKA 340

Insert 57

Sequence comparison between S2B clade short pAgo proteins with CrtSPARTA

	insert 57
CrtSPARTA	101 IRRYLFNASTHKRTYDLYTLFNCKIITANKNDEERVDWFVIVPEETYKYCRPNSVLPNE-LVQTKSLISKSKAKSFRYTPTLFEEFNKKLKEV KEARTYNYDAOFHDQLKARLLE 216
WP_092996166.1	115 LAHALR-GDAYQAFQRVVDLYSDAHLRLASRDLNRPDIVLNCIPDDVFSKVSSVERK-ATETERKRARE IRRARPSNQLDLFD-TLDEV COPPOF-LKRDLRLALKARALR 222
WP_008706031.1	115 LSEQLTKRDRFERFQEIVDIFVSGITSLAKRDTNRPDVVLICLPKSISLKARTVDRE-LSQEEKKVAKHIEDQQGLRQLELFD-LSEEIEQTKEDL-LKRDLRHAKALE 223
WP_202795867.1	117 IADA RSDEPLVRFTKVLGLYDDALKQLASRE INRPD IVLVC I PPDVRERAGNV TKE - LSDEAKAVAKSFAK RQKSRQLDLLD - LLETVEGPDDL - IKRDLHNALKAKALK 225
WP_218823222.1	110 LTDALAIQDDSLRFDTVLNLYNKGLEKLSTLESTRPNVIFCCLPENLVDACHHVERK-LTKDEKSELKKMAS-ANKPGFQFSLFDE-QIEDTEEDL-LYRDFRALKAKAIL 217
WP_029585865.1	111 FAEALETANPSQRFDLVLSMYQSSIRRLSKLESTRPDVVVCCVSDEVLVKCRNVTKT-LTREEKRAAEALKKRKASTQYDMFD-EMAIDEAQPEDL-LYRDFRRALKARAMK 219
WP_059000177.1	112 LEQALQL - KP I DR FQK VLEL YVHG I ER I ANLD - VR PD VVVCCL PDAVVRDCWSV TNHNL TPHELKK IW- QQ QRQP TVQLSL FE - I S - DV EKAEDL - L TRS FRALKAEVMK 217
WP_013581438.1	113 LDVALALVDPKLRFETVLDVFSTGIKRLAESEATEPDVIFCCIPDDLIAKCASIENS-LTEEDRTAAKALRKRKVDNQLALFE-AE-ALEEOPEDL-LRRDFRALKARAMR 220
CrtSPARTA	217 HTIPTOILRESTLAWRDFKNTFGAPIRDFSKIECHLAMTISTAAYYKAGGKPWKLCDIRPGVCYLCLVYKKIEKSKNPQNACCAAQMFLDNCDGTVFKGEVGPWYNPEKGEYHLKPK333
WP_092996166.1	223 NRLPIQLVTDALLSDTAKNQDPATRAWNFAVGLYYKAGGVPWRLPPTGPDTCFVCISFHHFRTTQRAIVRSSLAQAFSSDGEGFAIRGEGVPVEPEQGRNVHLSEQ 328

WP_092996166.1	223 NRLPIQLVTDALLS DTAKNQD	PATRAWNFAVGLY	YYKAGG VPWRLPPTGPDTCF	VGISFHHFRTTQRAIVRSSLAG	AFSSDGEGFAIRGEGVPVEPEQGRNVHLSEQ 328
WP_008706031.1	224 LRLPIQIVTPSLTE DSGTNQE	PATRAWN FSVAL)	YYKSGGVPWRLPVGEVETCF	VGISFHHFRTTMRHMVHSSLAG	AFSSEGEGFAIRGEAVPADPDQRRNTKLTER 329
WP_202795867.1	226 YRLPIQIVTRKLLH ETNESED	PATRAWN FSVGL∖	YYKSGG VPWRLRPEGPQTCF	VGVTFHHVERAKSHIVRSSLAF	AFSSDGEGFAIRGGGVPVEPGQALNVHLSYE 331
WP_218823222.1	218 VNIPIQIGRDKIFL DLADNQD	AAARAWNSSVALY	YYKAGG I PWRLRAHD VETCF	VGITFHHLRTNIKQVVKSCIAC	GFSSDGEGFALRGSDVEYNPRASRAVHLSSA 323
WP_029585865.1	220 EQMPIQIATGGLFVDGRSGQG	····SATRAWNSSVGL∖	YYKSGG I PWRLKTEGP ETCF	VGISFNHIKTTERHLVKSSIAG	AFSSQGEAFALRGGDIEWTEEQGRAVHLTGE 325
WP_059000177.1	218 HRMPIQIATTNLLVDGKANQD	AATRAWNSCLGIF	FYKSGG I PWRLALDGP ETCF	VGISFHHLKTNKRHLVYSSLAG	AFSTEGDGFALRGDFVPWDEDQGRTPHLSEE 323
WP_013581438.1	221 SRVPVQLATNGLVL DGASGQG	PATRAWNSCVGLY	YYKAGG I PWRLRANGPETCF	VGVSFHHLQTTKRHLVHSSIA	AFSNOGEGFALRGGSVDWSDEQGREVHLSSE 326

Sequence comparison between S1B clade short pAgo proteins with CrtSPARTA

CrtSPARTA	101 IRRYLFNASTHKRTYDLVTLFNDKIITANKNDEERVDW	MFVIVPEEIYKYCRPNSVLPNELVQTKSLISKSKAKSFRYTPTLFEEFNKKLKEVEKEAKTYNYDAQFHDQLKARLLE 216
WP_274305503.1	120 L	NQFSGDGDPQARL-FQAMDAALRRLDTMRNEFDVVLVHLPDSWGPATRAKFFDAHDALKALGAK 183
WP_056152151.1	119 L	GELPGDDDAQTRL-FQAMDTALRRLETVRNDFDVVLVHFPDSWAPATRGRFFDAHDALKALGAK 182
WP_175224223.1	121	YDLGQGDTVSERV-RAALHHALRRLEAARAEFDVALVYFPDRWLAHLRTKEFDAHDELKALGAQ 184
WP_056176878.1	120	GQLPGAGDPQARL-FRAMDAALRRLEALRNEFDVVLVHFPDAWLPVTRTQDFDAHDALKALGAK 183
WP_083842423.1	120	SELPGDGNPNAKL-FRAMDAALRRLDNVRNDFDVVLVHFPDSWASATRAKLFDAHDALKALGAK 183
WP_013078837.1	119 🗖	
WP_083592923.1	119 🗖	
WP 062341845.1	120 L	ADDGDGDDGARL-FLSMEAALRRLDSVRDTFDVALVHFPDAWAPATRSNVFDAHDALKALGAR 183
	-	·
CrtSPARTA	217 HTIPTQILRESTLAWRDFKNTFGAPIRDFSKIEGHLAW	TISTAAYYKAGGKPWKLGDIRPGVCYLGLVYKKIEKSKNPQNACCAAQMFLDNGDSTVFKGEVGPWYNPEKGEYH 329
- CrtSPARTA WP_274305503.1	217 HTIPTQILRESTLAWRDFKNTFGAPIRDFSKIEGHLAW 184 YN I PTQVINDRVFSFN	TISTAAYYKAGGKPWKLGDIR PGVCYLGLVYKK I EKSKNPQNACCAAQMFLDNGDGTVFKGEVG PWYNPE KGEYH 329 RLAIALYVKAAGTPWKLAPLKGVPVDTAY I GLAYALRGNQRDAHYVTCCSQVFDMDGGGMQFVAFEARDPVAD I AEARRNPF 288
- CrtSPARTA WP_274305503.1 WP_056152151.1	– 217 HT I PTQ I LRESTLAWRDFKNTFGAP I RDFSK I EGHLAW 184 YN I PTQVINDRVFSFN	TISTAAYYKAGGKPWKLGDIR PGVCYLGLVYKKIEKSKNPQNACCAAQMFLDNODGTVFKGEVG PWYNPE KGEYH 329 RLAIALYVKAAGTPVKLAPLKGVPVDTAYIGLAYALRGNQRDAHYVTCCSQVFDMDGGGMQFVAFEARDPVADIAEARRNPF 288 RLATALYVKAAGTPVKLAPLNGVPADTAYYGLAYALRGDQRNAHFVTCCSQVFDMDGGGMQFVAFEARDPVVDVAEARRNPF 287
CrtSPARTA WP_274305503.1 WP_056152151.1 WP_175224223.1	– 217 HTIPTQILRESTLAWRDFKNTFGAPIRDFSKIEGHLAW 184 YNIPTQVINDRVFSFN 183 YNIPTQVLNDRVFTFK 185 LGIPTQVINDRVFTFK 185 LGIPTQVINDKSLKFG	TISTÄÄ YYKÄÄGEK PWIKLEDIR POVCYLELVYKKIIEKSKNPONACCAAOMIFLDNODOTVFKOEVOS-PWYNPE KOEYH 329 RLAIALYVKAAOTPWIKLAPLKOVPVDTAYIIGLAVALRONORDAHYVTCCSOVFDMDOGGMOFVAFEARDPVADIAEARRNPF 288 RLATALYVKAAOTPVIKLAPLNOVPADTAYVGLAVALRODORNAHFVTCCSOVFDMDOGGMOFVAFEARDPVDVAEARRNPF 287 RLSVALYAKAOGTPVIKLAPLEGIPEDTAVIIGLAVAIRRSDEAHYVTCCSOVFDMDOGGMOFVAFEARDPVDVAEARRNPF 289
CrtSPARTA WP_274305503.1 WP_056152151.1 WP_175224223.1 WP_056176878.1	– 217 HTIPTQILRESTLAWRDFKNTFGAPIRDFSKIEGHLAW 184 YN IPTQVINDRVFSFN 183 YN IPTQVLNDRVFTFK 185 LGIPTQVINDKSLKFG 185 LGIPTQVINDKSLKFG 184 YN IPTQVLNDRVFTFP YKASLAW	TISTAA YYKAAGOK PWKLODIR PGVC YLGLVYKK IEKSKNPONACCAAOMFLDNODG TVFKGEVG PWYNPE KGEYH 329 RLAIAL YVKAAG TPWKLAPLKGVPVD TAY IGLAYALRONORDAH YYTCCSOVFDMDOGGMOFVAFE ARD PVAD IAEARRNPF 288 RLATAL YVKAAG TPWKLAPLNGVPAD TAY VGLAYALRODORNAH FVTCCSOVFDMDOGGMOFVAFE ARD PVVDVAEARRNPF 289 RLSYAL YAKAAG TPWKLAPLEG IPED TAY IGLAYALRODORDAH YVTCCSOVFDMEOGGMOFVAFE ARD PVADLAEARRNPF 289 RLATAL YVKAAG TPWKLAPLEG IPED TAY IGLAYALRODORDAH YVTCCSOVFDMEOGGMOFVAFE ARD PVADLAEARRNPF 289
CrtSPARTA WP_274305503.1 WP_056152151.1 WP_175224223.1 WP_056176878.1 WP_083842423.1	– 217 HTIPTQILRESTLAWRDFKNTFGAPIRDFSKIEGHLAW 184 YN IPTQVINDRVFSFN 183 YN IPTQVLNDRVFTFK 185 LGIPTQVINDKSLKFG 184 YN IPTQVLNDRVFTFP 184 YN IPTQVLNDRVFTFP HTASLAW	TISTAA YYKAGGK PWKLGDIR PGVC YLGLVYKK I EKSKNPONACCAAOMFLDNOD GTVFKGEVG PWYNPE KGEYH 329 RLAIAL YVKAAG TPWKLAPLKGVPVDTAY I GLAYALRCNORDAH YYTCCSOVFDMD GGGMOFVAF EARDPVAD I AEARRNPF 288 RLATAL YVKAAG TPWKLAPLNGVPADTAY I GLAYALRGDORNAH FVTCCSOVFDMD GGGMOFVAF EARDPVVD VAEARRNPF 289 RLATAL YVKAAG TPWKLAPLEG I PEDTAY I GLAYALRGDORDAH YVTCCSOVFDME GGMOFVAF EARDPVAD LAEARRNPF 288 RLATAL YVKAAG TPWKLAPLKGVPEDTAY I GLAYALRGDORDAH YVTCCSOVFDMD GGMOFVAF EARDPVADLAEARRNPF 288 RLATAL YVKAAG TPWKLAPLKGVPEDTAY I GLAYALRGDORDAH YVTCCSOVFDMD GGMOFVAF EARDPVADLAEARRNPF 288
CrtSPARTA WP_274305503.1 WP_056152151.1 WP_175224223.1 WP_056176878.1 WP_083842423.1 WP_013078837.1		TISTÄA YYKAAGEK PWKLEDIR PGVCYLELVYKKIEKSKNPQNACCAAOMFLDNODETVFKEEVE PWYNPE KGEYH 329 RLAIALYVKAAGTPVKLAPLKGVPVDTAYIGLAYALRGNQRDAHYVTCCSQVFDMDGGGMOFVAFEARDPVADIAEARRNPF 288 RLATALYVKAAGTPVKLAPLNGVPADTAYVGLAYALRGDQRNAHFVTCCSQVFDMDGGGMOFVAFEARDPVDVAEARRNPF 287 RLSVALYAKAGGTPVKLAPLEGIPEDTAYIGLAYALRGDQRDAHYVTCCSQVFDMEGGMOFVAFEARDPVDVAEARRNPF 288 RLATALYVKAAGTPVKLAPLRGVPEDTAYIGLAYALRGDRDAHYVTCCSQVFDMDGGGMOFVAFEARDPVADVAEARRNPF 288 RLATALYVKAAGTPVKLAPLKGVPADTAYIGLAYALRGDRDAHYVTCCSQVFDMDGGGMOFVAFEARDPVADVAEARRNPF 288 RLATALYVKAAGTPVKLAPLGVPADTAYIGLAYALRGDKRDAHYVTCCSQVFDMDGGMOFVAFEARDPVADVAEARRNPF 288 RLATALYVKAAGTPVKLAPLGVPADTAYIGLAYALRGDKRDAHYVTCCSQVFDMDGGMOFVAFEARDPVADVAEARRNPF 288
CrtSPARTA WP_274305503.1 WP_056152151.1 WP_056176878.1 WP_058176878.1 WP_083842423.1 WP_013078837.1 WP_083592923.1	T T	TISTÄÄ YYKÄÄGEK PWIKLEDIR POVCYLELVYIKKI EKSKNPONACCAADMIFLDNOD GTVIFKEEVG PWYNPE KGEYH 329 RLAIAL YVKÄÄGET PWIKLAPLKOVP DTAYI GLAYALRONORDAH YVTCCS OV FDMD OG GMOFVAFE ARD PV DVA LAEARRNPF 288 RLATAL YVKÄÄGET PWIKLAPL KOVP DTAYI GLAYALROD ORNAH FVTCCS OV FDMD OG GMOFVAFE ARD PV DVA EAARRNPF 289 RLATAL YVKÄÄGET PWIKLAPLROVP DTAYI GLAYALROD ORNAH SVTCCS OV FDMD OG GMOFVAFE ARD PV DVA EAARRNPF 289 RLATAL YVKÄÄGET PVIKLAPLROVP ED TAYI GLAYALROD ORDAH YVTCCS OV FDMD OG GMOFVAFE ARD PV AD LAEAARRNPF 288 RLATAL YVKÄÄGET PVIKLAPLROVP ED TAYI GLAYALROD ORDAH YVTCCS OV FDMD OG GMOFVAFE ARD PV AD LAEAARRNPF 288 RLATAL YVKÄÄGET PVIKLAPLROVP AD TAYI GLAYALROD ORDAH YVTCCS OV FDMD OG GMOFVAFE ARD PV AD LAEAARRNPF 288 RLATAL YVKÄÄGET PVIKLAPLOVP AD TAYI GLAYALROD ORDAH YVTCCS OV FDMD OG GMOFVAFE ARD PV AD LAEARRNPF 288 RLATAL YVKÄÄGET PVIKLAPLKOVP AD TAYI GLAYALROD ORDAH YVTCCS OV FDMD OG GMOFVAFE ARD PV AD LAEARRNPF 287

Sequence comparison between S1A clade short pAgo proteins with CrtSPARTA

	Insert 5/	
CrtSPARTA	102 RRYLFNASTH-KRTYDLVTLFNDKTITANKNDEE-RVDW/FVIVPEEIYKYCRPNSVLPNELVQTKSLISKSKAKSFRYTPTLFEEFNKKLKEVEKEAKTYNYDAGFHDOLKAR	213
WP_078814669.1	681 MLGLDDNL-I-GEPINAAKDLAQRICRSLDVIR-SLRPAAVVAIF	749
WP_075793711.1	688 AQN INDRV-E-GDRLEAAKSLAQRLCRALDN IR-SST-RPGAVTLIF	757
WP_200903946.1	684 /MINLDDSSLP-SNSLEAAKTLGRRICNELNVIR-SLN-PGSVVVIF	753
WP_059982501.1	686 MLDVDD I L - P - SDVLSAAKKLAQRIICRGLDA I R - SLN - PRAVA I VY	754
WP_101237571.1	678 / RF VDEARSA - QKA I DNAKNLANR I CYELDSLK - KLELRTGTV / I I Y	749
WP_067556984.1	702 /MTCPEPAARNAAEGSLEVARL DRS IEALQ-SSY - APD VLIY	770
WP_006746222.1	700 /VALCAEPSNTGGHTGQGPLSVAQH TGAIEALR-SSY-APH VLAF	770
WP_047866596.1	687 MASCSEPDAG-LAPERAGPELGREILAS IDSLE-SSANPNYVLVF	756
CrtSPARTA	214 LLEHTIPTOILRESTLAW-RDFKNTFGAPIRDFSKIEGHLANTISTAATYKAGGKPMKLGDIRPGVCYLGLVYKKIEKSK-NPONACCAAOMFLDNODGTVFKGEVGPWYNPE	324
WP_078814669.1	750 AARHGQSTOFIREKTTLG-SQACRTRWVLSLALYAKAQRTPVRLDCLDDETAFVGLGYSVDSAASLGNHILLGCSHLYNARGECLQFRLGRIENPVF	845
WP_075793711.1	758 AARAGLSTOFLRAETVQS-PQPCRVRWLSLALYAKALRTPMRDCIDDETAYVGIGYSIDQSADNGSHILLGCSHLYSSROEGLQFRLGRLENSII	853
WP_200903946.1	754 AARMGLSTOFIREETMAG-GQKCRIYWWLSLALYVKALRSPWRDCIDDQTAYVGIGYSIDQEASIGNHVLLGCSHLYSARGEGLQFRLGRIENPIY	849
WP_059982501.1	755 AARAGLSTOLIREKTAGS-TQ	850
WP_101237571.1	750 AAQQGISTOFIREKTVNS-FQSCRVKWWLSLAFYVKAMRTPWRLESIDNQTAFVGIGYSVNRTTYPENSKRIILGCSHIYSARQEGMQFQLGRIENPII	847
WP_067556984.1	771 CVQRGIATOFLNEDTLSD-NQQCRWWWLSLALTVKOMRTPALDNLADNAAFVGLGFSIDKSAQRODHVVLGCSHIYSARGEGLQYRLSKVENPII	866
WP_006746222.1	771 AVQRGVATQFLTEDTLSD-TQQCRWWWLSLALYVKGIRTPWVLSGLDEDAAYVGLGFSIDRNAAKGNHVVLGCSHIYSARGEGLQYRLSKVENPIM	866
WP_047866596.1	757 CVQRGIGTQFLRERTLGK-AHQGEISWWLALQCYVKAMRTPWLLADMDPDLAFIGLGFSLDPTAGRGRQVLMGCSHVYNSEGLGLSYKLGKLENSVM	852

Sequence alignment between short pAgo (CrtSPARTA) and long pAgo

CitSPARTA	98 DEETRRYLFN-ASTHKRTYDLVTLFNDKIITANKNDE-ERVDVWFVIVPEE 148
CbAgo	36 KYISKNLMNIGFKPSECIFESYKLGDITEYK-ATARKLKEHEKVGFVIAVIPDM 88
TtAgo	42 PEFLRRALLR.AFGASGASLRLHTLHAHPSQGLAFR.EALRMAKE.EGVQAVLVLTPPM 97
	Insert 57
CitSPARTA	147 IYKYCRPNSVLPNELVQTKSLISKSKAKSFRYTPTLFEEFNKKLKEVEKEAKTYNYDAQFHD 208
CbAgo	89 NELEVENPYN 98
TtAgo	98 AWEDRN 103
CitSPARTA	209 QLKARLLEHTIPTQILRESTLAWRDFKNTFGAPIRDFSKIEGHLAWTISTAAYYKAGGKPWK 270
CbAgo	99 PFKKVWAKLNIPSQMITLKTT ·· EKFKNIV ·· ·· D· · KSGLYYLHNIALNILGKIGGIPWI 151
TtAgo	104 RLKALLLREGLPSQILNVPLR ·· EEERHRW ·· ·· · E ·· NALLG ·· ·· ·· LLAKAGLQVVA 148

Supplementary Figure 7. Sequence alignment of CrtSPARTA pAgo with short and long pAgos. CrtSPARTA pAgo (of clade S2A) aligned with all short pAgo clades (S2A, S2B, S1B, and S1A), as well as long pAgos (*Clostridium butyricum* CbAgo and *Thermus thermophilus* TtAgo). Insert57 in the short pAgo clades is highlighted in a dashed red box. Sequence alignments were conducted using Jalview software¹.



Supplementary Figure 8. Conformational changes of the TIR domain in the SPARTA heterodimers. Comparison of the TIR and APAZ domains in the inactive Apo SPARTA heterodimer versus the heterodimers A-D in the activated SPARTA oligomer. In the heterodimers B and D, the TIR domain undergoes ~175° rotation, as shown in the figure. The location of the BB-loop is marked to identify the conformational change.



SPARTA^{TIR} AbTir^{TIR} (PDB: 7UXU)



His9 Tyr568

Arg569 Arg71

b. SPARTATIR modeled with NAD*



C. Sequence alignment of enzymatically active prokaryotic and eukaryotic TIR domains with CrtSPARTA

	1	10		20	30		40	5	0	60		70	80	90	100	
CrtSPARTA-TIR	MRNKIFI	**I* SHATPED	-DDFTR	-WLSLKLI	GLGYEV-		.FLDKGV	D FWS T	I Keirent	CKFLIVS	STAGN	KREGVL	KELAVATKV	KKHLQD-DMF	IIPLAIDENL -	syddi
TirS-WP_000114516.1	IEYDVFL	SHSSLDK	- EDYV S	-KISEKLI	EKGLKV-	• FEDVK	VFEIGK	SQTETM	MGILNSR	FVVVFLS	PNFIE	SGWSR	YEFLSFLNR	EINEEHVI	ILPIWH KV	SVEDVRA
TcpO-WP_074798936.1	KEYDIFV	SHSSEDK	- EDFVK	-EFVNLLK	QKGLSV-	WYDDD	IVKIGH	INLRKRIS	SKGIKSSN	IYAVVIFS	EDFFK	SKWTN	YEYDNIFL-I	DFYD-EEK	VLPILHDL·	TIEDLEM
BtpA-WP_004684737.1	EEYDFFI	SHASEDK	-EAFVQ	-DLVAALR	DLGAKI-	FYDAY	TLK∨GD	SLRRKID	QGLANSK	FGIVVLS	EHFFS	KQWPA	RELDGLTAM	EIGG-QTR	ILPIWH KV	SYDEVRF
AbTIR-EJB8469465.1	PEYDLFI	SHASEDK	-EDFVR	- PLAETLG	QLGVNV-	WYDEF	TLKVGD	SLRQKID	SGLRNSK	YGTVVLS	TDFIK	KDWTN	YELDGLVAR	EMN G - HKM	ILPIWHKI-	TKNDVLD
ThsB-WP_001129548.1	KRVFF	SFHYQDV	- IDFRVNVVR	NHWVTKLN	QSAAGVFE	DASLWEDAK	-KTSD I	ALKRLIN	IGGLNNTS	VTCVLIG	SQTFN	RRWVR	YEIMKSIE-	K - GNK	IIGIHI NAF	FKDKYGNIKS
BdTIR-XP_003560074.3	SRYEVEI	NHRGVD T	KRTVAR	- LLYDRLA	QAGLRG-	FLDNM	ISMRPGD	RLEERIC	SAIRECT	VAVAIFS	PSYCD	SEYCL	RELAMLVE -	S-RKA	IIPIFYDI-	KPSDLLL
BtTIR-WP_195383607.1	KQYDFFI	SHASEDK	-DD I V R	-DLAEALR	NNGFEV-	· WYDEF	ELKIGD	SLRKKID	YGLSNAN	IYGIVIIS	PSFVK	KNWTE	YELNGMVAR	EMN G - HK V	ILPIWHKI-	TKDEVLR
RUN1-KAJ9687320.1	TTYDVFL	SFRGEDT	RFNFTD	-HLYSALG	RRGIST-	FRDDK	- LSRGE	AIAPELL	NATEKSR	SSVIVES	ENYAR	SRWCL	DELVKIMEC	HKD L - GHA	VEPIEYHV-	DPSHVRK
RPP1-NP_001326419.1	WKHDVFP	SFHGADV	RRTFLS	-HIMESFR	RKGIDT-	F I DNN	I - I ERSK	SIGPELP	EATKGSK	TATVLLS	RKYAS	sswcL	DELAEIMKC	RQM V - GQ I	VMTIFYEV-	DP TD I KK
SARM1-NP_055892.2	DTPDVFI	SYRRNSG	-SQLAS	-LLKVHLG	LHGFSV-	FIDVE	KLEAGK	- FEDKL	I QS VMGAR	NEVLVLS	PGALDKCN	1QDHDCKDWVH	KEIVTALS-	C - GKN	IVPIIDGF-	EVVP E P Q \

Supplementary Figure 9. Comparison of TIR active sites and modeling of NAD⁺. (a) Structural superposition of SPARTA active site formed across the TIR_A and TIR_B heterodimers with that in AbTir (PDB:7UXU) (left) and SARM1 (PDB: 7NAK) (right) containing bound 3AD. (b) TIR domains of SPARTA heterodimers modeled with NAD⁺ and a close-up view of the active site showing the putative residues interacting with NAD⁺. (c) Sequence alignment of enzymatically active prokaryotic and eukaryotic TIR domains with SPARTA^{TIR}. The active site residues are highlighted with asterisks (*) and show conservation among members of enzymatically active TIR domains from bacterial (TirS, TcpO, BtpA, AbTIR, ThsB, BdTIR, BtTIR), plant (RUN1, RPP1), and human (SARM1). Sequence alignments were carried out using Jalview software¹.



Supplementary Figure 10. Structural basis for pAgo-mediated oligomerization. Close-up view of the pAgo dimer interface showing a positively charged pocket on $pAgo_A$ receiving residues from $pAgo_B$. Residues GIn35 and Tyr37 of $pAgo_B$ are involved in additional interactions with $pAgo_A$.



Supplementary Figure 11. Mass photometry data for the SPARTA deletion mutant. Mass photometry data for the SPARTA deletion mutant, in which residues from the loop10-9 were deleted (pAGOΔAA322-327), incubated with guide RNA (gRNA) and target DNA (tDNA). The mass distribution plot shows that the majority of the population (~93%) is monomeric SPARTA bound to the gRNA/tDNA, with an average MW of ~134.6 kDa (close to the expected MW of 132.6 kDa) with only a very small fraction forming SPARTA dimeric-gRNA/tDNA complexes (~6%), with an average MW of ~273 kDa (close to the expected MW of 265.2 kDa).

	Apo-SPARTA (PDB: 8U7B)				
Data collection					
Space group	P6₅22				
Cell dimensions					
a, b, c (Å)	197.18 Å, 197.18 Å 183.4 Å,				
α, β, γ (°)	90.00 90.00 120.00				
Resolution (Å)	170.76-2.66 (2.99-2.66)*				
R _{sym} or R _{merge}	14.8 (176.7)				
R _{pim}	4.2 (55.4)				
No. of unique reflections	40914				
Ι/σΙ	13.7 (1.7)				
Completeness (%)	96.2 (73.9)				
(ellipsoidal)					
Redundancy	13.3 (10.7)				
CC(1/2)	1.00 (0.57)				
Refinement					
Resolution (Å)	49.71-2.66				
No. reflections	40895				
Rwork / Rfree	18.6/24.0				
No. atoms					
Macromolecules	7420				
Ligands/Ions	2				
Water	91				
B-factors					
Macromolecules	69.0				
Ligands/Ions	67.0				
Water	63.9				
R.m.s. deviations					
Bond lengths (Å)	0.003				
Bond angles (°)	0.61				
Ramachandran Plot					
Favored (%)	93.0				
Allowed (%)	6.5				
Outliers (%)	0.5				

Supplementary Table 1. X-ray data collection and refinement statistics for Apo-SPARTA

Supplementary Table 2. CryoEM data collection, model refinement and validation statistics

SPARTA-gRNA-tDNA-Mg²⁺

PDB ID:8U72 EMDB IDs: EMD-41945, EMD-41947, EMD-41948, EMD-41959, EMD-41966

Data collection and processing

Magnification (kV)	x81000
Voltage (kV)	300
Pixel Size (Å/pixel)	1.083
Electron dose (e ⁻ /Å ²)	50.79
Defocus range (µm)	0.8 - 2.5
Number of Micrographs	13,884
Tilt angle	30°, 45°
Number of Particles	238,432
Nominal Map resolution (Å)	3.35
FSC threshold	0.143
Refinement (Phenix)	
Model composition	
Non-hydrogen atoms	29715
Protein residues	3469
DNA/other	148/4
R.M.S. deviations	
Bond lengths (Å)	0.003
Bond Angles (°)	0.596
Validation	
Molprobity score	1.90
Clashscore	7.07
Rotamer outliers (%)	0.33
Cβ outliers (%)	0.00
Ramachandran Statistics (%)	
Favored	91.16
Allowed	8.78
Outliers	0.06

Supplementary References.

1. Waterhouse, A. M., Procter, J. B., Martin, D. M., Clamp, M. & Barton, G. J. Jalview Version 2-a multiple sequence alignment editor and analysis workbench. *Bioinformatics* **25**, 1189-1191 (2009).