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RNase E-dependent guide generation and activation of the SPARMA defense system

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Abstract

The evolutionary arms race between prokaryotes and their invaders has driven extensive diversification of defense systems. Prokaryotic Argonautes (pAgos) comprise a diverse family of defense systems in which nucleic acid-guided DNA-targeting proteins are coupled to different effector domains. Here, we demonstrate that short pAgo/Mrr-APAZ (SPARMA) systems mediate anti-plasmid and anti-phage defense: RNA-guided invader DNA recognition catalytically activates the SPARMA Mrr-like effector domains to induce collateral DNA degradation and consequential abortive infection. Using a genome-wide CRISPR interference screen supported by biochemical experiments, we identify RNase E as the principal host factor required for guide RNA biogenesis. Finally, in-solution analyses and cryo-EM structures expose that, upon guide RNA-mediated target binding, SPARMA dimerizes *en route* to the formation of higher-order catalytically active SPARMA filaments in a concentration-dependent manner. Together, these findings provide key insights into the conserved principles that underlie pAgo-mediated immunity, including RNase E-dependent guide RNA biogenesis and concentration-dependent control of activation.

Keywords: Ago; Argonaute; Mrr; abortive infection; host defense; prokaryotic immunity; anti-plasmid; anti-phage; short pAgo; small RNA; RNase E

Main

Prokaryotes face continuous invasion by plasmids and viruses¹. The resulting evolutionary arms race^{2,3}, yielded a diverse defense system repertoire comprising over 200 characterized defense systems^{4,5}. Argonaute proteins form a heavily diversified family of nucleic acid-guided proteins involved in defense in both prokaryotes and eukaryotes⁶. Eukaryotic Agos (eAgos) are the key component of RNA-guided RNA silencing pathways that are crucial to regulate gene expression, but can also interfere with viral RNA and transposon activity⁷⁻⁹. Unlike eAgos, however, most pAgos characterized to date target DNA rather than RNA¹⁰⁻¹⁷.

Based on phylogeny, prokaryotic Argonautes (pAgos) are divided into four major clades: long-A, long-B, SiAgo-like, and short pAgos¹⁸. Long-A pAgos share their structural architecture (N-L1-PAZ-L2-MID-PIWI domain)¹⁹ with eAgos, exhibit various guide type specificities, and generally cleave invading plasmid or bacteriophage DNA using a catalytic site in the PIWI domain^{10,11,15,20-22}. For DNA-guided long-A pAgos, it is known that guide DNAs are generated by DNA repair complexes RecBCD and AddAB^{15,23}. In contrast, long-B, SiAgo-like, and short pAgos strictly mediate RNA-guided invader DNA targeting¹⁹. A central outstanding question is how the RNAs that guide these pAgos are generated. RNA-guided pAgos appear to sample the transcriptome for guide RNAs^{14,17,24} and preferentially load 5'-phosphorylated guides with AU nucleotides on the first two positions (5'-P-AU guides)^{14,17,24-27}. While crucial for their activity, however, it remains completely unclear how these guide RNAs are generated and whether specific host factors contribute to guide RNA biogenesis.

Long-B, SiAgo-like, and short pAgos have lost the catalytic residues in the PIWI domain (indicated as PIWI*). These pAgos function as guide RNA-programmable “sensors”, while the co-encoded proteins function as catalytic “effectors”. For example, long-B pAgos function in conjunction with effector proteins harboring nuclease, NADase, or membrane-disrupting activities^{27,28}, and SiAgo-like pAgos with membrane-depolarizing proteins²⁹. Short pAgos are comprised of the MID and PIWI* domains only^{6,30,31} and are co-encoded in an operon with (or fused to) a protein comprised of an APAZ domain (analog of PAZ; structurally resembles the N-L1-L2 domains of long Agos¹⁸) which is fused to one of various effector domains. Together, they form a heterodimeric complex in which the short pAgo controls the activity of the APAZ-associated effector domain^{12,14,17,24}. Ultimately, activation of effector domains in long-B, SiAgo-like, and short pAgos triggers cell death, thereby constituting an abortive infection mechanism³².

Short pAgos are subdivided into four subclades: S1A, S1B, S2A, and S2B (Figure 1A). S1A and S1B subclades contain ‘SPARSA’ (short pAgo/SIR2-APAZ) systems in which the short pAgo is co-encoded with (S1A) or fused to (S1B) a SIR2-APAZ protein¹⁷. Subclade S2A contains ‘SPARTA’ systems in which the short pAgo is co-encoded with a TIR-APAZ protein¹². Subclade S2B contains short pAgos co-encoded with an APAZ domain fused to one of various effector domains, including DREN (SPARDA²⁵), HNH (SPARHA²⁴), and others¹⁸. In short pAgo systems, guide RNA-mediated target DNA binding triggers conformational changes that activate the effector domain through various mechanisms: Target DNA binding induces distinct tetramerization forms to activate the SPARSA SIR2 domain³³ or the SPARTA TIR domain^{12,34-37}, or filamentation to activate the SPARDA DREN or SPARHA HNH domains^{24,25,38}. These distinct activation mechanisms underscore the complex co-evolution of pAgos and their associated effector domains.

Mrr-like nucleases (including Mrr and SMEK nucleases) comprise a subfamily of the PD-(D/E)XK nuclease superfamily³⁹ (Extended Data Fig. 1a) that act as effector domains in Type IV Restriction-Modification, Avs, and Lamassu systems^{40–42}. In these systems, Mrr-like domains are typically activated by oligomerization. For example, in the Avs4 system, phage portal protein binding triggers a monomer-to-tetramer transition of Mrr that remodels its active site to enable double-stranded DNA cleavage⁴¹. According to their structural architecture and phylogeny, Mrr-like domains associated with short pAgos in at least three distinct evolutionary events (Figs. 1a, 1b, and Extended Data Fig. 1a): twice with S2B pAgos, and once with S2A pAgos. In clade S2B short prokaryotic Argonaute/Mrr-APAZ (SPARMA) systems, the Mrr-like domain is directly fused to APAZ (Figs. 1a, 1b, and Extended Data Fig. 1a). A previous study reported that S2B SPARMA from *Kordia jejudonensis* (KjAgo) acts as a nuclease⁴³. However, the function and molecular mechanisms of Mrr-like (from hereon: Mrr)-associated pAgo systems remain unclear.

Here, we describe the functional and mechanistic characterization of the *Pseudomonas plecoglossicida* SPARMA (PspSPARMA) system. SPARMA mediates RNA-guided invader DNA recognition which induces Mrr-mediated collateral DNA degradation. This provides population-based immunity against both plasmids and phages. Using a genome-wide CRISPR interference (CRISPRi) screen corroborated by *in vitro* assays, we establish Ribonuclease E (RNase E) as the general host factor that is essential for guide RNA generation. As such, we reveal the biogenesis pathways of guide RNAs that underlie the sequence-specific activity of RNA-guided pAgos. Finally, we reveal how target DNA binding induces conformational changes that result in step-wise activation of SPARMA. This requires SPARMA dimerization *en route* to Mrr-domain activation through filamentation in a concentration-dependent manner. As such, these findings provide crucial insights into the conserved principles that underlie pAgo-mediated immunity.

Results

SPARMA protects against plasmid DNA and dsDNA phages

To investigate whether SPARMA functions as a defense system, it was expressed in *Escherichia coli* from a single-copy bacterial artificial chromosome (BAC) (Fig. 1b). In the absence of invading DNA, SPARMA expression does not reduce fitness (Extended Data Fig. 2a). However, expression of SPARMA in the presence of a high copy-number pUC plasmid (500-700 copies⁴⁴) drastically reduces cell viability (Fig. 1c and Extended Data Fig. 2a). This effect is abolished in cells expressing structural alignment-based catalytic Mrr mutant (SPARMA^{Mrr-APAZ-D67A-Q78A}) (Fig. 1c and Extended Data Figs. 1b, 2a and 2b). This indicates that the SPARMA Mrr domain induces cell death in the presence of plasmid DNA.

SPARMA-mediated cell death is reduced in the presence of medium copy number (pET; 15-20 copies⁴⁵) and low copy number (pSC101; 5 copies⁴⁶) plasmids (Fig. 1c). Inducing T7 polymerase-mediated transcription of a plasmid-encoded gene enhances cell death (Extended Data Fig. 2b). As observed for SPARTA¹², this implies that both the invading DNA copy number and its transcription level stimulate SPARMA activation.

Epifluorescence microscopy reveals that cells in which SPARMA is activated retain intact membranes but lose detectable genomic DNA signal (Fig. 1d). This indicates that SPARMA triggers cell death through non-specific collateral DNA degradation. Abortive infection mechanisms protect bacteria by eliminating infected cells, preventing the spread of invading DNA³². To assess population-level dynamics, cells containing a pUC plasmid (99% of cells) were mixed with plasmid-free cells (1% of cells). Plasmids persist in bacterial populations containing cells that do not express SPARMA or that express the catalytic mutant SPARMA^{Mrr-APAZ-D67A-Q78A} (Fig. 1e). In contrast, within two days, plasmid-containing cells are nearly completely removed from cultures in which cells express SPARMA (Fig. 1e). This demonstrates that SPARMA mediates abortive infection that purges plasmid-infected cells from bacterial populations.

To determine if SPARMA also protects against phages, *E. coli* expressing SPARMA was challenged with 78 dsDNA phages (Basel collection⁴⁷ and classic *E. coli* phages: T2, T3, T4, T5, T6, T7, N4, λ_{vir} , P1_{vir}, P2_{vir}) and two ssDNA phages (M13, Φ X174) (Extended Data Fig. 2c). SPARMA, but not the catalytic mutant SPARMA^{Mrr-APAZ-D67A-Q78A}, provides strong (10^2 - 10^4 -fold) resistance against phages λ_{vir} , P1_{vir}, and P2_{vir} (Fig. 1f and Extended Data Fig. 2d). Consistent with an abortive infection mechanism, SPARMA protects *E. coli* from λ_{vir} infection at a multiplicity of infection (MOI) of 0.1 in liquid culture, whereas complete culture collapse is observed at an MOI of 1 (Fig. 1g). Combined, this demonstrates that SPARMA provides defense against plasmids and certain dsDNA phages by inducing abortive infection.

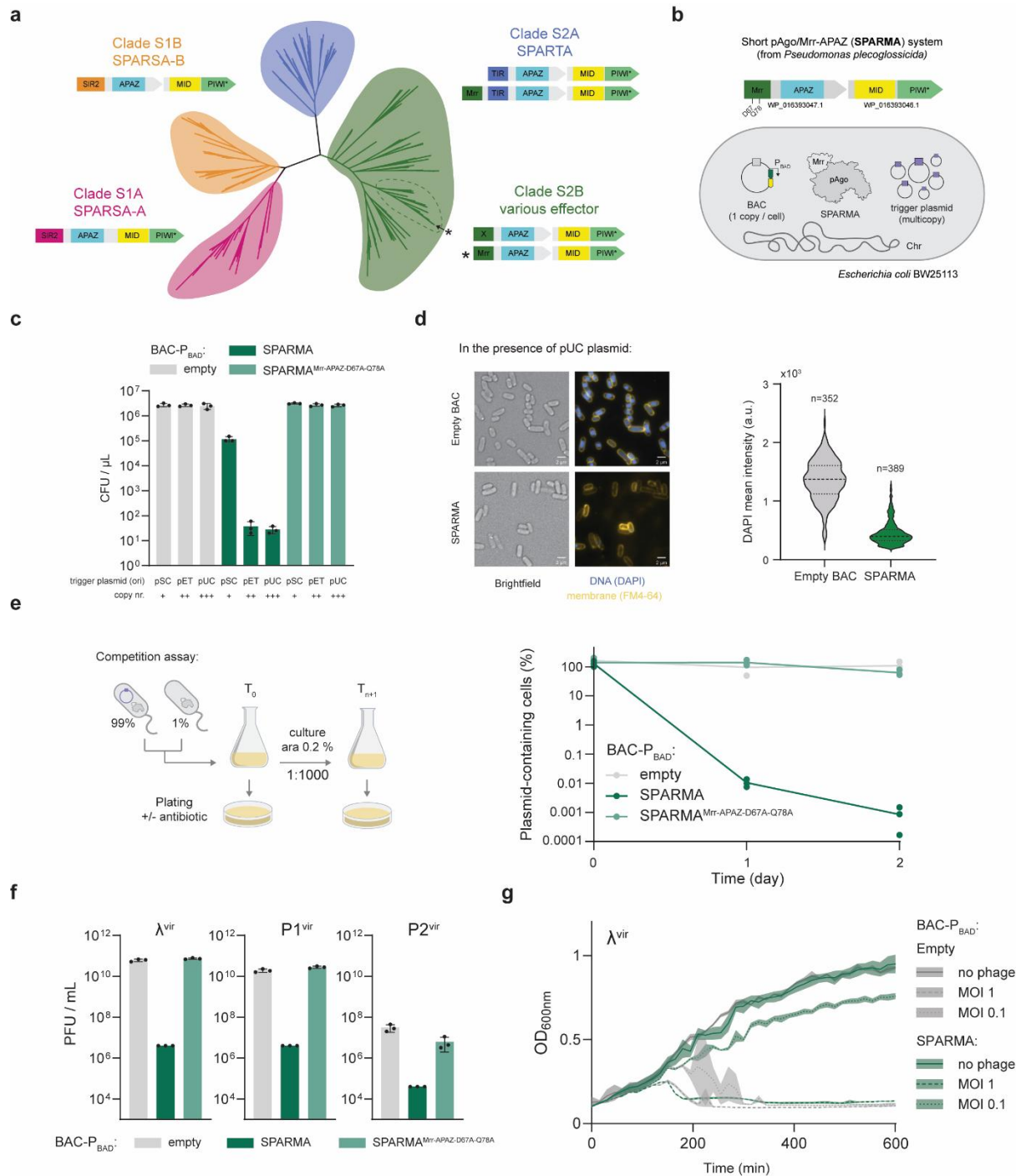


Fig. 1: SPARMA interferes with invading DNA.

a. Maximum-likelihood unrooted phylogenetic tree (adapted from¹⁸). Clade S1A: SIR2-APAZ-pAgo fusion (SPARSA-A). Clade S1B: short pAgo in operon with SIR2-APAZ (SPARSA-B). Clade S2A: short pAgo in operon with TIR-APAZ (SPARTA) or Mrr-TIR-APAZ (SPARMTA). Clade S2B: short pAgo in operon with and X-APAZ, where X denotes one of various effector domains, including Mrr (SPARMA), DREN (SPARDA), HNH (SPARHA), DHS-like, or other domains. PspSPARMA (S2B) system described in this study is indicated with an asterisk (*). **b.** Top. Schematic gene organization of *Pseudomonas plecoglossicida* (Psp)SPARMA operon. Predicted catalytic residues in the Mrr nuclease domain are indicated. See also Figure S1A. Bottom. Schematic representation of SPARMA experiments in *Escherichia coli* BW25113. PspSPARMA system is expressed from a bacterial artificial chromosome (BAC) in the presence of multicopy trigger plasmids. **c.** SPARMA triggers cell death in the presence of plasmids. Cell viability of *E. coli* expressing PspSPARMA or PspSPARMA^{Mrr-APAZ-D67A-Q78A} in the presence of plasmids with different origins of replication (copy number: high - ori_{pUC}, medium - ori_{pET}, low - ori_{pSC101}). Experiments were performed in biological triplicate. Dots indicate single experiments; error bars indicate standard deviation. **d.** SPARMA degrades intracellular DNA. Left: Representative epifluorescence microscopy images of *E. coli* harboring an empty BAC or a BAC expressing PspSPARMA in the presence of a pUC

plasmid. Genomic DNA was stained with DAPI, cell membranes were stained with FM4-64. Right: Quantification of mean DAPI fluorescence intensity per cell; the number of cells analyzed is indicated above each graph. Data are derived from three independent biological replicates. **e.** SPARMA purges plasmid-harboring cells from bacterial cultures. Left: Schematic representation of the mixed-culture competition experiment. Right: Cultures of PspSPARMA-expressing *E. coli* cells that contain a pUC plasmid and plasmid-free cells were mixed in a 99:1 ratio and cultivated in LB. The percentage of plasmid-containing cells was determined daily. Cultures of *E. coli* with an empty BAC or a BAC expressing PspSPARMA^{Mrr-APAZ-D67A-Q78A} were used as controls. Dots indicate single experiments; graph lines indicate the average of three biological replicates. **f.** SPARMA provides innate immunity against various bacteriophages. *E. coli* expressing PspSPARMA or PspSPARMA^{Mrr-APAZ-D67A-Q78A} were challenged with 10-fold serial dilutions of λ_{vir} , P1_{vir}, and P2_{vir} bacteriophages. Bar graphs indicate the average of three biological replicates; dots indicate single experiments. See also Extended Data Figs. 1c and 1d. **g.** Growth curves of *E. coli* culture expressing PspSPARMA or PspSPARMA^{Mrr-APAZ-D67A-Q78A} in the presence of λ_{vir} bacteriophage at various multiplicity of infection (MOI). λ_{vir} was added at timepoint $t = 0$ min. Experiments were performed in biological triplicate. Lines indicate averages; shaded areas indicate standard deviations.

SPARMA samples the transcriptome for 5'-P-AU guide RNAs

Co-expression and purification of SPARMA using an N-terminal His-MBP tag on the Mrr-APAZ subunit results in co-purification of the untagged pAgo subunit (Fig. 2a). This suggests that, akin to other short pAgo systems^{12,14,17,18}, the two subunits form a heterodimeric complex. To determine the guide/target preferences of SPARMA, we analyzed co-purified nucleic acids (Fig. 2b). Small RNAseq reveals that SPARMA associates with 5' phosphorylated (5'-P) 18-21 nt small RNAs that have a strong bias for an adenosine (A) and uracil (U) at positions 1 and 2 (5'-P-AU; Fig. 2c and 2d). A 5'-P-AU bias has also been reported for pAgos from the S1A and S1B¹⁷, S2B^{14,24}, and long-B^{20,27} pAgo clades. The widespread preference for 5'-P-AU guides suggests that a conserved mechanism for guide RNA generation and/or loading exists.

Consistent with observations for other short and long-B pAgos^{12,14,17,24,27,48}, SPARMA-associated guide RNAs share their sequences with the most abundant small and long RNA species in the cell (Fig. 2e). This suggests that the transcriptome-wide degradome is sampled for guide RNAs, with the 5'-P-AU end as primary determinant for selective loading. Notably, plasmid-encoded mRNAs reach levels that are 100-1000-fold higher than genome-encoded mRNAs. Consistent with this, sequencing of SPARMA-associated DNA fragments (presumed target DNA) reveals that plasmid genes are targeted 100-1000-fold more frequently than chromosomal genes (Fig. 2e). Such targeting pattern might result in local high concentrations in the cell at locations where invaders replicate⁴⁹.

RNA-guided DNA target binding triggers collateral DNA degradation

To verify the observation that SPARMA exhibits collateral nuclease activity *in vivo* (Fig. 1d), purified SPARMA was incubated with guide RNA, a complementary target ssDNA, and various collateral substrates. In the presence of guide RNA and target ssDNA, SPARMA exhibits *in trans* cleavage activity toward dsDNA and ssDNA (but not ssRNA) substrates (Extended Data Fig. 3a). Similarly, SPARMA degrades *E. coli* genomic DNA in a guide RNA/target ssDNA-dependent manner (Fig. 2f). Sequencing of degradation products shows no sequence bias at the cleavage site (Extended Data Fig. 3b), which indicates that Mrr-mediated collateral DNA cleavage occurs at random sequences.

To define the biochemical constraints governing SPARMA activation, we systematically assessed the effects of guide RNA sequence, length, and guide/target complementarity using DNaseAlertTM, a substrate that emits a fluorescent signal upon DNA degradation (Fig. 2g). Consistent with 5'-P-AU preferences *in vivo* (Fig. 2d), adenine and uracil at guide positions 1 and

2 are preferred for activation of the system (Extended Data Fig. 4a). Guide RNAs 17-23 nt in length activate SPARMA most efficiently, while shortening guide RNAs reduces activity progressively, with no detectable activation using 11 nt guide RNAs (Extended Data Fig. 4b). Single guide/target mismatches have modest effects on SPARMA activation, double mismatches at positions 1–2 or 7–8 reduce activity (Extended Data Fig. 4c, 4d). Combined with the analysis of nucleic acids that co-purified with SPARMA (Figs. 2a-e), these experiments demonstrate that SPARMA is activated upon guide RNA-mediated target ssDNA binding. Consistent with our *in vivo* microscopy observations (Fig. 1d), this results in Mrr effector domain activation and consequential collateral DNA degradation.

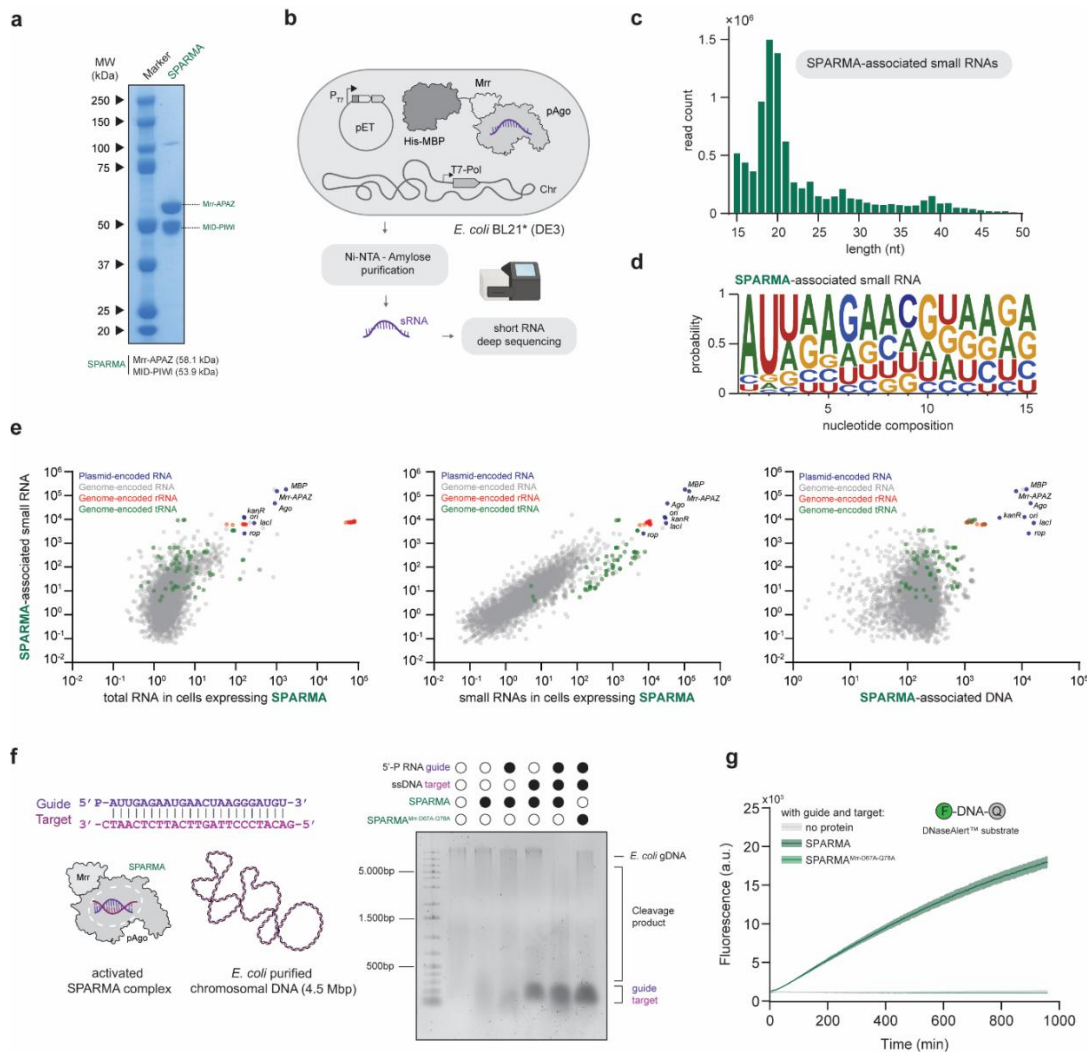


Fig. 2: SPARMA uses 5'-P-AU guide RNA to trigger collateral DNA degradation.

a. Mrr-APAZ and short pAgo subunits form a SPARMA complex. SDS-PAGE analysis of co-purified Mrr-APAZ/short pAgo proteins. **b.** Schematic representation of the purification of small RNAs bound to SPARMA. **c.** Length distribution of small RNA copurified with PspSPARMA. **d.** Small RNAs associated with PspSPARMA are enriched in sequences with 5'-AU nucleotides. **e.** SPARMA-associated guide RNA abundance corresponds to the abundance of total cellular RNA transcripts (left), cellular small RNA transcripts (middle), and SPARMA-associated DNA (right). **f.** SPARMA degrades genomic DNA *in trans*. PspSPARMA or PspSPARMA^{Mrr-APAZ-D67A-Q78A} were incubated with guide RNA and complementary ssDNA target, after which *E. coli* genomic DNA was added as substrate. Genomic DNA degradation products were resolved by agarose gel electrophoresis and visualized with SYBR Gold staining. **g.** *In trans* cleavage activity kinetics of SPARMA. PspSPARMA and PspSPARMA^{Mrr-APAZ-D67A-Q78A} were incubated with guide RNA, complementary ssDNA targets, and DNaseAlertTM. DNaseAlertTM fluorescence was measured over time. Data points represent the average of three technical replicates; shaded areas indicate standard deviations.

CRISPR interference screen identifies host factors involved in pAgo-mediated immunity

To identify host factors required for short pAgo-mediated immunity, we performed a genome-wide CRISPR interference (CRISPRi) screen. The EcoWG1 CRISPRi library contains 21417 plasmids each encoding a dCas9 and a single guide (sgRNA) that facilitate transcriptional silencing of an individual *E. coli* gene, allowing silencing of 4.444 of 4.519 genes in individual *E. coli* cells⁵⁰. The EcoWG1 library was introduced into *E. coli* cells that genomically encode a SPARMA and harbor a pUC trigger plasmid. Induction of SPARMA expression in these cells leads to cell death upon trigger plasmid detection (Fig. 3a). However, cells in which genes required for SPARMA activation are silenced escape cell death and become enriched. By comparing sgRNA-coding sequence abundance before and after SPARMA expression, genes essential for SPARMA activation were identified (Fig. 3b).

The CRISPRi screen recovered expected internal controls: CAP-cAMP pathway components (*cyaA*, *crp*) and *araC*, required for pBAD-driven SPARMA expression) and pUC plasmid maintenance genes (*priA*, *nrdA*, *dnaB*, *polA*, *pcnB*) were identified in the screen (Fig. 3b). Furthermore, the screen identified DNA supercoiling genes *gyrB* and *topA*, which suggests that plasmid supercoiling plays a role in dsDNA targeting. Finally, *rne*, which encodes Ribonuclease E (RNase E), was identified. RNase E is best known for its role in rRNA/tRNA processing and global RNA turnover⁵¹, which makes it a prime candidate for guide RNA generation.

To validate their importance for SPARMA activation, *priA*, *nrdA*, *dnaB*, *gyrA*, *gyrB*, *pcnB*, and *rne* were selected for individual CRISPRi-mediated knockdown. Except for *nrdA*, silencing of each gene significantly reduces SPARMA-mediated cell death (Fig. 3d). Notably, silencing *rne* completely abolishes SPARMA activation. To disentangle the role of RNase E in guide RNA generation or RNase E-dependent ColE1 plasmid replication⁵², we replaced the RNase E-dependent ColE1 origin of the pUC trigger plasmid with a pSC101-RepA^{E93R} origin⁵³. Using this trigger plasmid, silencing of *rne* still abolishes SPARMA-mediated cell death (Fig. 3c). These results imply that RNase E is crucial for SPARMA activation and suggest that it is involved in guide RNA biogenesis.

Short pAgos require RNase E-mediated guide RNA generation

RNase E cleaves RNA at RN↓WUU motifs, generating fragments with 5'-P-(A/U)U-sequences^{54,55}. This product matches the 5'-P-AU guide RNAs preferentially bound by long-B and short pAgos^{12,14,17,24,27}, including SPARMA (Fig. 2). To confirm that RNase E is the main host factor responsible for guide RNA biogenesis, purified RNase E (lacking its C-terminal membrane anchor⁵⁶) was incubated with a 28-nt 5'-P-UA pre-guide RNA containing an internal RNase E cleavage motif (5'-...AG/AUU...-3'). Consistent with its design, this produces a ~23-nt 5'-P-AU guide RNA (Fig. 3d). Next, a one-pot assay was performed with SPARMA, pre-guide RNA, and target ssDNA. While the pre-guide fails to activate SPARMA due to its incompatible 5'-P-UA sequence, addition of RNase E (but not catalytic mutant RNase E^{D303A}) results in SPARMA-mediated collateral DNase activity (Fig. 3e). Together, these results demonstrate that RNase E generates functional guide RNAs that are used by SPARMA for DNA detection. In combination with the CRISPRi screens (Fig. 3b and 3c), the available literature on pAgo 5'-P-AU preferences, and its strict conservation in bacteria⁵⁷, this identifies RNase E as the main host factor responsible for biogenesis of guide RNAs that guide pAgo activity.

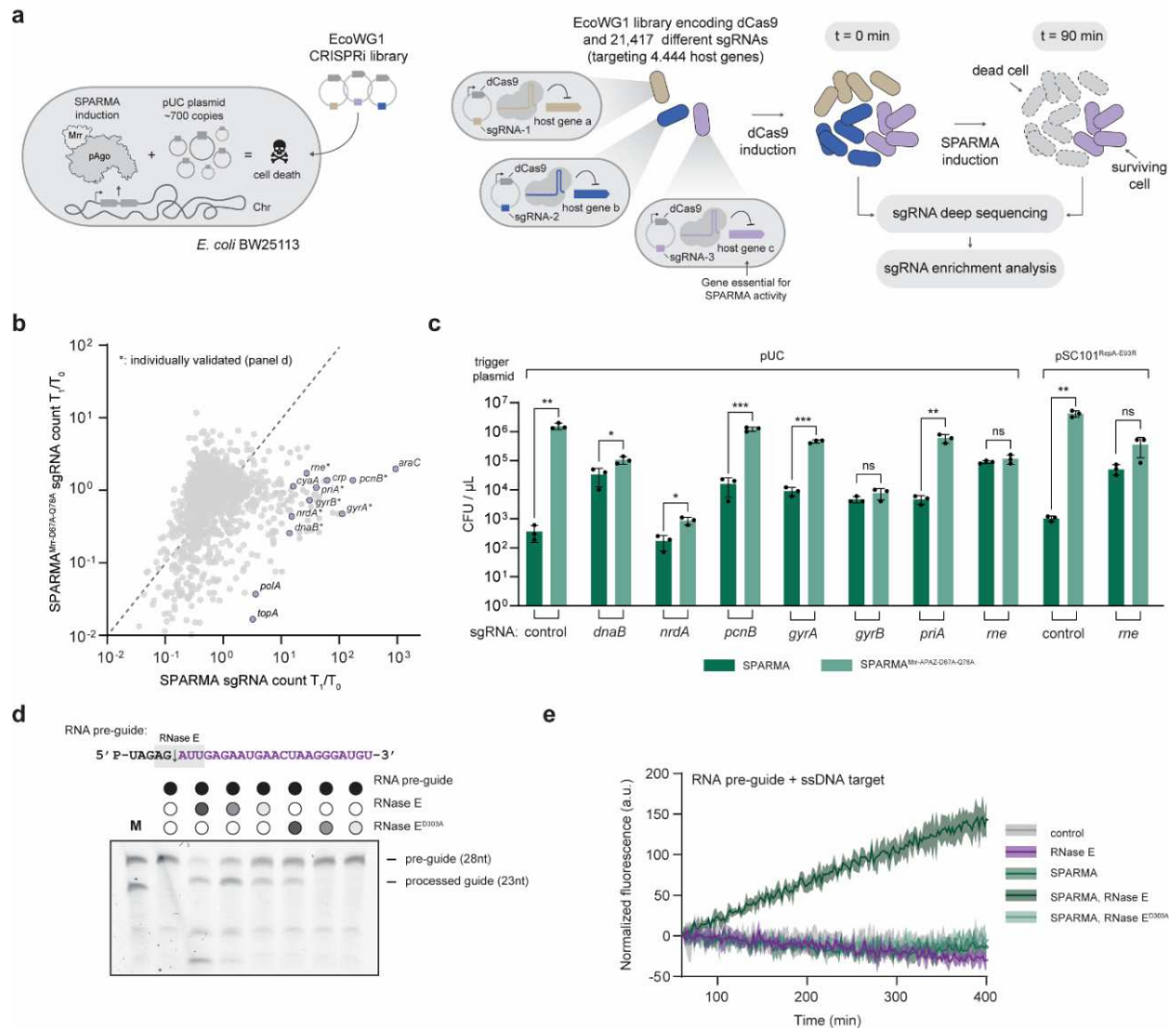


Fig. 3: RNase E is responsible for guide RNA generation.

a. Schematic representation of the SPARMA CRISPR interference screen. PspSPARMA expression triggers cell death in pUC plasmid-containing cells. These cells were transformed with the EcoWG1 library, which comprises plasmids encoding dCas9 and one of 21,417 sgRNAs targeting *E. coli* genes. After dCas9 and sgRNA expression, SPARMA expression was induced for 90 minutes. Cells in which genes crucial for SPARMA activity become enriched are identified by comparing next-generation sequencing data of sgRNA-coding regions in the EcoWG1 plasmids before and after SPARMA induction. **b.** Enrichment analysis of the SPARMA CRISPR interference screen. For each sgRNA-coding gene, reads were counted and normalized at $t = 0$ min (before SPARMA induction) and at $t = 90$ min (after SPARMA induction). A strain with PspSPARMA^{Mrr-APAZ-D67A-Q78A} was used as a control. Genes selected for further validation (panel 3c) are indicated with an asterisk (*). **c.** Individual validation of CRISPRi hits. Survival assays of *E. coli* following CRISPRi-mediated knockdown using single gene-specific sgRNAs or a non-targeting control sgRNA. SPARMA system activation was tested in the presence of pUC (left) or pSC101-RepA^{E93R} origin plasmids (right). Data represent relative cell viability following dCas9-mediated transcriptional silencing and subsequent expression of PspSPARMA or PspSPARMA^{Mrr-APAZ-D67A-Q78A}. Experiments were performed in biological triplicate; dots indicate results of independent experiments; error bars indicate mean $\pm$ SD. Student's t-test was used to evaluate significance (*: $p < 0.05$; ***: $p < 0.005$; ns, not significant). **d.** RNase E-mediated processing of a pre-guide RNA substrate. Top: Sequence of the 28-nt 5'-phosphorylated (5'-P) pre-guide RNA substrate. The predicted RNase E cleavage motif (5'-...AG/AUU...-3') is indicated in a grey box; the cleavage position is indicated with an arrow. Bottom: Denaturing PAGE analysis of RNase E pre-guide RNA cleavage. The pre-guide RNA substrate was incubated with increasing concentrations of RNase E or a catalytic mutant thereof (RNase E^{D303A}). RNA marker lane (M) contains the 28-nt pre-guide RNA and the expected 23-nt 5'-P-AU guide RNA product. **e.** One-pot reconstitution of RNase E-dependent SPARMA activation. PspSPARMA and RNase E or catalytic mutant RNase E^{D303A} were incubated with the pre-guide RNA and a complementary ssDNA target. PspSPARMA activation was determined by measuring DNaseAlertTM fluorescence over time. Curves represent the average of three technical replicates; shaded areas indicate standard deviations.

RNA-guided DNA targeting triggers SPARMA dimerization

Most pAgos are activated through various forms of oligomerization^{12,24,25,38,58,59}. To understand how SPARMA is activated, we assessed its oligomeric state under different conditions. Mass photometry, which is constrained to nanomolar concentrations (10–30 nM SPARMA), reveals that guide RNA-bound SPARMA exists as a heterodimeric complex (1 Mrr-APAZ:1 MID-PIWI*:1 guide RNA) that, upon DNA target binding, transitions to a dimer-of-dimers (2 Mrr-APAZ:2 MID-PIWI*:2 guide RNA:2 target DNA) (Fig. 4a). In contrast, high-concentration SEC-MALS analysis (2 μ M SPARMA) reveals that target DNA binding induces formation of ~16.6 MDa SPARMA oligomers (Fig. 4b). This suggests that target DNA binding triggers hierarchical assembly: the SPARMA complex forms a dimer *en route* to higher-order oligomerization at high concentrations of activated SPARMA complexes.

To define the structural basis of target DNA binding-induced SPARMA activation, we determined a cryo-EM reconstruction of the SPARMA dimer at 2.9 Å resolution (Fig. 4c). In the SPARMA dimer, both protomers are associated with a guide RNA/target DNA duplex. In each protomer, the APAZ, MID, and PIWI* domains form a 1163 Å² interaction surface. Together, they form a positively charged channel that coordinates the guide/target duplex (Extended Data Fig. 5a). The guide 5'-P is sequestered by a MID domain pocket and coordinated by a Mn²⁺ cation^{24,25,38} (Fig. 4d). Guide nucleotide 1 (g1)A is sequestered in a hydrophobic pocket and forms a hydrogen bridge with Ago^{N178}, while g2U is contacted by Ago^{N220} and Ago^{Q212}, which prevents base pairing with the target DNA. These interactions explain the 5'-P-AU guide preference of SPARMA (Fig. 2d).

In the SPARMA dimer, the APAZ, MID, and PIWI* domains of each protomer broadly conform to cyclic C2 symmetry. This arrangement resembles dimer-protomers within fully activated SPARDA-²⁵, SPARHA-²⁴ and SPARTA-gRNA:tDNA³⁴ oligomers (Extended Data Fig. 5b). Mutations at the Ago::Ago interface (SPARMA^{Ago-R142E-D145K}) (Fig. 4e and Extended Data Fig. 5c) prevent dimerization, abolish anti-phage defense, and attenuate collateral nuclease activity *in vitro* (Fig. 4f-h). This shows target DNA binding-induced dimerization is essential for SPARMA activation.

Beta-relay remodeling and Mrr::Mrr interactions are essential for SPARMA activation

Within the SPARMA dimer, the two Ago protomers exhibit structural differences in the guide/target duplex, beta-relay (BR) region, and Mrr domains. The BR is a conserved regulatory element within the PIWI domain that comprises three distinct loops connected to two beta-strands²⁵. During activation, the small sensory loop (SSL) and the large sensory loop (LSL) together sense the bound guide/target duplex and, through the beta-strands, transduce an allosteric recognition signal that remodels the response loop (RL) at the Ago surface to either organize the active site or facilitate dimerization and oligomerization²⁵.

Within our dimeric complex, one of the SPARMA sub-complexes has 16 base pairs of the guide/target duplex resolved, while its BR assumes an 'activate' conformation. In contrast, the other SPARMA sub-complex has only 10 base pairs resolved, while its BR assumes an 'inactive' conformation (Extended Data Fig. 5d). This asymmetry contrasts with typical pAgo structures in which all protomers transition simultaneously to an active conformation upon target engagement^{24,25,38}. Therefore, our structure likely represents an activation intermediate, in which BR switching occurs as two distinct events as opposed to a conserved transition across each sub-complex. Underscoring the importance of the BR, mutating any BR loop (Δ SSL = Ago^{K255:L258>GG}; Δ LSL = Ago^{R289:Y294}; Δ RL = Ago^{E272:H275>GGG}) abolishes SPARMA anti-phage activity (Extended Data Fig. 5e). Combined, this shows that guide/target-binding induces BR remodeling which consequently allows for the Ago::Ago dimerization required for catalytic activation.

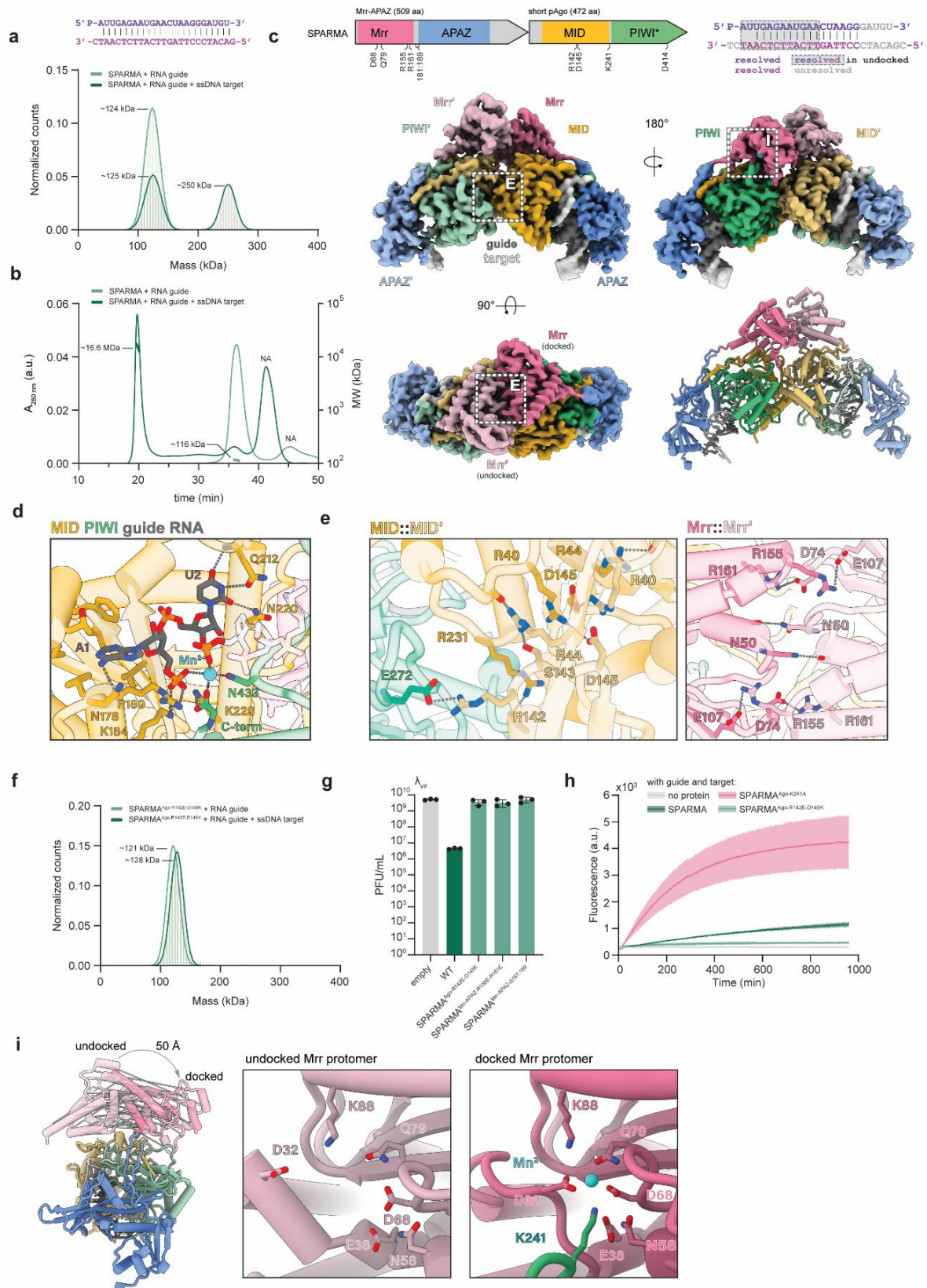


Fig. 4: SPARMA dimerizes upon target DNA recognition before higher-order oligomerization.

a. SPARMA dimerizes upon guide RNA-mediated target DNA binding. Mass photometry measurement of PspSPARMA in complex with guide RNA only or with guide RNA and target DNA. **b.** SPARMA forms higher-order oligomers upon guide RNA-mediated target DNA binding. SEC-MALS measurement of PspSPARMA in complex with guide RNA only or with guide RNA and target DNA. **c.** SPARMA dimer structure in complex with guide RNA and target DNA. Top: Schematic representation of PspSPARMA proteins and bound nucleic acids. Bottom: Cryo-EM density (left) and structure (right)

of dimeric SPARMA complex (2 Mrr-APAZ:2 MID-PIWI:2 guide RNA:2 target DNA). **d.** SPARMA recognizes the 5' -P-AU end of guide RNAs. Close-up view of the MID-domain, the first two bases of the 5'-P end of the guide RNA, and the residues that underly the recognition. **e.** SPARMA-SPARMA dimerization interfaces. Close-up of MID::MID' (left) and Mrr::Mrr' (right) interfaces. **f.** MID::MID' interactions are essential for dimerization. Mass photometry measurement of PspSPARMA^{Ago-R142E-D145K} in complex with guide RNA only or with guide RNA and target DNA. **g.** MID::MID' and Mrr::Mrr' interfaces are essential for anti-phage activity. λ_{vir} phage drop tests with *E. coli* expressing PspSPARMA, interface mutants thereof (PspSPARMA^{Ago-R142E-D145K}; PspSPARMA^{Mrr-APAZ-R155E-R161E}), or a shortened Mrr-linker mutant (PspSPARMA^{Mrr-APAZ-Δ181:189}). Experiments were performed in biological triplicate. Dots indicate single experiments; error bars indicate standard deviation. **h.** MID::MID' and Mrr::Mrr' interfaces control SPARMA activation. PspSPARMA or mutants thereof (PspSPARMA^{Ago-K241A}; PspSPARMA^{Ago-R142E-D145K}) were incubated with guide RNA and complementary ssDNA target, after which a DNaseAlertTM substrate was added to follow *in trans* cleavage activity over time. Data points represent the average of three technical replicates; shaded areas indicate standard deviations. **i.** Mrr domains from each SPARMA protomer adopt distinct conformations. The SPARMA structure is pseudo-symmetrical: Mrr is docked on the PIWI* domain whereas Mrr' does not interact with the Ago subunit. PIWI* residue K241 interacts with the docked-Mrr catalytic site.

The Mrr domains also deviate markedly from C2 symmetry: one Mrr domain adopts a 'docked' configuration against the PIWI* domain of its own protomer, while the other remains undocked (Figs. 4c, 4i and Extended Data Fig. 6a). We observed cryo-EM density likely corresponding to a divalent cation within the docked Mrr domain active site, coordinated by Mrr catalytic residues D68 and Q79, and Ago MID domain residue K241, whereas no equivalent density is observed in the undocked Mrr domain (Fig. 4i). As the docked Mrr domain contacts Ago^{K241}, we hypothesized that this residue stabilizes the intermediate dimeric state and attenuates SPARMA activation. Expression of a SPARMA^{Ago-K241A} mutant induces cell death (Extended Data Fig. 6d) and purified SPARMA^{Ago-K241A}, activated by guide RNA/target DNA binding, displays elevated collateral nuclease activity compared to WT SPARMA (Fig. 4h and Extended Data Fig. 6e). This confirms that Ago^{K241} serves as a kinetic brake on SPARMA activation.

Furthermore, the SPARMA dimer appears to be stabilized by Mrr::Mrr interactions (Fig. 4i). The Mrr domain is tethered to APAZ through a flexible linker that is poorly resolved. Negative-stain EM imaging of SPARMA:gRNA complexes confirmed that, in the monomeric state, Mrr is highly mobile and does not adopt a defined position relative to the rest of the protomer (Extended Data Fig. 6b). To test whether Mrr::Mrr engagement is required for activation, we introduced charge-swapping substitutions at the Mrr::Mrr interface (Mrr-APAZ^{R155E-R161E}; Fig. 4e) and a linker truncation intended to restrict Mrr mobility without disrupting the pAgo dimer itself (Mrr-APAZ^{Δ181:189} (Extended Data Fig. 6c)). Mutagenic disruption of the Mrr::Mrr interface or linker truncations abolished phage defense (Fig. 4f), which implies that, next to pAgo::pAgo interactions, Mrr::Mrr interactions constitute a second, essential checkpoint in the SPARMA activation pathway.

Transition to higher-order oligomers activates SPARMA

We next determined a cryo-EM structure of SPARMA^{Ago-K241A} in complex with guide RNA and target DNA at 2.8 Å resolution (Fig. 5a). Multiple assembly states, including SPARMA dimers, tetramers, and hexamers, were resolved using this dataset. The SPARMA^{Ago-K241A} dimer structure assumes a similar fold to the WT SPARMA dimer, but the dimerized APAZ and MID-PIWI* domains now exhibit strict C2 symmetry: both protomers adopt the 'activated' BR conformation (Extended Data Fig. 5d). Consistent with the increased conformational flexibility of Mrr upon mutating Ago^{K241}, the Mrr domains are not resolved in the cryo-EM map, which indicates that the Mrr domains are 'released' (Fig. 5a). Similarly, the protomers in tetrameric and hexameric SPARMA assemblies assume activated conformations (Figs. 5b and 5c). These assemblies represent further oligomerization of SPARMA dimers through a lateral, side-by-side interface formed through MID::PIWI* contacts

between neighboring protomers (Fig. 5b). These higher-order assemblies similarly exhibit diffuse Mrr density insufficient for confident atomic modelling. Combined, these structures reveal a stepwise oligomeric hierarchy required for SPARMA activation: guide RNA-mediated target DNA binding drives progression from an asymmetric SPARMA dimer intermediate to a symmetric SPARMA dimer and eventually to higher-order assemblies.

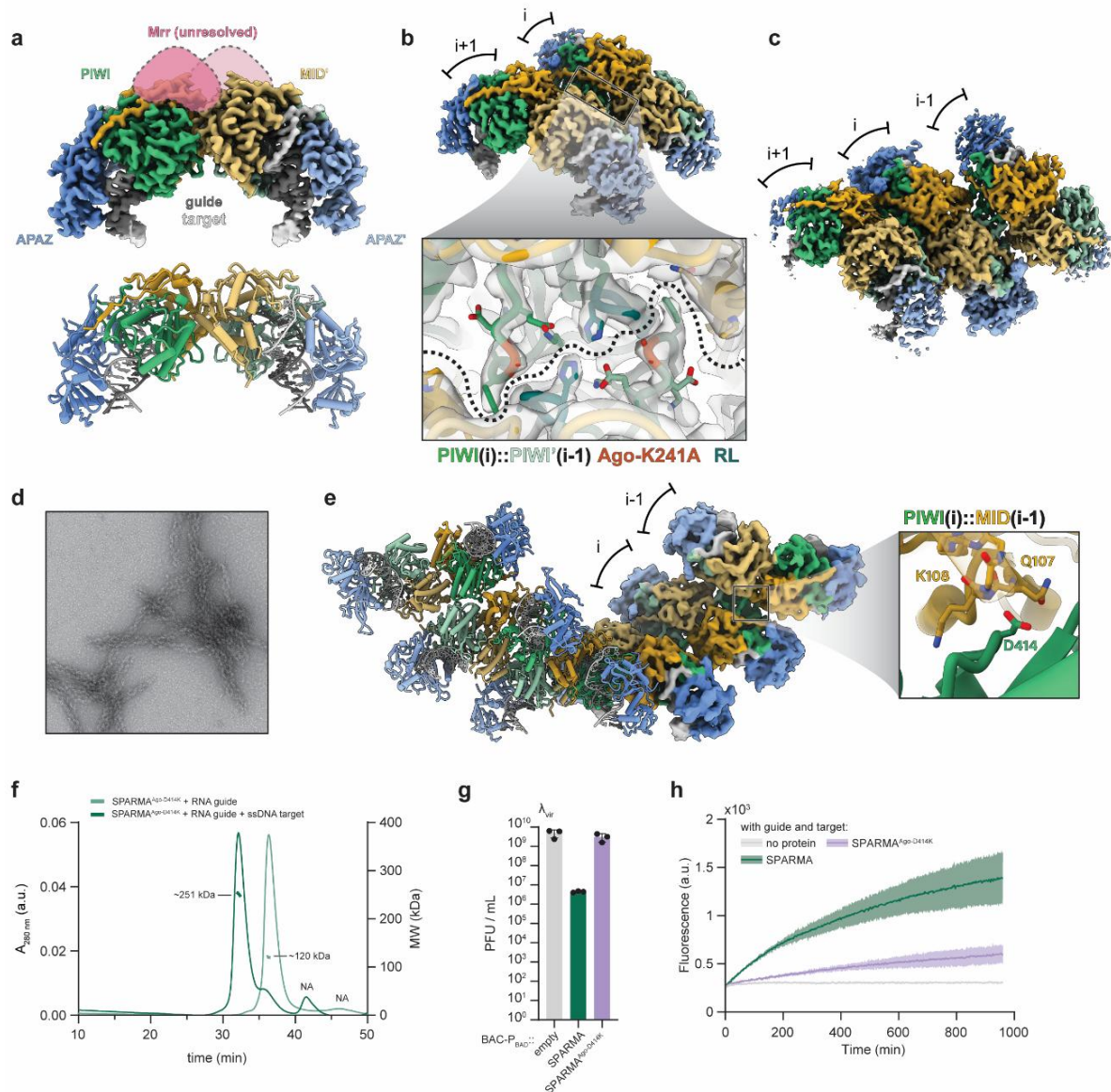


Fig. 5: Activated SPARMA oligomerizes into a filament assembly.

a. Cryo-EM structure of the PspSPARMA^{Ago-K241} dimer in complex with guide RNA and target DNA. Cryo-EM density (top) and structure model (bottom). Mrr is flexible and unresolved (indicated in pink). **b-c.** Cryo-EM densities for tetrameric (B) and hexameric (C) PspSPARMA^{Ago-K241} in complex with guide RNA and target DNA. Bottom: details of the interaction interface between two PspSPARMA dimers. **d-e.** SPARMA forms filaments. Negative stain electron micrograph of PspSPARMA in complex with guide RNA and target ssDNA (5d) and fitting of the PspSPARMA^{Ago-K241A} hexamer in the cryo-EM density of the PspSPARMA filament (5e). Mrr is flexible and unresolved. Close-up inset shows filament-forming PspSPARMA-PspSPARMA interactions. **f.** Filament interface mutant PspSPARMA^{Ago-D414K} prevents filament formation. SEC-MALS measurement of PspSPARMA^{Ago-D414K} in complex with guide RNA only or with guide RNA and target ssDNA. **g-h.** Filament formation is essential for SPARMA activity. G: λ_{vir} phage drop tests with *E. coli* expressing PspSPARMA or PspSPARMA^{Ago-D414K}. Experiments were performed in biological triplicate. Dots indicate single experiments; error bars indicate standard deviation. H: PspSPARMA or PspSPARMA^{Ago-D414K} was incubated with guide RNA and target ssDNA, after which a DNaseAlert™ substrate was added to follow *in trans* cleavage activity over time. Data points represent the average of three technical replicates; shaded areas indicate standard deviations.

Finally, we determined a cryo-EM structure of right-handed SPARMA filaments at 4.3 Å resolution (Figs. 5d and 5e). The conformation of SPARMA dimers within these filaments is identical to that observed in SPARMA^{Ago-K241A} assemblies. A charge-swapping mutation in a PIWI* domain residue involved in filamentous side-by-side interactions, but not in SPARMA dimerization or Mrr-docking (SPARMA^{Ago-D414K}), prevents SPARMA filamentation (Fig. 5f), abolishes phage defense (Fig. 5g), and abrogates collateral DNase activity *in vitro* (Fig. 5h). This demonstrates that dimerization alone is not sufficient; further SPARMA oligomerization is essential for SPARMA activation.

Mrr docking on PIWI* (Fig. 4c) is not compatible with filament formation (Extended Data Fig. 6f). This implies that SPARMA filamentation drives the release and consequential catalytic activation of the Mrr domain. Within the filament, Mrr domain density remains diffuse at standard resolution thresholds, though it becomes discernible upon low-pass filtering of the maps (Extended Data Fig. 6g), as observed for SPARHA²⁴. This might reflect conformational heterogeneity of the Mrr domain within the filament.

We further observed that multiple SPARMA filaments can come together to form higher-order filaments, in which the proto-filaments periodically associate through an interface at the tip of their APAZ domains and through interactions mediated by their associated nucleic acids (Extended Data Fig. 6h). The resolution at these interfaces is too low to accurately model the regions that underlie these interactions, and it remains to be determined whether they contribute to SPARMA functionality.

Combining all insights, we propose that SPARMA activation proceeds through three sequential checkpoints (Fig. 6): 1) guide RNA-mediated target DNA recognition rearranges the BR to license SPARMA dimerization through Ago::Ago interactions; 2) SPARMA dimers form but are kept in an inactive state by docking Mrr onto Ago; 3) at critical SPARMA dimer concentrations, SPARMA filaments assemble. Filamentation coincides with Mrr domain release, resulting in catalytic activation. This requirement for sequential molecular events restricts spurious activation in the absence of multiple colocalized *bona fide* targets; only at high local concentrations of activated SPARMA, filaments can form. Given the 100-1000-fold higher propensity to target invader DNA compared to the genome (Fig. 2e), this suggests that required concentrations can only be achieved on invader DNA and not on the host genome. As such, these data provide a structural basis for how pAgos limit autoimmune effects.

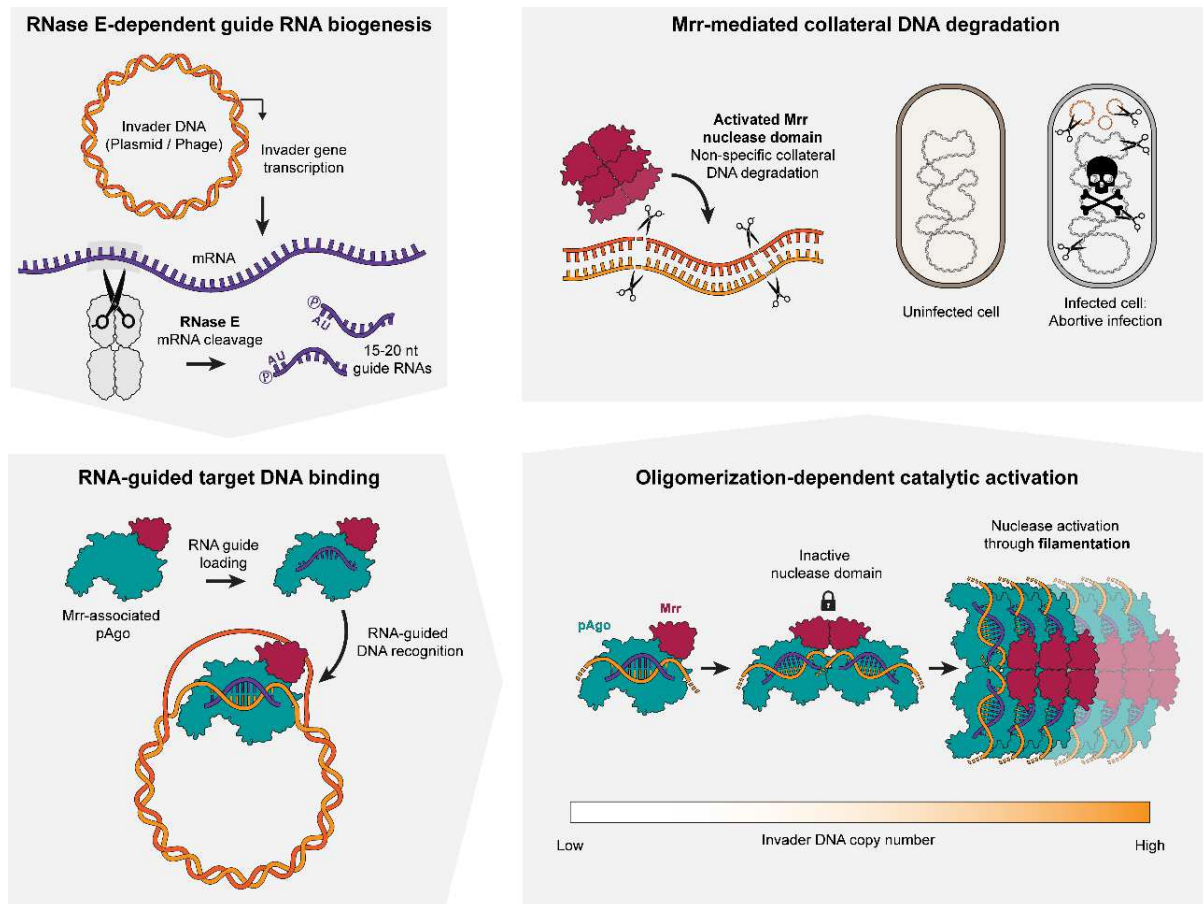


Fig. 6: Proposed model for SPARMA-mediated immunity.

Host RNA transcripts, including transcripts from invading plasmids or phages, are processed by host RNase E into small guide RNAs. These guides are loaded into the apo SPARMA complex to direct sequence-specific DNA recognition. A single guide/target-bound SPARMA complex is inactive. However, because invader DNA is highly transcribed and therefore preferentially targeted, high local concentrations of activated SPARMA arise. This allows for SPARMA dimerization (inactive) *en route* to SPARMA oligomerization into active filaments. In these filaments, Mrr autoinhibition is released which facilitates Mrr activation and unleashes non-specific collateral degradation of cellular DNA to trigger abortive infection. The requirement for filamentation establishes a threshold that prevents autoimmunity at low target abundance.

Discussion

In this manuscript, we show that SPARMA protects against both plasmid DNA (Figs. 1c-e) and against a narrow range of phages (λ_{vir} , P1_{vir}, and P2_{vir}; Fig. 1f, Extended Data Fig. 2d). This limited anti-phage spectrum is consistent with other pAgo systems: CbAgo selectively protects against phage P1_{vir} but not T7¹⁵, and SPARSA protects against phage λ_{vir} but not T4 or T7¹⁷. A notable commonality among phage λ_{vir} , P1_{vir}, and P2_{vir} is their negatively supercoiled circular DNA topology^{60,61}, akin to plasmid DNA⁶². Unlike CRISPR-Cas systems, which unwind target dsDNA duplex upon PAM binding⁶³, pAgos lack an intrinsic mechanism to open the DNA duplex prior to guide-target hybridization. Therefore, we hypothesize that pAgo systems depend on target DNA supercoiling for efficient targeting. Negative supercoiling might facilitate local DNA unwinding, thereby lowering the kinetic barrier for R-loop formation and target recognition. In line with this, long-A pAgos efficiently cleave negatively supercoiled plasmid DNA only^{10,11}. Our hypothesis is further supported by our CRISPRi screen, which identified subunits of DNA gyrase, the primary enzyme to maintain negative supercoiling in bacteria, as essential factors for SPARMA-mediated immunity (Fig. 3c).

A major outstanding question about RNA-guided pAgo systems was how their guide RNAs are generated. Our CRISPRi screen and corroborating *in vitro* assays identify endoribonuclease RNase E as the primary host factor responsible for guide RNA biogenesis (Fig. 3c). Coupling pAgo activity to this widely conserved bacterial mRNA degradation factor generates a bias toward targeting highly transcribed elements: rapidly replicating phages and high copy-number plasmids produce abundant mRNA substrates that are processed by RNase E into guide RNAs (Fig. 3). Recruitment of host factors for guide RNA maturation mirrors established paradigms in other RNA-guided defense systems. For example, Type II CRISPR-Cas systems rely on the host factor RNase III to process trans-activating CRISPR (cr)RNAs (tracrRNAs) and precursor crRNAs⁶⁴. Utilization of RNase E by SPARMA represents an analogous evolutionary solution, underscoring the reliance of bacterial defense systems on the co-option of essential housekeeping factors. Beyond DNA gyrase and RNase E, our screen identified multiple other host factors that contribute to SPARMA-mediated cell death. These results highlight the utility of CRISPRi screens to systematically uncover host factors essential for defense system function.

Leveraging the host mRNA processing machinery enables the generation of a diverse and adaptable guide pool but also introduces a risk of autoimmunity through incorporation of host transcripts (Fig. 2). Yet, SPARMA induces no autoimmunity in the absence of invading DNA (Extended Data Fig. 2a). We propose that pAgo activation is constrained by the requirement for oligomerization^{12,14,17,24,59,65,66}. Oligomerization of guide/target-bound SPARMA is concentration-dependent: SPARMA filament assembly is observed at ~10 μ M, but not at ~0.5 μ M (Figs. 4a and 4b). This implies activation requires high local concentrations of guide/target-bound complexes to surpass an assembly threshold. Invading plasmid-encoded genes are ~10²-10³-fold more likely to be targeted than host genes (Fig. 2e). We speculate that this contributes to generating a high local concentration of SPARMA at invader DNA, but not at host genomic DNA. The spatial organization of plasmids, which are excluded from the nucleoid and localize to the bacterial poles⁴⁹, might contribute to this model. As such, the requirement for oligomerization prevents autoimmunity while enabling defense against highly transcribed multicopy invader DNA.

Abortive immunity effectors mediate diverse enzymatic activities, including NAD(P)ase activity (e.g., SIR2 and TIR), membrane depolarization (e.g., TM and phospholipases), nucleotide-pool modification (e.g., deaminases), and nuclease activity (e.g. HEPN, HNH, TOPRIM, and PD-(D/E)XK). PD-(D/E)XK superfamily nucleases are particularly prominent⁶⁷; this superfamily includes the nuclease domains Cap4⁶⁸, DREN¹⁴, Cas4-like⁶⁹, Mrr and SMEK domains^{41,70}. PD-(D/E)XK domains (including Mrr) emerge as primary hits in anti-phage screens⁷¹, and machine-learning-based tools underscore this prevalence: PD-(D/E)XK domains occur in 9% of uncharacterized defense systems, making it the most abundant effector superfamily in the bacterial ‘defense-ome’⁷². In these diverse contexts, various PD-(D/E)XK nuclease domains utilize different scaffolding strategies to facilitate higher-order oligomerization required for activation^{68 25,38}.

In SPARMA systems, activation of the PD-(D/E)XK family Mrr nuclease domain is linked to RNA-guided DNA detection by the short pAgo subunit. We propose that by integrating RNase E-mediated guide RNA biogenesis, DNA topology, and concentration-dependent activation, SPARMA and other pAgo systems avoid autoimmunity and are activated by high-copy-number invaders only. As such, our findings reveal a unifying principle in immunity, whereby effector domains are adapted to function with sensory domains that enforce stringent invader-specific activation of the associated effector.

Methods

Bacterial strains

The *Escherichia coli* strains utilized in this study and their respective applications are detailed below. *E. coli* BW25113 was used as the primary host for *in vivo* assays, including mixed-culture competition assays, phage defense assays, and the genome-wide CRISPRi screen. The lysogenic derivative *E. coli* BW25113 (DE3) was employed for plasmid-mediated abortive infection assays. For heterologous protein expression and purification, *E. coli* BL21* (DE3) was used. For nucleic acid pull-downs, *E. coli* BL21 (DE3) was used. For plasmid propagation, *E. coli* DH5 α was used for routine molecular cloning, and *E. coli* π 3813 was used for cloning R6K ori-containing plasmids. Finally, *E. coli* β 3914 served as the donor strain for conjugative transfer during *attTn7* chromosomal transposition.

Bacterial growth conditions

All experiments were conducted in Lysogeny Broth media (LB). For the maintenance of various genetic constructs, the medium was supplemented with antibiotics at the following final concentrations: 12.5 μ g/mL chloramphenicol (Cam) for bacterial artificial chromosomes (BAC), 25 μ g/mL for pFR56-derived CRISPRi vectors, 100 μ g/mL carbenicillin (Carb), 25 μ g/mL kanamycin (Kan), and 50 μ g/mL spectinomycin (Spec). To regulate gene expression under the P_{BAD} promoter, 1% (w/v) glucose (Glu) was added to ensure tight transcriptional repression, while 0.2% (w/v) L-arabinose (Ara) was used to induce gene expression. All liquid cultures were incubated at 37 °C with continuous shaking unless otherwise specified.

Phylogenetic analysis of Mrr-containing proteins

Mrr-containing proteins were identified by searching complete prokaryotic genomes from the prokaryotic NCBI Refseq database (downloaded April 11, 2026)⁹⁶ using HMMER3 v3.4 (<http://hmmer.org>). Two Hidden Markov Model (HMM) profiles corresponding to Mrr and Mrr-like domains (PF04471, PF21941; from hereon: Mrr) were used for the searches. DefenseFinder v3.0⁹⁷ was used to identify pAgos based on gene neighborhoods ($\pm$ 5 genes).

To examine the evolutionary relationships of Mrr domains of pAgo proteins, we performed a phylogenetic analysis on Mrr and APAZ (n=80) domains. Each domain (Mrr and APAZ) was aligned separately with MAFFT L-INS-i⁹⁸ (--localpair --maxiterate 1000) and trimmed using TrimAl v1.4 (-gt 0.2)⁹⁹. The trimmed alignments were then concatenated and analyzed as two partitions under maximum-likelihood with IQ-tree2 v2.3.5¹⁰⁰. Substitution models were selected by ModelFinder with partition merging (MFP+MERGE), yielding Q.pfam+R5 for Mrr and Q.pfam+R4 for APAZ domains. Branch support was determined with 1000 ultrafast bootstrap replicates (-B 1000 with --bnni) and 1000 SH-aLRT replicates (-alrt 1000); branches were considered well supported at SH-aLRT $\geq$ 80 and UFBoot $\geq$ 95. The tree was visualized using iTOLv7.6¹⁰¹.

Protein purification

The genes encoding the SPARMA system from *Pseudomonas plecoglossicida* (NCBI accessions: WP_016393046, WP_016393047) were synthesized as codon-optimized gBlocks for expression in *E. coli*. These sequences were cloned into a pET-based vector under the control of a T7 promoter. SPARMA complex with N-terminal 6x-His-MBP-tags (on APAZ protein) was expressed in *E. coli* BL21* (DE3) and purified in 4 steps: Ni²⁺-affinity, Amylose-affinity, heparin cation

exchange, and size exclusion chromatography. pET plasmids used for protein expression were transformed into *E. coli* BL21* (DE3). A single colony was used to inoculate a 10 mL LB culture supplemented with kanamycin (Kan) (25 µg/mL), which was incubated o/n at 37 °C at 160 rpm. The o/n culture was used to inoculate 12x500 mL LB supplemented with Kan (25 µg/mL), which was incubated at 37 °C at 150 rpm. When an OD_{600 nm} of ~0.25 was reached, the temperature of the incubator was switched to 18 °C, and after 1 h (when OD_{600 nm} reached ~0.6), protein expression was induced by the addition of IPTG (0.5 mM) and took place for 16 hours. Cells were harvested by centrifugation at 6000 x g at 4 °C for 10 min; pellets were stored at -80 °C until used for protein purification. Pellets were resuspended in NiNTA lysis buffer (500 mM NaCl, 20 mM Imidazole, cOmplete™ protease inhibitor pill (Roche), a pinch of DNase I recombinant (Roche), 20 mM HEPES (pH 7.5 at 25 °C)). Cells were lysed by sonication (QSONICA Q700A-220 sonicator with ½” tip; amp 50%, 1 s ON/2 s OFF, 5 minutes total ON time) and the lysate was centrifuged for 30 min at 30,000 x g at 4 °C. The clarified lysate was applied to a 5 mL HisTrap HP column (Cytiva Life Sciences). The column was washed with ~6 column volumes (CV) NiNTA buffer A (500 mM NaCl, 5 mM Imidazole, 20 mM Tris-HCl (pH 8 at 25 °C)) or until the A_{280 nm} of the eluate was below 250 a.u. The protein was eluted in NiNTA buffer B (500 mM NaCl, 350 mM Imidazole, 20 mM Tris-HCl (pH 8 at 25 °C)). Fractions containing the protein of interest were pooled and loaded on a 20 mL Amylose resin (New England Biolabs) column. The column was washed with 3x CV Amylose wash buffer (500 mM NaCl, 1 mM DTT, 20 mM Tris-HCl (pH 8 at 25 °C)) or until the A_{280 nm} was 0 a.u. The protein was eluted in Amylose elution buffer (500 mM NaCl, 1 mM DTT, 10 mM maltose, 20 mM Tris-HCl (pH 8 at 25 °C)). Fractions containing the protein of interest were pooled, EDTA was added to a final concentration of 2 mM, and TEV protease was added in a 1:50 (w/w) ratio. The sample was dialyzed in SnakeSkin dialysis tubing (10 kDa MWCO, Thermo Scientific) against dialysis buffer (250 mM KCl, 1 mM DTT, 2 mM EDTA, 20 mM HEPES (pH 7.5 at 25 °C)) for 16 h. Cleavage of the MBP tag by TEV was confirmed by SDS-PAGE and Coomassie Brilliant Blue (CBB) staining. The sample was diluted 1:1 with 20 mM HEPES (pH 7.5 at 25 °C), and loaded on a HiTrap Heparin column (Cytiva Life Sciences). The column was washed with 98.7% ion-exchange buffer I (100 mM KCl, 1 mM DTT, 8 mM HEPES (pH 7.5 at 25 °C)) and 1.3% ion-exchange buffer II (2.5 M KCl, 1 mM DTT, 20 mM HEPES (pH 7.5 at 25 °C)) until the A_{280 nm} was 0. The protein was eluted with ion-exchange buffer II by applying a gradient from 1.3% to 50% over a total volume of 60 mL. Peak fractions were analyzed by SDS-PAGE and CBB staining, and fractions containing the protein of interest were combined. The sample was concentrated to 1 mL using centrifugal filter units (Amicon) according to the protocol of the manufacturer. The sample was centrifuged for 10 min at 16,000 g at 4 °C, and the protein was fractionated on a custom 200 mL Superdex 200 Prep Grade resin (Cytiva Life Sciences) column, which was equilibrated in and eluted with SEC buffer (500 mM KCl, 1 mM DTT, 20 mM HEPES (pH 7.5 at 25 °C)). Peak fractions were analyzed by SDS-PAGE and CBB staining, and fractions containing the protein of interest were combined. The sample was concentrated to the desired concentration (around 10 mg/mL) using centrifugal filter units (Amicon) according to the protocol of the manufacturer. Samples were aliquoted, flash frozen in liquid nitrogen, and stored at -80 °C.

Plasmid abortive infection assay

E. coli BW25113 (DE3) was transformed with BAC-SPARMA (under P_{BAD}) and selected on LB Cam. To repress leaky expression, all media were supplemented with Glu (1% w/v). Trigger plasmids with varying copy numbers or +/- P_{T7} promoters were introduced via heat shock, with transformants selected on LB Carb/Cam/Glu. Colonies were picked and grown overnight in LB (Cam/Carb/Glu), washed once in fresh LB to remove residual Glu, and used to inoculate (1:100) into LB (Cam/Carb). Upon reaching an OD_{600 nm} of 0.5, systems were induced with 0.2% (w/v) L-

arabinose. For P_{T7} containing - trigger plasmids, 0.25 mM IPTG was added simultaneously. Cell viability was assessed via 10-fold serial dilution spot assays. Samples were collected 3 hours (SPARMA) post-induction and spotted (4 µL) onto LB agar supplemented with Cam, Carb, and Glu (1% w/v) to repress further system expression. Colony-forming units (CFUs) were quantified after overnight incubation at 37 °C.

To measure the impact of SPARMA expression on bacterial fitness, growth curves were conducted in the presence or absence of a high-copy pUC trigger plasmid. Starter cultures were grown overnight in LB medium supplemented with Cam and Glu to ensure tight repression of the P_{BAD} promoter. Cells were harvested by centrifugation and washed once in LB to remove residual Glu. The cultures were then inoculated at a 1:1000 dilution into 200 µL of LB medium containing Cam (for BAC maintenance), Carb (for pUC trigger plasmid maintenance, where applicable), and Ara to induce SPARMA expression. Growth was monitored using a BioTek Synergy H1 plate reader (Agilent) with continuous shaking at 37 °C. OD_{600 nm} was recorded every 5 min for a duration of 16 hours. All growth experiments were performed in biological triplicate.

Genomic DNA degradation *in vitro* assay

Genomic DNA from *E. coli* BW25113 was extracted from 1 mL of overnight culture grown in LB medium using the DNeasy Blood and Tissue Kits (Qiagen). DNA concentration was quantified using the Qubit dsDNA BR Assay Kit (Thermo Fisher). Reactions (20 µL total volume) were assembled in 10 mM HEPES-KOH (pH 7.5 at 25 °C), 125 mM KCl, and 5 mM MnCl₂, containing 1 µM protein complex (SPARMA), 1 µM guide RNA, 1 µM single-stranded DNA target, and 200 ng of purified *E. coli* genomic DNA. Protein and guide RNA (TNS206: 5'-AUUGAGAAUGAACUAAGGGAUGU) were pre-incubated for 5 min at 37 °C, followed by the addition of the target ssDNA (TNS273: 5'-GACATCCCTTAGTTCATTCTCAATC) and incubation for an additional 5 min at 37 °C. Purified *E. coli* genomic DNA was then added, and the reaction was incubated for 1 h at 37 °C. Reaction products were analyzed by electrophoresis on a 0.8% (w/v) agarose gel in 0.5x TBE buffer at 100 V for 1 h. Gels were stained with SYBR[™] Gold Nucleic Acid Gel Stain (Invitrogen; 1x concentration in 0.5x TBE) for 15 min at room temperature with gentle shaking and imaged using a Typhoon FLA 7000 laser scanner (GE Healthcare) with Cy2 channel.

Epifluorescence microscopy

E. coli BW25113 cells co-harboring the single-copy BAC-P_{BAD}::SPARMA and the high-copy pUC-derived trigger plasmid were grown overnight at 37 °C in LB broth supplemented with Cam/Carb/Glu. The following day, 1 mL of culture was pelleted (5 min, 6 000 g, 4 °C), washed once with antibiotic-free LB to remove residual Glu, and resuspended in 1 mL fresh LB. Washed cells were used to inoculate (1:100) 5 mL LB containing Cam and Carb without Glu. The culture was incubated at 37 °C with shaking (160 rpm) until mid-log phase (OD_{600 nm} ≈ 0.5), at which point Ara was added to induce expression of SPARMA from the pBAD promoter. After 4 h of induction, a 1 mL sample of the culture was harvested (5 min, 6 000 g) and the supernatant discarded. The pellet was resuspended in 1 mL 1x PBS; 100 µL was moved to a new 1.5 mL tube and stained with 1 µL DAPI stock (0.5 mg/mL) and 1 µL FM 4-64 stock (5 mg/mL), giving final concentrations of 5 µg/mL and 50 µg/mL, respectively. Samples were vortexed briefly and incubated for 15 min in the dark at room temperature. Stained cells were washed once with PBS (5 min, 6 000 g) and finally resuspended in 100 µL 1x PBS. For imaging, 1 µL of the washed cell suspension was spotted onto a 700 µL pre-cast 1.4 % (w/v) agarose pad in 1x PBS, air-dried for approximately 5 min, and overlaid with a glass coverslip. Images were then acquired on a Nikon ECLIPSE Ti2 inverted epifluorescence microscope equipped with a 100×/1.45 NA Plan-Apo λ oil-immersion objective,

and Nikon Type A immersion oil ($n_D = 1.515$) was used. Fluorescence was recorded using fixed exposure times of 2 ms (DAPI channel) and 100 ms (FM 4-64 channel) for all samples; bright-field images were also taken. Images were processed in Fiji⁷³ without altering fluorescence intensity or contrast. For each condition, at least two fields of view were analyzed from each of three biological replicates. Cell masks were generated from the FM 4-64 membrane channel using the following pipeline: Otsu auto-threshold (dark background) → Binary Close → Fill Holes. Particles were retained if their area was $0.5\text{--}8\ \mu\text{m}^2$ and circularity $0.2\text{--}1.0$ (Analyze Particles command). The mean DAPI fluorescence intensity was then extracted for every segmented cell, yielding ≥ 300 individual cells per condition.

Competition assay

Overnight LB–Glu precultures of *E. coli* strains carrying the single-copy pBAC P_{BAD} –SPARMA either alone (pBAC strain) or together with a high-copy pUC trigger plasmid (pBAC + pUC strain) were grown at 37 °C in the presence of Cam and, where appropriate, Carb. 1 mL of each culture was harvested (5 min, $6,000 \times g$), washed once in antibiotic-free LB, resuspended in 1 mL LB, and combined in a 1 : 100 ratio (pBAC : pBAC + pUC). The mixture was serially ten-fold diluted in PBS, and 4 μL of each dilution were spotted (“drop-test”) onto LB-agar + Cam with or without Carb to determine the baseline proportion of pUC-positive cells (day 0). The mixed culture was then diluted 1 : 1000 into 10 mL fresh LB containing Cam and Ara (no Carb) and incubated for 24 h at 37 °C with shaking (160 rpm). This passage was repeated once more under identical conditions. After each 24 h growth cycle (days 1 and 2), samples were serially diluted, and 4 μL drops were plated on LB-Cam and LB-Cam + Carb plates. Colonies in drops containing 5–50 CFU were counted the next day; plasmid retention was calculated as:

$$pUC \text{ containing cells (\%)} = \left(\frac{CFU \text{ on carb and cam plates}}{CFU \text{ on cam plates}} \right) * 100$$

Three independent biological replicates were performed for each strain.

Phage assays

For phage drop test assays, *E. coli* BW25113 was transformed either with empty pBAC (control) or a pBAC carrying SPARMA operon under P_{BAD} promoter. Three independent colonies were picked and grown overnight in LB broth supplemented with Cam. The overnight culture was used to inoculate LB supplemented with Cam (and incubated at 37 °C in a shaker incubator. When the culture reached an $OD_{600\text{ nm}}$ of 0.5, pAgo expression was induced by adding Ara, and the induced cells were returned to the incubator. After 1 h of incubation and expression, 500 μL of *E. coli* culture at an $OD_{600\text{ nm}}$ of 1.5 was mixed with 10 mL molten top agar supplemented with Cam Ara, 20 mM MgSO_4 , and 5 mM CaCl_2 . The mixture was poured onto a solidified bottom LB agar plate containing the same supplements. Once the top agar solidified, 2.5 μL of serially diluted phage stocks (ten-fold dilutions) of a collection of phages (λ_{vir} , P1_{vir}, P2_{vir}, T3, T4, T5, T6, T7, N4, Bas01-69) were spotted onto the surface. Plates were incubated at 30 °C for 24 h. Plaque formation was assessed by counting plaques, and images of the plates were taken. All experiments were performed in biological triplicate. For phi174 phage, *E. coli* C3000 strain was used. For the phage M13, *E. coli* BW25113 F⁺ was used.

For liquid culture infection with λ_{vir} , *E. coli* BW25113 was transformed either with empty pBAC (control) or a pBAC carrying SPARMA under P_{BAD} . Three independent colonies were picked and grown overnight in LB broth supplemented with Cam. The overnight cultures were diluted in fresh

LB to an OD_{600 nm} of 0.075 and supplemented with Cam, Ara, 20 mM MgSO₄, and 5 mM CaCl₂. Phage titers were determined by a drop test on an *E. coli* control strain lacking the SPARMA system. Phage stocks were then diluted to different multiplicities of infection (MOI) and added to the different *E. coli* strains in a 96-well plate. Strains were cultivated in a BioTek Synergy H1 plate reader (Agilent) with shaking at 30 °C. OD_{600 nm} was measured every 5 min for 10 h. All experiments were performed in biological triplicate.

Mass photometry

Mass photometry measurements were performed using a Refeyn TwoMP instrument (Refeyn Ltd.). Measurement coverslips with silicone cassettes were assembled using the MassGlass™ UC Sample Prep Kit (Refeyn Ltd.). The PspSPARMA complex for mass photometry analysis was assembled in assembly buffer (125 mM KCl, 2 mM MgCl₂, 1 mM DTT, and 10 mM HEPES (pH 7.5 at 25°C)) by first mixing the protein (0.5 μM) with the guide RNA (0.5 μM; TNS206), followed by incubation at 37 °C for 5 min. Where indicated, the target ssDNA (0.5 μM; TNS273) was subsequently added and incubated at 37 °C for an additional 5 min. For droplet-dilution measurements, samples were diluted immediately before imaging in silicone wells to final concentrations of 15–35 nM using either 1x PBS (pH 7.2 at 25 °C; Gibco) or assembly buffer to ensure single-molecule detection. Adsorption of biomolecules was recorded for 60 s using AcquireMP software (version 2024 R2; Refeyn Ltd.). Ratiometric contrast values were converted to molecular mass using MassFERENCE™ P1 Calibrant (Refeyn Ltd.) for calibration. Acquired mass photometry movies were subsequently analyzed using DiscoverMP software (version 2024 R2; Refeyn Ltd.).

Size exclusion chromatography – multi-angle light scattering

The PspSPARMA complex for Size-exclusion chromatography with a multi-angle light scattering (SEC-MALS) analysis was assembled in 200 μL assembly buffer (125 mM KCl, 2 mM MgCl₂, and 10 mM HEPES (pH 7.5 at 25°C)) by first mixing the protein (2 μM) with the guide RNA (2 μM; TNS206), followed by incubation at 37 °C for 5 min. Where indicated, the target DNA (2 μM; TNS273) was subsequently added and incubated at 37 °C for an additional 10 min. Sample analysis was carried out at room temperature on the HPLC system (Waters Breeze) using a Superdex 200 Increase 10/300 GL column (Cytiva) equilibrated with buffer (125 mM KCl, 2 mM MgCl₂, 1 mM DTT, 10 mM HEPES (pH 7.5 at 25 °C)), at a flow rate of 0.4 ml/min. The light-scattering signals were monitored using a miniDawn TREOS II detector (Wyatt), and the concentrations of all samples were measured with an Optilab T-rEX refractive index detector (Wyatt). Data acquisition and evaluation were performed using ASTRA 7 software (Wyatt).

Nucleic acid pull-down, isolation, and sequencing

PspSPARMA was expressed and purified as described above, with the following modifications: cells were grown at 28 °C for 4.5 hours after induction, the NaCl concentration in all purification buffers was reduced to 125 mM, all purification buffers were supplemented with 2 mM MnCl₂ and 2 mM MgCl₂, and only NiNTA and amylose purification steps were performed. After purification, samples were concentrated to 10 μM using centrifugal filter units (Amicon) and stored at -70°C until further use. To extract total RNA from the same cultures as were used for protein purification, 5 mL was collected before lysis, pelleted, and stored at 70°C. These samples were sonicated before nucleic acid extraction, as detailed below.

For nucleic acid extraction and library preparation, all centrifugation steps were performed in a tabletop centrifuge cooled to 4 °C, at 16000 x g. To extract nucleic acids, 10 mM EDTA and 40 μL

proteinase K (New England Biolabs) were added to 360 μ L of purified protein or lysed cell culture, and incubated for two hours at RT. Next, 1 volume of Roti phenol/chloroform/isoamyl alcohol was added, followed by vortexing and centrifugation for 15 min. The upper aqueous phase was transferred to a fresh tube, and 0.1 volumes of 1 M sodium acetate, 3 volumes of 100% ethanol, and 10 μ L of linear polyacrylamide (Alfa Aesar) were added. The mixture was briefly vortexed and incubated at -20°C for 16 h. Samples were then centrifuged for 30 min, and the supernatant was carefully removed without disturbing the pellet. The pellet was washed with 500 μ L of 70% ethanol pre-cooled to -20°C and centrifuged for 15 min. The ethanol was carefully removed, taking care not to disturb the pellet. Pellets containing the copurified nucleic acids were dried at 50°C for 10 min before being resuspended in 50 μ L of RNase-free water.

For small RNA and long RNA sequencing, libraries were generated by GenomeScan (Leiden, NL). For small DNA sequencing, samples were treated subsequently with RNase A (Thermo Scientific) and T4 PNK (Thermo Fisher, EK0031), with PCI extraction steps as detailed above after both enzymatic treatments. Next, library preparation was carried out according to the protocol described by²⁰. The DNA oligonucleotides used in this protocol are listed in Table S1. In brief, the 5'- and 3'-adapters were annealed to their respective bridge oligonucleotides in the presence of 10 mM KCl by heating the mixture to 95°C for 2 min in a thermocycler, followed by gradual cooling to room temperature. Next, 4 μ L of DNA was ligated to the annealed adapter oligonucleotides in a total reaction volume of 15 μ L. Subsequently, 5 μ L of the ligation reaction was amplified using barcoded primers compatible with Illumina sequencing platforms (Table S1).

All libraries were sequenced by GenomeScan on an Illumina NovaSeq 6000 platform using paired-end sequencing with a read length of 150 bp.

Fragmented genomic DNA sequencing

Reactions (60 μ L total volume) were assembled in 10 mM HEPES-KOH (pH 7.5 at 25°C), 125 mM KCl, and 5 mM MnCl_2 , containing 2 μ M protein complex (SPARMA), 2 μ M guide RNA (TNS206), 2 μ M DNA target (TNS273) and 200 ng of purified *E. coli* genomic DNA. Protein and guide RNA were pre-incubated for 5 min at 37°C , followed by addition of the target DNA and incubation for an additional 5 min at 37°C . Purified *E. coli* genomic DNA (200 ng) was then added and the reaction was incubated for 2 h at 37°C . Fragmented DNA products were purified with AMPure XP beads (Beckman Coulter) at 1X concentration to remove oligonucleotides. DNA concentration was measured using Qubit dsDNA BR kit. DNA fragments were sequenced by Novogene on an Illumina NovaSeq 6000 platform using paired-end sequencing with a read length of 150 bp.

Collateral cleavage assay (DNaseAlert)

Collateral nuclease activity was monitored using the DNaseAlert™ substrate (Integrated DNA Technologies). Reactions (20 μ L total volume) were prepared in 10 mM HEPES-KOH (pH 7.5 at 25°C), 125 mM KCl, and 5 mM MnCl_2 , containing 1 μ M protein, 1 μ M guide RNA, 1 μ M ssDNA target, and 1 μ M DNaseAlert™ substrate. Protein and guide were pre-incubated for 5 min at 37°C , followed by the addition of the target DNA and incubation for an additional 5 min at 37°C . The DNaseAlert™ substrate was then added, and fluorescence was recorded in a 384-well microplate using a Synergy plate reader (BioTek) with excitation at 536 nm and emission at 556 nm. Fluorescence was measured every 2 min for 16 hours at 37°C with continuous shaking.

Chromosomal integration in the *attTn7* site

Chromosomal integration of the PspSPARMA systems was performed using a dual plasmid Tn7-based transposition system⁷⁴. Briefly, *E. coli* BW25113 was first transformed with a helper plasmid (pMVM1), which contains a thermosensitive pSC101 origin of replication and encodes the *tnsABCD* transposase genes under the control of an arabinose-inducible pBAD promoter. The cargo (containing SPARMA system) was cloned into a pMP234-derived suicide vector flanked by Tn7R and Tn7L sites and containing a SpecR gene (*aadA7*). The shuttle vector was delivered into the pMVM1-bearing recipient strain via conjugation. Transposition into the specific *attTn7* site was induced by the expression of the TnsABCD complex and selected on Spec plates at 42°C to induce the loss of the thermosensitive helper plasmid. Successful integration was verified by colony PCR and sequencing of the *attTn7* locus.

Generation of the CRISPRi library

To generate the CRISPRi library, the *E. coli* BW25113 strain was used as the recipient strain. These strains carried either PspSPARMA or the catalytically inactive PspSPARMA^{Mrr-D67A-Q78A} variant under the control of a P_{BAD} promoter, integrated at the *attTn7* chromosomal site. Both recipient strains were first transformed with the pBK146 trigger plasmid (pUC21 origin) via heat shock and selected on LB medium supplemented with Carb and Glu to ensure repression of the P_{BAD} promoter. The EcoWG1 library was transferred via conjugation from a donor MFD-pir *E. coli* strain (a diaminopimelic acid [DAP] auxotroph). The donor harbored the pFR56::EcoWG1 plasmid, which encodes dCas9 under a PhIF promoter (inducible by DAPG) and the sgRNA library under a constitutive promoter⁵⁰. For library preparation, a 500 µL cryo-tube of the donor strain was thawed and used to inoculate 50 mL of LB medium supplemented with Cam (25 µg/mL) and DAP (300 µM) and grown overnight. Simultaneously, recipient overnight cultures were grown in LB supplemented with Carb and Glu. On the day of conjugation, the donor library was diluted 1:100 into 10 mL of fresh LB supplemented with Cam (25 µg/mL) and DAP (300 µM) and grown at 37°C with shaking (160 rpm) until reaching an OD_{600 nm} of 1.0. To remove residual antibiotics, 1 mL of the donor day culture and 1 mL of the recipient overnight cultures were harvested by centrifugation (5,000 g for 5 min) and resuspended in 1 mL of LB supplemented with DAP (300 µM) and Glu. Donor and recipient cells were mixed at a 1:1 ratio (1 mL of each), concentrated by centrifugation (5000 x g for 5 min), and resuspended in 100 µL of LB supplemented with DAP (300 µM) and Glu. The mixture was spotted onto a 0.22 µm membrane filter placed on an LB agar plate supplemented with DAP and Glu. Following a 2 h incubation at 37°C, filters were transferred to 50 mL tubes, and cells were recovered in 2 mL of LB supplemented with Carb, Cam (25 µg/mL), and Glu. Transconjugant efficiency was determined by serial dilution and colony-forming unit (CFU) counts via drop tests (4 µL per drop) on LB agar plates supplemented with Carb, Cam (25 µg/mL), and Glu. Total transconjugants for both strains exceeded 5*10⁷ ensuring a library coverage of 2000X. The remaining recovery culture was concentrated (5,000 g for 5 min), and resuspended in 100 µL of LB and plated onto 12 cm² square LB agar plates supplemented with Carb/Cam (25 µg/mL)/Glu and incubated overnight at room temperature. To harvest the library, 5 mL of LB was added to the square plates, and the bacterial lawn was thoroughly scraped and recovered. The resulting library was stored in cryo-tubes with 10% (v/v) DMSO at -80 °C.

CRISPRi library induction and screening

To start the CRISPRi screen, a 500 µL glycerol stock of the previously generated library was used to inoculate 50 mL of LB medium supplemented with Carb and Cam (25 µg/mL). The culture was grown at 37 °C with shaking at 160 rpm. Upon reaching an OD_{600 nm} of 1.0, 1 mL of this pre-culture was used to inoculate 100 mL of fresh, pre-warmed LB medium containing DAPG (50 µM) for dCas9 induction. This step was performed without antibiotics. The culture was incubated for 3

hours at 37 °C with shaking (160 rpm) to establish the CRISPRi knockdown. Following the 3 h dCas9 induction period, a 50 mL aliquot of the culture was harvested as the T0 sample. Cells were collected by centrifugation (5,000 g for 5 min at 4 °C) and the cell pellet was stored at –20 °C for subsequent plasmid DNA extraction. To the remaining 50 mL of culture, Ara was added to induce the expression of either PspSPARMA or the PspSPARMA^{Mrr-D67A-Q78A} variant. The cultures were then incubated for an additional 90 min at 37 °C. The cultures were transferred to 50 mL tubes and harvested by centrifugation (5,000 g for 5 min at 4°C). The resulting pellet was washed once with 50 mL of LB supplemented with Carb/Cam (25 µg/mL)/Glu to remove the inducers. After a final centrifugation step (5,000 g for 5 min at 4 °C), cells were resuspended in 1 mL of LB Carb/Cam (25 µg/mL)/Glu. The concentrated suspension was divided and plated onto ten 12 cm x 12 cm square LB agar plates supplemented with Carb/Cam (25 µg/mL)/Glu. Plates were incubated overnight at room temperature to allow for the recovery of surviving clones. These surviving cells were pooled by adding 5 mL of LB to the surface of each plate and thoroughly scraping the bacterial lawn. This pooled population, designated as the T1 sample, was harvested and stored at –20 °C for subsequent plasmid DNA extraction.

Preparation of CRISPRi libraries for Next-Generation Sequencing

Plasmid DNA was extracted from the harvested cell pellets (T0 and T1 samples) using the NucleoSpin Plasmid kit (Macherey-Nagel) according to the manufacturer's instructions. DNA concentration was quantified using the Qubit BR dsDNA assay (Thermo Fisher Scientific). Illumina sequencing libraries were generated via a two-step, consecutive PCR approach to amplify the sgRNA region from the pFR56 plasmid. The first PCR step (PCR1) was designed to add the i5 index and generate a 290 bp amplicon. Each 20 µL reaction contained 100 ng of plasmid DNA, 0.4 µM of each forward and reverse primer, and 10 µL of Kapa HiFi HotStart ReadyMix (Roche). The amplification was performed using the following thermocycling conditions: 98 °C for 5 min; 2 cycles of 98 °C for 30 s, 60 °C for 90 s, and 72 °C for 30 s; followed by a final extension at 72 °C for 5 min. To remove residual primers and single-stranded DNA, 0.5 µL of Exonuclease I (Thermo Scientific) was added directly to the PCR1 product and incubated at 37 °C for 15 min, followed by heat inactivation at 85 °C for 15 min. The reaction was then purified using AMPure XP beads (Beckman Coulter) at a 1.8x ratio. Briefly, 36 µL of beads were added to the reaction, incubated for 5 min, and washed twice with 70% ethanol on a magnetic rack. The purified DNA was eluted in 14 µL of MQ water. The second PCR step (PCR2) was used to append the i7 index and the P5/P7 adapters, yielding a 354 bp amplicon. The 30 µL reaction consisted of 15 µL of Kapa HiFi HotStart ReadyMix, 9 µL of the purified PCR1 product, and 1 µM of each indexing primer. The thermocycling conditions were: 98 °C for 5 min; 14 cycles of 98 °C for 30 s, 60 °C for 90 s, and 72 °C for 30 s; followed by a final extension at 72 °C for 5 min. The final libraries were purified again using AMPure XP beads (1.8x ratio) and quantified using the Qubit HS dsDNA assay to ensure a final concentration >1 ng/µL. Libraries were sequenced on an Illumina platform using paired-end reads. A minimum depth of 2.1×10^6 reads per sample was targeted to ensure at least 100x coverage of the sgRNA library.

Quantification and analysis of sgRNA abundance

The resulting sequencing data were demultiplexed based on the i5 and i7 index sequences. Quantification of sgRNA abundance was performed using the MAGeCK (Model-based Analysis of Genome-wide CRISPR-Cas9 Knockout) software package⁷⁵ implemented on the Galaxy platform⁷⁶. The count function was used to generate a count matrix from the FASTQ files for both the T0 and T1 samples, which served as the basis for downstream enrichment and depletion analysis.

Validation of CRISPRi targets using a single sgRNA assay

To generate a vector for single-spacer cloning, the spacer region of the pFR56 plasmid was replaced with a 20 bp sequence containing two BsaI restriction sites (pTNS18). This modified pFR56 served both as the cloning backbone for Golden Gate assembly and as a non-targeting sgRNA negative control, as the 20 bp sequence lacked homology to the *E. coli* BW25113 genome. Individual spacers targeting genes of interest were cloned into this destination vector using BsaI-HF v2 (New England Biolabs) according to standard Golden Gate protocols. For the *in vivo* survival assays, *E. coli* strains harboring genome-integrated, P_{BAD}-inducible PspSPARMA, PspSPARMA^{Mrr-D67A-Q78A} were used. These strains were first transformed with a trigger plasmid and selected on LB agar supplemented with Carb (100 µg/mL). Subsequently, the strains were transformed with either the control pFR56 plasmid or pFR56 carrying a specific-target sgRNA. Transformants were selected on LB agar plates containing Cam (25 µg/mL), Carb, and Glu to ensure repression of the SPARMA systems. Three independent colonies were inoculated into LB medium Carb/Cam (25 µg/mL)/Glu and grown overnight at 37°C. To remove residual Glu, 1 mL of each overnight culture was harvested by centrifugation (5 min at 5,000 g) and washed once with 1 mL of fresh LB. The cells were then used to inoculate 5 mL of LB Carb/Cam (25 µg/mL) at a 1:20 dilution and grown at 37 °C until the OD_{600 nm} reached 1.0. To induce the CRISPRi knockdown, 50 µL of this culture was transferred into 5 mL of fresh LB supplemented with DAPG (50 µM) and incubated for 3 h at 37 °C with shaking (160 rpm). Following the 3 h of dCas9 induction, Ara was added to trigger the expression of the SPARMA systems. Induction was maintained at 37 °C for 1.5 h. Cell viability was quantified by performing ten-fold serial dilutions in LB, followed by drop tests (4 µL per drop) on LB agar plates supplemented with Carb/Cam (25 µg/mL)/Glu. Colony-forming units (CFU) were counted after overnight incubation at 37°C.

In vitro RNase E digestion assays

The catalytic domain of *E. coli* RNase E (residues 1–529) and its catalytically inactive variant (RNase E^{D303A}) were expressed with an N-terminal 6xHis-MBP tag and purified using a sequential four-step chromatographic protocol. Proteins were initially captured via Ni-NTA affinity chromatography, followed by amylose affinity chromatography. The 6xHis-MBP tag was removed by TEV protease cleavage, and the proteins were further purified using heparin affinity and a final step via size-exclusion chromatography (SEC). Purified proteins were stored at high concentrations (>10 mg/mL) in SEC buffer (20 mM HEPES-KOH (pH 7.5 at 25°C), 500 mM NaCl, and 1 mM DTT) at -80°C. Cleavage assays were performed using a 28-nucleotide 5'-phosphorylated pre-guide RNA substrate (TNS501: 5'-UAGAGAUUGAGAAUGAACUAAGGGAUGU-3') in a reaction buffer consisting of 20 mM HEPES (pH 7.5 at 25°C), 250 mM KCl, and 10 mM MnCl₂. For each reaction, 2 µM of the RNA substrate was incubated at 37°C for 5 min with WT RNase E or RNase E^{D303A} mutant at final concentrations of 0.05 µM, 0.005 µM, or 0.0005 µM. Reactions were stopped by the addition of 2x RNA loading buffer (95% (v/v) deionized formamide, 5 mM EDTA, 0.025% (w/v) SDS, 0.025% (w/v) bromophenol blue, and 0.025% (w/v) xylene cyanol) followed by incubation at 95 °C for 5 min. Cleavage products were resolved by 20% (w/v) denaturing polyacrylamide gel electrophoresis (7 M urea, 0.5x TBE buffer) at 100 V for 2 h. Gels were subsequently stained with SYBR Gold (Thermo Fisher Scientific) and visualized using a Typhoon FLA 7000 laser scanner (GE Healthcare).

One-Pot coupled RNase E and SPARMA *in trans* cleavage activity

To evaluate the coupled pre-guide processing by RNase E and subsequent activation of the SPARMA complexes, one-pot nuclease assays were performed using a DNaseAlertTM (IDT)

fluorescent readout. Pre-incubation reactions were conducted in a buffer consisting of 20 mM HEPES (pH 7.5 at 25°C), 250 mM KCl, and 10 mM MnCl₂. Initially, 2 μM of SPARMA complex WT or catalytic mutant) was mixed with 0.05 μM of RNase E (wild-type or D303A mutant). To this mixture, 2 μM of the pre-guide RNA (TNS501: 5'-UAGAGAUUGAGAAUGAACUAAGGGAUGU) was added and incubated at 37 °C for 5 min to allow for RNA processing. Subsequently, 2 μM of a single-stranded DNA (ssDNA) target complementary to the generated guide (TNS273: 5'-GACATCCCTTAGTTCATTCTCAATC) was added and incubated for an additional 5 min at 37 °C. The reaction was then initiated by mixing 10 μL of this pre-incubated mixture with 10 μL of 2 μM DNaseAlert™ substrate in a 384-well black plate. Fluorescence kinetics were monitored every 2 min for 16 h at 37 °C using a microplate reader (BioTek Synergy). All experiments were performed in triplicate.

Negative stain EM sample preparation and data acquisition

Flash-frozen SPARMA was incubated at 2 μM concentration with 2.5 μM RNA guide (TNS206: 5'-AUUGAGAAUGAACUAAGGGAUGU) for 10 minutes at 37 °C in 10 mM HEPES (pH 7.5), 125 mM KCl, and 5 mM MnCl₂. Then, 2.5 μM DNA target (TNS207: 5'-ATGATTCTACTTACGACATCCCTTAGTTCATTCTCAATCTCTAACTGTCTCAACA), or MQ, was added and samples were incubated for 10 more minutes. Samples were diluted to lower the concentration to 0.25 μM SPARMA, and 4 μL of each sample was applied to Formvar/carbon-coated 300-mesh copper grids that had been glow-discharged for 1.5 m (25 mA current, 7.0 × 10⁻¹ mbar vacuum). After blotting of the sample, grids were twice stained with 2% uranyl acetate. Grids were imaged on a Tecnai T12 using a pixel size of 4.66 Å with ~2.5 μM defocus. Data was analyzed using the same software as Cryo-EM data, detailed below.

Cryo-EM sample preparation and data acquisition

To obtain structures of SPARMA and SPARMA^{Ago-K241A}, the flash-frozen proteins were incubated at 5 μM concentration with 6 μM RNA guide (TNS206: 5'-AUUGAGAAUGAACUAAGGGAUGU) for 10 minutes at 37 °C in 10 mM HEPES (pH 7.5), 200 mM KCl, 5 mM MnCl₂, and 5% glycerol. Subsequently, 6 μM DNA target (JBO-0234: CGACATCCCTTAGTTCATTCTCAATCT) was added followed by 10 min incubation.

For the structure of activated SPARMA filaments, SPARMA was incubated as described above, but 5 mM MgCl₂ was used instead of MnCl₂, the salt concentration was lowered to 125 mM KCl, and incubation with the DNA target was extended to 30 minutes. After incubation, the samples were centrifuged for 10 minutes at maximum speed in a cooled tabletop centrifuge, and the lower fraction containing a higher concentration of filaments was used to prepare grids. Samples prepared this way were used both for cryo-EM and negative stain EM (detailed above).

For each of these samples, a 4 μL volume of sample was applied to continuous carbon-coated Quantifoil R1.2/1.3 holey carbon grids that had been glow discharged for 10 s (23 mA current, 7.0 × 10⁻¹ mbar vacuum). Grids were blotted with a Vitrobot Mark IV (Thermo Fisher) for 2 s, blot force 25, at 4 °C and 100% humidity, and plunge-frozen in liquid ethane. Data for all four datasets were collected on a 200 kV FEI/Thermo Fisher Glacios cryo-TEM equipped with an X-FEG electron source and a Falcon 4i direct electron detector. Data were collected in EPU v2.11, with a pixel size of 1.182 Å, a defocus range of -1.2 to -2.2 μm, and a total exposure time of ~7 s per movie, resulting in a total accumulated dose of 40 electrons Å⁻² split into 40 electron event representation (EER) fractions. A total of 3,320 movies were collected for the SPARMA complex, 3,754 for the SPARMA^{Ago-K241A} mutant, 1,583 for the SPARMA filament.

Cryo-EM data processing, model building ,and figure preparation

All cryo-EM data processing was carried out in cryoSPARC v4.4.1⁸⁷. For the SPARMA complex, SPARMA^{Ago-K241A} mutant datasets, particles were picked using the blob picker, and extracted particles were subjected to 2D classification. For the SPARMA filament dataset, regions of the grid were manually selected during collection to enrich for density features corresponding to large, amorphous aggregates visible at low resolution. Suitable 2D classes were selected and used for *ab initio* reconstruction (three classes) and heterogeneous refinement, using the *ab initio* models as templates. After multiple rounds of *ab initio* reconstruction and heterogeneous refinement, and once a homogeneous subset of particles had been curated, non-uniform refinement⁸⁸ was used to determine a high-resolution consensus reconstruction for each map, resolving the SPARMA complex to 2.9 Å and the SPARMA^{Ago-K241A} mutant dimer to 2.8 Å. Further classification of the filament dataset resolved a tetramer to 3.2 Å and a hexamer to 3.0 Å. Initial models for the WT SPARMA complex and the SPARMA^{Ago-K241A} mutant were built using ModelAngelo⁸⁹ directly into the respective cryo-EM maps, with AlphaFold3⁹⁰ used to fill gaps in regions of local flexibility or weak density. Divalent cations were placed manually in Coot v0.9.1⁹¹. Models were then refined using ISOLDE v1.8⁹² to improve the fit of the model to the map, followed by real-space refinement as implemented in Phenix v1.21.2⁹³ to optimize model geometry. For the higher-order tetramer and hexamer reconstructions, the refined symmetric SPARMA^{Ago-K241A} mutant dimer model was used as a starting template: additional copies were generated by rigid-body rotation and translation to match the oligomeric arrangement observed in each map, then fitted and refined against the corresponding density using ISOLDE and Phenix real-space refinement as described above. All structural figures were generated using ChimeraX v1.8⁹⁴. Modevector arrows were created using the PDBarrows script⁹⁵ and are used in the Supplementary Information.

Cryo-EM data collection, refinement, and validation statistics are provided in Supplementary information Table S1.

All data processing pipelines are illustrated in Supplementary information Figures S1-S3.

Data availability

- The unprocessed image files, raw microplate reader data, and RNA/DNA-seq analyses used to prepare the figures in this manuscript have been deposited in Mendeley Data. Reviewer link: <https://data.mendeley.com/preview/sxnbw2xr96?a=a3d341f3-f535-4634-8157-870d836f8385>.

- Raw next-generation sequencing data from pull-downs, genomic DNA digestion and from the CRISPRi screen are deposited in the Sequence Read Archive (SRA; BioProject: PRJNA1504088).

Reviewer link:

<https://dataview.ncbi.nlm.nih.gov/object/PRJNA1504088?reviewer=hf5i7e3eej34sjoqvdr13t1hr8>.

- Cryo-EM density maps are deposited in the Electron Microscopy Data Bank (EMDB) with accession codes EMD-59177 (SPARMA dimer, composite map), EMD-59173 (SPARMA dimer, consensus map), EMD-59174 (SPARMA dimer, local refinement), EMD-59175 (SPARMA dimer, local refinement), EMD-59195 (SPARMA dimer, local refinement), EMD-59154

(SPARMA^{Ago-K241A}, dimer), EMD-59178 (SPARMA^{Ago-K241A}, tetramer), EMD-59195 (SPARMA^{Ago-K241A}, hexamer), EMD-59157 (SPARMA filament, consensus map), EMD-59159 (SPARMA filament, local refinement), EMD-59158 (SPARMA filament, local refinement).

- Atomic coordinates are deposited in the Protein Data Bank with accession codes 3ZUH (SPARMA dimer), 3ZTP (SPARMA^{Ago-K241A}, dimer), 3ZUI (SPARMA^{Ago-K241A}, tetramer), 3ZVD (SPARMA^{Ago-K241A}, hexamer)

Code availability

- This paper does not report original code.

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Competing interests

D.C.S. and B.K. are inventors of patents describing the utilization of short pAgo systems for nucleic acid detection.

Author contributions

T.N., B.K., and D.D. conceived and performed experiments under supervision of M.Z., J.B., and D.C.S.. T.N. and B.K. generated strains and purified proteins. T.N. performed *in vitro* and *in vivo* experiments. T.N. and B.K. performed RNA/DNAseq analyses. T.N. performed the CRISPR interference screen and validation thereof. B.K. and J.B. determined cryo-EM structures and performed structure analyses. D.D. performed Mass Photometry, D.D., D.Z., and S.L. performed SEC-MALS experiments. J.J. and T.N. performed microscopy experiments. A.S.A. performed bioinformatics analysis. M.Z., J.B., and D.C.S. supervised the project. T.N., B.K., J.B., and D.C.S. wrote the manuscript with input from all other authors.

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