nature portfolio

Peer Review File

The PAZ Pocket and Dimerization Drive CpAgo's Guide-Independent and DNA-Guided Dual Catalysis

Corresponding Author: Professor Kaiming Zhang

This file contains all reviewer reports in order by version, followed by all author rebuttals in order by version.

Version 0:

Reviewer comments:

Reviewer #1

(Remarks to the Author)
Liu and colleagues reports a comprehensive investigation into the structural and functional mechanisms of the prokaryotic Argonaute protein (CpAgo). The study combines cryo-EM structural analysis, biochemical assays, and in vivo experiments to elucidate CpAgo's guide-independent and DNA-guided cleavage activities. While the findings sheds lights on prokaryotic immune systems and offering potential biotechnological applications, several issues need to be addressed before acceptance for publication.

Major Comments:

1. Does CpAgo form a dimer after binding to gDNA? The authors could incubate CpAgo with gDNA and then run it through a size-exclusion chromatography to observe whether a dimer exists. If available, they can take the protein corresponding to the dimer and monomer forms, respectively, and validate their tDNA cleavage activity.

2. The guide-independent cleavage activity of CpAgo is convincingly demonstrated. However, the mechanism remains unclear because the density of nucleic acids cannot be accurately modeled. The authors propose that the PAZ pocket contributes to this versatility. What is the mechanism by which mutating the positively charged amino acids of the PAZ cavity to negatively charged ones shifts CpAgo's target preference from DNA to RNA? The authors could verify the affinity through EMSA experiments and discuss it in the results.

3. The dimerization-dependent activity of CpAgo is a key finding, but the structural basis for why dimerization is essential for catalysis remains speculative. The authors propose conformational changes in the PIWI domain loops but do not provide direct evidence. Validating the impact of PIWI loop region mutations on dimer formation and cleavage activity is necessary.
- The functional assays for dimer interface mutants (Fig. 4e) are convincing, but the authors should clarify whether dimerization is also required for guide-independent activity or if it is specific to guide-dependent cleavage.

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2. What about SL5 RNA, what are its sequence and structure like?

3. In Extended Data Fig. 1c, size-exclusion chromatography shows the presence of four peaks. Are peaks 1 to 3 also different oligomeric states of the CpAgo protein, and do they have cleavage activity?

4. The statistical analysis of in vivo experiments (e.g., error bars in Fig. 5b,d) should be explicitly described (e.g., SD vs. SEM, number of replicates).

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Reviewer #2

(Remarks to the Author)
In this manuscript, Liu et al. report the cryo-EM structures of CpAgo in complex with various guide and target DNAs, revealing that the dimeric form of CpAgo represents the catalytically active state. Notably, the authors uncover a surprising guide-independent plasmid cleavage activity and identify a positively charged pocket within the PAZ domain as critical for both guide-dependent and guide-independent functions. Since the role of the PAZ domain in plasmid cleavage has not been previously recognized, additional experimental evidence is required to strengthen this claim.
Major Concerns:
1. Please include TtAgo as a negative control in the plasmid cleavage assays shown in Figure 1. This will help rule out the possibility that the observed activity arises from contaminating nucleases.
2. The study would be significantly strengthened by identifying the specific catalytic residues within the PAZ domain responsible for the observed cleavage activity.
3. The conformational differences between the PIWI domains of the monomeric and dimeric CpAgo are subtle. It is currently unclear how these minor changes could lead to a complete loss of catalytic activity in the monomeric form. Please provide additional structural or biochemical rationale to support this conclusion.
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1. Many of the structural models—especially those at high resolution—exhibit relatively high clash scores and notable outliers in the Ramachandran plots. Please refine the models to reduce the clash scores (ideally below 10) and improve the overall geometry.
2. Please carefully proofread the manuscript to correct grammatical errors throughout.

Reviewer #3

(Remarks to the Author)
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Reviewer #4

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In this study, Liu et al. investigated the catalytic activity and immune function of a mesophilic prokaryotic Argonaute (pAgo) protein derived from Clostridium perfringens. CpAgo exhibits dual catalytic activity at physiological temperatures: guide-independent degradation of plasmids and RNAs, and DNA-guided cleavage of dsDNA. Structural analyses reveal a positively charged pocket within the PAZ domain that is critical for substrate binding targeted mutations in this region modulate substrate specificity between DNA and RNA. Catalytic activity is shown to rely on dimerization, mediated by interaction at the N-PAZ, PIWI-PIWI, and MID-PIWI interfaces. In E. coli, CpAgo degrades plasmids through guide-independent activity and targets invading DNA using self-generated short interfering DNA (siDNA) guides. High-resolution cryo-EM structures reveal conformational shifts that facilitate substrate recognition. These findings establish CpAgo as a bacterial defense protein with promising biotechnological applications in genome editing. I support the publication of this manuscript, provided that the authors adequately address the questions listed below.
1. In Figure 1: The study does not include catalytic site-specific amino acid substitutions as negative controls to validate guide-independent nuclease activity. While Figure 2 shows reduced cleavage efficiency with PAZ domain charge-reversal mutants, presumably due to impaired nucleic acid binding, the identity of the active site responsible for guide-independent catalysis remains unknown.

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3. Line 65-67: The claim that plasmid-derived DNA fragments serve as guides for subsequent cleavage lacks experimental support. There is no evidence provided that these fragments are loaded into CpAgo or function as guides in vitro or in vivo. This statement should be moderated.

4. While dimerization is shown to be essential for activity, this study does not investigate how different nucleic acid substrates influence dimer formation or stability. Moreover, the functional relevance of the monomeric CpAgo state observed

in cryo-EM structures remains unexplained.

5. There is a labeling discrepancy in Figure 3. Panel 3d is referenced but appears to be missing from the figure layout.

6. The manuscript does not adequately describe the content or significance of Extended Data Figures 8 and 9.

Version 1:

Reviewer comments:

Reviewer #1

(Remarks to the Author)
The authors have addressed all raised concerns in their revised paper. I think this paper in current form is acceptable for publication.

Reviewer #2

(Remarks to the Author)
The authors have fully addressed my concerns. I am satisfied with the revision.

Reviewer #3

(Remarks to the Author)
I co-reviewed this manuscript with one of the reviewers who provided the listed reports. This is part of the Nature Communications initiative to facilitate training in peer review and to provide appropriate recognition for Early Career Researchers who co-review manuscripts.

Reviewer #4

(Remarks to the Author)
The authors have addressed all my concerns, especially about the model. The current version of this revised manuscript is suitable for publication in this journal.

REVIEWER COMMENTS

Reviewer #1 (Remarks to the Author):

Liu and colleagues reports a comprehensive investigation into the structural and functional mechanisms of the prokaryotic Argonaute protein (CpAgo). The study combines cryo-EM structural analysis, biochemical assays, and in vivo experiments to elucidate CpAgo's guide-independent and DNA-guided cleavage activities. While the findings sheds lights on prokaryotic immune systems and offering potential biotechnological applications, several issues need to be addressed before acceptance for publication.

Major Comments:

1. Does CpAgo form a dimer after binding to gDNA? The authors could incubate CpAgo with gDNA and then run it through a size-exclusion chromatography to observe whether a dimer exists. If available, they can take the protein corresponding to the dimer and monomer forms, respectively, and validate their tDNA cleavage activity.

Response: We thank the reviewer for the insightful suggestion. To investigate whether CpAgo forms a dimer upon binding to gDNA, we incubated purified CpAgo_WT with gDNA at a 1:2 molar ratio for 20 min and analyzed the mixture using Superdex 200 Increase 10/300GL size-exclusion chromatography (SEC) (Fig. R1a). The chromatogram revealed that the CpAgo-gDNA binary complex existed predominantly in the monomeric form, with a minor fraction corresponding to the dimer and excess free gDNA. Next, we collected the monomeric and dimeric fractions and evaluated their tgDNA cleavage activity (Fig. R1b). Only the monomeric fraction exhibited detectable substrate cleavage activity, while the dimeric fraction showed no catalytic function, suggesting that the dimeric fraction is in a dysfunctional state.

To further assess whether the functional dimer (Fig. 4 and Extended Data Figs. 6, 7) forms upon substrate binding, we incubated the SEC-purified CpAgo-gDNA monomer fraction with tg_ssDNA in the presence of Ca^{2+} to prevent cleavage. A second round of SEC analysis revealed the emergence of a distinct dimer peak corresponding to the ternary complex (Fig. R1c),

confirming that catalytically active CpAgo dimers form only after binding to both guide and target strands. These results support a substrate-induced dimerization model in which CpAgo exists as a mixture of monomer and dysfunctional dimer upon gDNA binding, and only the monomer can transition into a catalytically competent dimer upon target engagement. These new results have now been added in the Extended Data Fig. 9a-c and described in the revised text (Page 9).

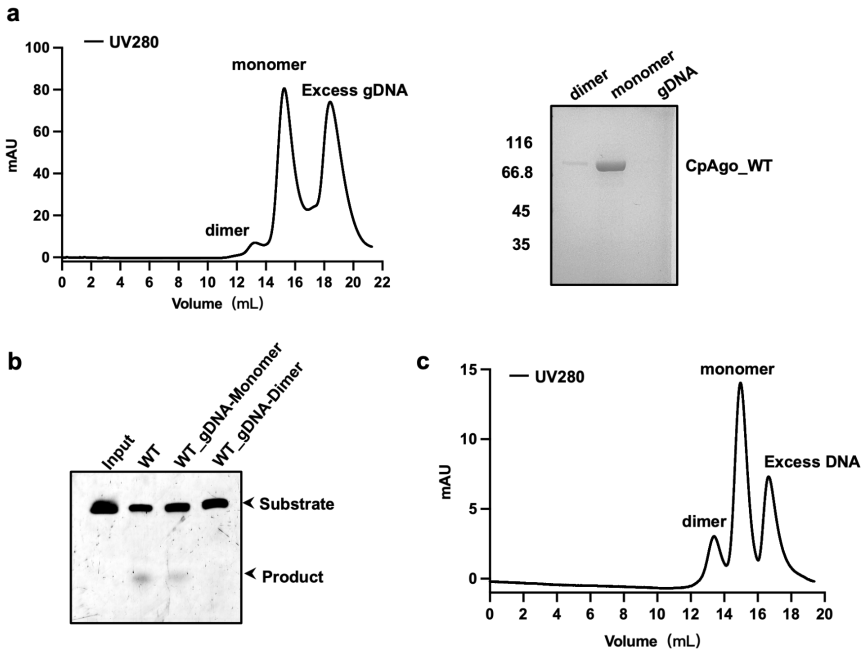


Fig. R1: Substrate-induced functional dimerization of CpAgo.

a SEC profile (left) and SDS-PAGE analysis (right) of CpAgo_WT pre-incubated with gDNA at a 1:2 molar ratio for 20 min. The SEC profile shows three peaks corresponding to CpAgo-gDNA dimer, monomer, and excess gDNA. SDS-PAGE confirms the presence of CpAgo in the dimer and monomer fractions.

b Target DNA cleavage assay comparing the catalytic activity of CpAgo-gDNA monomer and dimer fractions. Only the monomer fraction retained cleavage activity.

c SEC profile of ternary complex formation. CpAgo-gDNA monomer was incubated with tg_ssDNA in the presence of Ca^{2+} to prevent cleavage, followed by SEC analysis. A new dimer peak emerged, confirming that functional dimerization is induced upon target engagement.

2. The guide-independent cleavage activity of CpAgo is convincingly demonstrated. However, the mechanism remains unclear because the density of nucleic acids cannot be accurately modeled. The authors propose that the PAZ pocket contributes to this versatility. What is the mechanism by

which mutating the positively charged amino acids of the PAZ cavity to negatively charged ones shifts CpAgo's target preference from DNA to RNA? The authors could verify the affinity through EMSA experiments and discuss it in the results.

Response: We appreciate the reviewer's insightful question. To investigate the mechanism by which PAZ pocket charge-reversal mutants shift CpAgo's target preference from DNA to RNA, we performed EMSA assays to compare the RNA-binding affinity of wild-type CpAgo and PAZ mutants using a 29-nt ssRNA. As shown in Extended Data Fig. 4, PAZ mutants exhibit significantly enhanced binding to ssRNA, forming detectable ternary complexes at a 1:1:1 molar ratio (CpAgo:gDNA:ssRNA), whereas wild-type CpAgo requires at least a 16:16:1 ratio to achieve faint shifts. These results demonstrate that replacing the positively charged residues in the PAZ pocket with negatively charged ones increases CpAgo's affinity for RNA, thereby shifting its substrate preference from DNA to RNA. We have added this clarification to the revised Results section (Page 6).

3. The dimerization-dependent activity of CpAgo is a key finding, but the structural basis for why dimerization is essential for catalysis remains speculative. The authors propose conformational changes in the PIWI domain loops but do not provide direct evidence. Validating the impact of PIWI loop region mutations on dimer formation and cleavage activity is necessary.

- The functional assays for dimer interface mutants (Fig. 4e) are convincing, but the authors should clarify whether dimerization is also required for guide-independent activity or if it is specific to guide-dependent cleavage.

Response: We thank the reviewer for highlighting the importance of clarifying the structural basis underlying the dimerization-dependent activity of CpAgo. To explore the mechanistic basis for this, we conducted a detailed comparison between the dimeric ternary complex and the monomeric ternary complex. Our structural analysis identified two key features that differentiate the catalytically active dimer from the inactive monomer: 1) Angular shifts in two PIWI domain loops surrounding the catalytic tetrad (D544): The PIWI loops, located at the PIWI-PIWI interface (Fig. 4d), play a dual role: they are essential for both dimer formation and catalytic activation. In the dimeric state, angular shifts of these loops reposition D544 into an active conformation (Fig. 4f, g), while in the monomer, misalignment prevents catalysis. Mutating dimer interface residues—including those near the PIWI loops—disrupted dimerization (Extended Data Fig. 6b) and

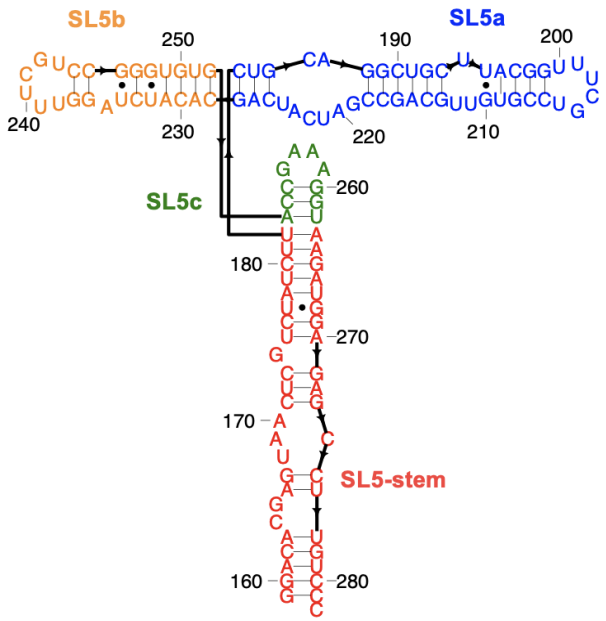
enzymatic activity (Fig. 4e), directly linking dimerization and enzymatic function. 2) Twisting of the guide-target duplex termini near the N domain in the dimer: This conformational change allows additional stabilizing interactions in the dimer, notably between t17'/t18' and Y37/K42 in the N domain, and between g14 and G579 in the PIWI domain (revised Fig. 4h, i). Disruption of these contacts via alanine substitution impaired cleavage activity and specificity (revised Fig. 4j). Together, these results provide direct structural evidence that dimerization is essential for CpAgo's catalytic mechanism. We have now clarified these mechanistic insights in the revised manuscript (Page 8), and appreciate the opportunity to strengthen the interpretation of this central finding.

Additionally, in our study, we found that CpAgo's catalytic activity—both guide-dependent and guide-independent—is dependent on its dimeric state. Functional assays (Fig. 4e and revised Fig. 4k) showed that mutations disrupting dimer interface interactions—located at the N-PAZ, PIWI-PIWI, and MID-PIWI regions—abolished cleavage activity for both tgDNA and plasmid/SL5 RNA substrates, strongly indicating that dimerization is required not only for target DNA cleavage but also for guide-independent degradation.

Minor Comments

1. In line 43, the term "siDNA" is introduced without definition.
Response: Done.

2. What about SL5 RNA, what are its sequence and structure like?
Response: The SL5 RNA is a structured RNA with a long stem and three stem-loops (SL5a, SL5b, SL5c), and its secondary structure is now provided in the Revised Fig. 1b.



Revised Fig. 1b: The secondary structure of the SL5 RNA.

3. In Extended Data Fig. 1c, size-exclusion chromatography shows the presence of four peaks. Are peaks 1 to 3 also different oligomeric states of the CpAgo protein, and do they have cleavage activity?

Response: Thank you for the question. We repeated the size-exclusion chromatography experiment. In revised Extended Data Fig. 1c, Peak 1 (p1) is the void volume, representing high-molecular-weight aggregates or misfolded protein species; peak 2 (p2) is largely composed of contaminating proteins as seen on the gel. Peak 3 (p3) corresponds to properly folded CpAgo, as indicated by its expected elution volume and prominent band at the correct molecular weight on SDS-PAGE. Only this fraction exhibited robust nucleic acid cleavage activity in downstream assays.

4. The statistical analysis of in vivo experiments (e.g., error bars in Fig. 5b,d) should be explicitly described (e.g., SD vs. SEM, number of replicates).

Response: We have revised them as suggested.

5. Line 172, Fig.3d? There is no “d” map in Figure 3.

Response: We thank the reviewer for pointing out this oversight. The correct citation should be Fig. 3c, not 3d. We have revised the text accordingly in the revised manuscript.

6. Line 264, CpAgo directly binds and processes host-derived exogenous mRNA into small fragments. What is the "host-derived exogenous mRNA", it means host mRNA or exogenous mRNA?

Response: Thank you for the question. The term "host-derived exogenous mRNA" refers to RNA transcribed from exogenous plasmids after they enter the host cell. We have revised our proposed model to avoid overinterpretation (Pages 10, 11).

7. The sample concentration used for cryo-EM needs to be specified in the methods section.

Response: We have revised the method part as suggested (Page 15).

Reviewer #2 (Remarks to the Author):

In this manuscript, Liu et al. report the cryo-EM structures of CpAgo in complex with various guide and target DNAs, revealing that the dimeric form of CpAgo represents the catalytically active state. Notably, the authors uncover a surprising guide-independent plasmid cleavage activity and identify a positively charged pocket within the PAZ domain as critical for both guide-dependent and guide-independent functions. Since the role of the PAZ domain in plasmid cleavage has not been previously recognized, additional experimental evidence is required to strengthen this claim.

Major Concerns:

1. Please include TtAgo as a negative control in the plasmid cleavage assays shown in Figure 1. This will help rule out the possibility that the observed activity arises from contaminating nucleases.

Response: We thank the reviewer for the helpful suggestion to include TtAgo as a negative control to rule out the possibility of contaminating nucleases in the plasmid degradation assays. Although TtAgo was not available to us, we performed the guide-independent plasmid cleavage assay using

another mesophilic prokaryotic Argonaute, IbAgo, which was readily available. The results showed that IbAgo exhibited similar guide-independent plasmid degradation activity, though with markedly lower efficiency compared to CpAgo (Extended Data Fig. 1e). To further exclude the possibility of nonspecific nuclease contamination introduced during protein purification, we purified an unrelated protein, T7 RNA polymerase, using the same purification protocol as CpAgo. The T7 polymerase showed no plasmid degradation activity under identical conditions (Extended Data Fig. 1e), supporting that the observed cleavage by CpAgo is not due to nuclease contamination. These additional controls have now been included in the Extended Data Fig. 1e and clarified in the revised text (Page 4).

2. The study would be significantly strengthened by identifying the specific catalytic residues within the PAZ domain responsible for the observed cleavage activity.

Response: We thank the reviewer for this important point. To identify residues responsible for CpAgo's guide-independent nuclease activity, we examined the PAZ pocket for candidate catalytic residues near the extra cryo-EM density. Based on the conserved DEDX catalytic motif in pAgos, we identified three aspartates (D80, D181, D249) as potential candidates. Alanine substitutions were introduced at these sites, and plasmid degradation assays were conducted *in vitro*. As shown in Fig. R2a, b (Fig. 2h), D80A and D249A significantly impaired guide-independent plasmid cleavage, whereas D181A had no effect. We further validated these findings *in vivo* using the plasmid clearance assay. The D80A and D249A mutants showed significantly reduced plasmid elimination efficiency in bacterial cells Fig. R2c, d (Fig. 5a, b), confirming that D80 and D249 are functionally important for CpAgo's guide-independent catalytic activity. These results help pinpoint the putative catalytic residues beyond the canonical PIWI tetrad. We have added these new results in the revised manuscript (Page 10).

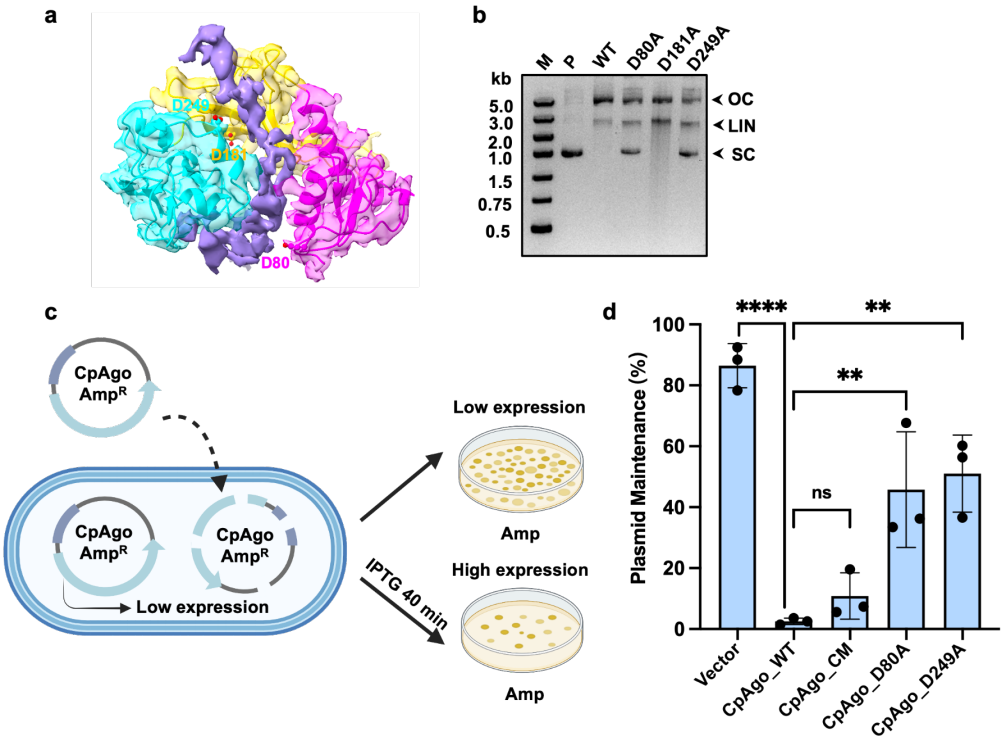


Fig. R2: Identification of residues critical for CpAgo's guide-independent nuclease activity.
a Structural model of CpAgo highlighting three aspartate residues (D80, D181, D249) near the PAZ pocket selected for mutational analysis.
b *In vitro* plasmid degradation assay showing that D80A and D249A mutants exhibit impaired guide-independent activity compared to wild-type CpAgo, while D181A has minimal effect.
c Schematic of *in vivo* plasmid clearance assay under low and high expression conditions.
d Quantification of plasmid maintenance in *E. coli* expressing CpAgo variants. Error bars represent standard deviation from three biological replicates. P values were determined using one-way ANOVA: ns, not significant; **, $P < 0.01$; ****, $P < 0.0001$.

3. The conformational differences between the PIWI domains of the monomeric and dimeric CpAgo are subtle. It is currently unclear how these minor changes could lead to a complete loss of catalytic activity in the monomeric form. Please provide additional structural or biochemical rationale to support this conclusion.

Response: We thank the reviewer for highlighting this important point. Through detailed comparison of the monomeric and dimeric CpAgo ternary complexes, we identified two key structural differences explaining the loss of catalytic activity in the monomer. First, angular shifts

in two PIWI domain loops at the PIWI-PIWI interface (Fig. 4d) reposition the catalytic residue D544 for catalysis in the dimer, while in the monomer, D544 is misaligned (Fig. 4f, g). Mutations at dimer interface residues—including those near the PIWI loops—disrupted both dimer formation (Extended Data Fig. 6b) and enzymatic activity (Fig. 4e), linking these loop shifts to catalysis. Second, twisting of the guide-target duplex termini near the N domain creates new stabilizing contacts in the dimer (revised Fig. 4h, i), whose disruption impaired cleavage efficiency (revised Fig. 4j). Together, these findings provide direct structural and biochemical evidence that dimerization is essential for CpAgo activation. We have clarified this mechanistic model in the revised manuscript (Page 8).

Minor Concerns:

1. Many of the structural models—especially those at high resolution—exhibit relatively high clash scores and notable outliers in the Ramachandran plots. Please refine the models to reduce the clash scores (ideally below 10) and improve the overall geometry.

Response: We thank the reviewer for the careful assessment of the structural models and for highlighting the importance of model quality and validation. Following your suggestion, we have re-refined all cryo-EM structures, with particular attention to reducing clash scores and optimizing geometry. As a result of these refinements: 1) Clash scores for five high-resolution models have been reduced to below 10, and in the other two cases, they are now within the range of 11-12, comparable to high-quality cryo-EM structures at similar resolutions. 2) Ramachandran plot statistics have also been improved significantly, with outlier percentages reduced. We have updated the Extended Data Tables 1-4 with revised model validation statistics and replaced the previous structure files with the refined versions in the manuscript submission. We appreciate the reviewer's valuable feedback, which helped us improve the rigor and reliability of our structural interpretations.

2. Please carefully proofread the manuscript to correct grammatical errors throughout.

Response: Done.

Reviewer #3 (Remarks to the Author):

I co-reviewed this manuscript with one of the reviewers who provided the listed reports. This is part of the Nature Communications initiative to facilitate training in peer review and to provide appropriate recognition for Early Career Researchers who co-review manuscripts.

Reviewer #4 (Remarks to the Author):

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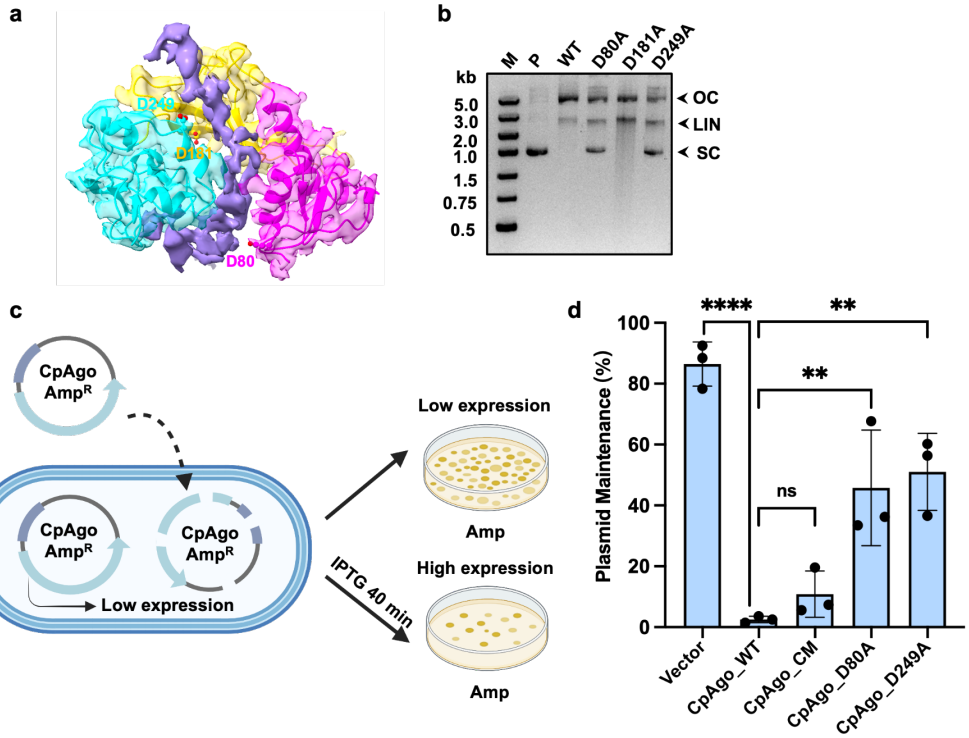

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Response: Thank you for the thoughtful comment. The apparent discrepancy between Figures 5a-b and Figures 5c-d arises from differences in experimental design and selection strategy. Figures 5a-b assess CpAgo's guide-independent plasmid degradation activity by directly transforming *E. coli* with plasmids expressing CpAgo or its mutants, followed by plating on Ampicillin to quantify colony formation. In this setup, CpAgo expression begins upon transformation, and subsequent induction exacerbates plasmid degradation, leading to substantial colony loss due to plasmid clearance. In contrast, Figures 5c-d evaluate CpAgo's guide-dependent plasmid interference using a two-plasmid system. Cells are co-transformed with CpAgo and target DNA plasmids and selected on Amp-Kanamycin plates. These strains are then made electrocompetent and used in a separate electroporation experiment, where guide DNA is introduced. Throughout this process, the cells are grown under dual antibiotic selection, which ensures plasmid maintenance. Thus, CpAgo expression occurs in a more controlled background, and the cleavage is only induced upon guide addition, allowing for visible colony growth on Amp-only plates and reduced growth on Amp-Kan plates. This setup is optimized to specifically capture guide-mediated interference rather than overall plasmid toxicity. To clarify this distinction, we have revised the figure in Fig. 5c to include a schematic illustrating the dual-plasmid selection step.

We also thank the reviewer for the valuable comment regarding the proposed model. While our study did not directly include phage infection assays, previous research on *Clostridium butyricum* Argonaute (CbAgo)—a close evolutionary homolog of CpAgo—demonstrated its ability to mediate plasmid and phage resistance in *E. coli*, effectively protecting cells from M13 and P1

phage infection (DOI: 10.1038/s41586-020-2605-1). Given the high sequence similarity between CpAgo and CbAgo, along with CpAgo's robust guide-independent plasmid degradation activity observed in our study, we infer that CpAgo may similarly contribute to antiphage defense via clearance of plasmid or foreign DNA. However, in the absence of direct experimental evidence, we have revised the proposed model to avoid overinterpretation (Pages 10, 11).

3. Line 65-67: The claim that plasmid-derived DNA fragments serve as guides for subsequent cleavage lacks experimental support. There is no evidence provided that these fragments are loaded into CpAgo or function as guides in vitro or in vivo. This statement should be moderated.
Response: We thank the reviewer for pointing out the need to clarify our interpretation regarding the function of plasmid-derived DNA fragments. In our study, we observed that CpAgo degrades plasmid DNA into ~21-nucleotide fragments (Fig. 1a), a length consistent with functional guide DNAs in Argonaute systems. While this observation provides a mechanistic rationale for the potential use of these fragments as guides in subsequent cleavage events, we acknowledge that direct evidence of their loading into CpAgo and their functional role in guiding target cleavage is not demonstrated in the current study. To address this, we have revised the corresponding statement in the manuscript to moderate the claim. The revised sentence now reads: “While it displayed no substrate preference between DNA and RNA, CpAgo generated ~21-nucleotide DNA fragments during plasmid degradation, suggesting a possible source of guide strands for targeted activity.” We believe this revision more accurately represents the scope of our findings and aligns with the reviewer’s concern.

4. While dimerization is shown to be essential for activity, this study does not investigate how different nucleic acid substrates influence dimer formation or stability. Moreover, the functional relevance of the monomeric CpAgo state observed in cryo-EM structures remains unexplained.
Response: Thank you for the insightful question. To investigate how different nucleic acid substrates influence CpAgo dimerization, we performed SEC assays using a catalytically inactive mutant (CpAgo_CM) preloaded with 21-nt gDNA, followed by incubation with target ssDNA (tg_ssDNA) of varying lengths (11, 16, 21, 29, and 58 nt). The results showed that ternary complex dimer formation was observed for 11–21 nt tg_ssDNA, but not for 29- or 58-nt tg_ssDNA (Fig. R3), suggesting that only shorter ssDNA triggers CpAgo dimerization. We then hypothesized that

the dsDNA substrates used for cryo-EM (29 bp and 58 bp) may still only expose ≤21 nt of single-stranded sequence for pairing with gDNA, allowing dimer formation. To test this, we incubated CpAgo_CM-gDNA with the same 29 bp and 58 bp dsDNA used in our structural studies and analyzed the complexes by SEC. As expected, clear dimer peaks corresponding to the ternary complex were detected (Fig. R3), supporting our model that dimer formation depends on the length of the single-stranded gDNA-target pairing region, rather than total duplex length.

Regarding the functional relevance of the monomeric CpAgo state observed in cryo-EM, particularly in ternary complexes, we hypothesize that this monomeric ternary state represents an intermediate configuration on the path to full activation. While the catalytically active CpAgo adopts a dimeric conformation, the monomeric ternary state likely serves as an initial recognition intermediate, where guide and target strands are aligned but the conformational rearrangements necessary for catalytic activation—particularly in the PIWI domain—have not yet occurred. This state may allow CpAgo to probe base-pairing fidelity and substrate geometry before committing to dimerization and cleavage. Such a checkpoint would prevent off-target activity and ensure that only properly matched substrates trigger dimer formation and catalysis. Thus, the monomeric ternary structure may play a critical regulatory role in CpAgo's mechanism, acting as a pre-cleavage surveillance state.

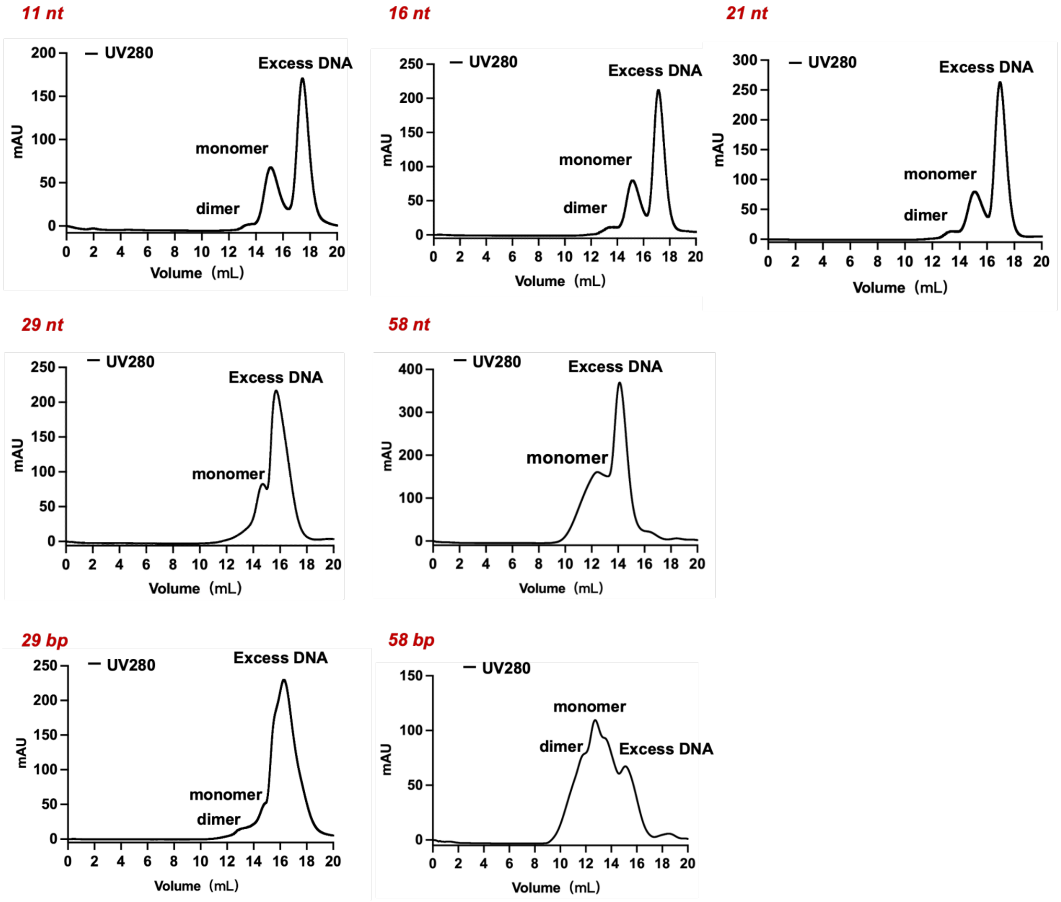

Fig. R3: SEC analysis of CpAgo_CM–gDNA complexes incubated with ssDNA or dsDNA targets of varying lengths.

5. There is a labeling discrepancy in Figure 3. Panel 3d is referenced but appears to be missing from the figure layout.

Response: We thank the reviewer for pointing out this oversight. The correct citation should be Fig. 3c, not 3d. We have revised the text accordingly in the revised manuscript.

6. The manuscript does not adequately describe the content or significance of Extended Data Figures 8 and 9.

Response: We appreciate the reviewer's valuable suggestion. We have now revised the corresponding section of the manuscript to explicitly describe the content and significance of original Extended Data Figures 8 and 9 (now revised Extended Data Figures 9 and 10) (Page 9).

REVIEWERS' COMMENTS

Reviewer #1 (Remarks to the Author):
The authors have addressed all raised concerns in their revised paper. I think this paper in current form is acceptable for publication.
Response: We sincerely thank the reviewer for the time, thoughtful comments, and positive assessment. We appreciate your support and are pleased that the revised manuscript meets the standards for publication.

Reviewer #2 (Remarks to the Author):
The authors have fully addressed my concerns. I am satisfied with the revision.
Response: We greatly appreciate the reviewer's thoughtful feedback and are grateful for your positive evaluation. Thank you for your support and for recognizing the improvements made in our revised manuscript.

Reviewer #3 (Remarks to the Author):
I co-reviewed this manuscript with one of the reviewers who provided the listed reports. This is part of the Nature Communications initiative to facilitate training in peer review and to provide appropriate recognition for Early Career Researchers who co-review manuscripts.
Response: We sincerely thank the reviewer and their co-reviewer for their valuable time and constructive feedback.

Reviewer #4 (Remarks to the Author):
The authors have addressed all my concerns, especially about the model. The current version of this revised manuscript is suitable for publication in this journal.
Response: We sincerely thank the reviewer for their thoughtful comments and support. We are grateful for the opportunity to improve our manuscript and are pleased that the current version meets the expectations for publication.