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Peer Review File

Dynamic monomer–dimer transition regulates DNA-guided RNA cleavage by MbpAgo

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)
The manuscript entitled “Dynamic monomer-dimer transition regulates DNA-guided RNA cleavage by MbpAgo” presents a series of high-resolution cryo-EM structures of MbpAgo in its apo, binary, and ternary states, together with comprehensive biochemical validation. The work provides novel and convincing mechanistic insight into how a prokaryotic Argonaute achieves DNA-guided RNA cleavage through a dynamic monomer-dimer transition, a mechanism that expands our current understanding of the functional versatility of pAgos. The work is well executed, and the overall quality of the structural and biochemical data is impressive. I believe the manuscript is suitable for publication in Nature Communications after the authors address several important issues detailed below to strengthen the conclusions and clarify mechanistic details.
1. Dimeric states have also been reported for PfAgo and CpAgo (Wang et al., Mol Cell, 2024; Liu et al., Nat Commun, 2025). The authors are encouraged to provide a systematic comparison between MbpAgo and these Argonautes, highlighting similarities and differences in their dimerization interfaces to better emphasize the unique features of MbpAgo.
2. Dimerization in PfAgo and CpAgo has been shown to stabilize catalytic loops (Wang et al., 2024; Liu et al., 2025). Does MbpAgo exhibit similar conformational rearrangements associated with activation?

3. The apo, binary, and ternary structures are all determined at high resolution, but did the authors attempt to apply symmetry constraints (such as C2) to further improve the binary complex resolution? Please clarify why the final reconstruction was performed with C1 symmetry.

4. Is the ternary structure completely monomeric? Were any minor dimeric subpopulations observed during 2D or 3D classification?

5. The Excess mutant shows reduced activity even with single-stranded DNA guides. Does this suggest that these residues are not only involved in stabilizing the “auxiliary density” but may also influence gDNA binding? The authors could perform EMSA or similar assays to further distinguish between effects on binding and catalysis.

6. Please provide local-resolution information for the auxiliary density to support the reliability of its interpretation in that region.

Reviewer #2

(Remarks to the Author)
Prokaryotic Argonaute (pAgo) proteins are nucleic acid–guided endonucleases. Unlike most other pAgos, MbpAgo is unique in that it uses DNA guides to specifically cleave RNA. In this study, the authors present cryo-EM structures of MbpAgo in three functional states: the apo form (without nucleic acids), the binary complex (with guide DNA), and the ternary complex (with guide DNA and target RNA). Structural comparisons reveal that upon binding guide DNA, MbpAgo forms a dimer through multiple protein–protein and protein–DNA interfaces. Binding the target RNA induces dimer dissociation, and

the resulting DNA–RNA duplex adopts an A-form like structure suited for RNA recognition and cleavage.

Overall, this work is interesting, and both the cryo-EM and functional data appear solid. However, I have two comments that the authors should address:

1. The authors claim that loop truncations do not alter the structure of MbpAgo based on AlphaFold3 predictions. This argument is not convincing. The authors should express and purify these truncation mutants and use biochemical methods (such as mass photometry, analytical ultracentrifugation (AUC), or SEC-MALS) to confirm that the truncations do not affect the folding or biochemical behavior of MbpAgo.

2. Similarly, the authors should perform biochemical or biophysical experiments (e.g., mass photometry, AUC, or SEC-MALS) to demonstrate that guide DNA binding indeed induces MbpAgo dimerization. Cryo-EM is not an ideal technique for characterizing dimerization, as particle selection may be biased and the air–water interface can potentially disrupt protein dimers.

Reviewer #3

(Remarks to the Author)
This manuscript reports a series of high-resolution cryo-EM structures of Mucilaginibacter paludis Argonaute (MbpAgo) in its apo, guide-bound, and guide–target states. The structures reveal distinct monomeric and dimeric conformations, suggesting a “functional cycle” in which MbpAgo transitions between oligomeric states during DNA-guided RNA cleavage.

The structural work is technically rigorous and significantly expands understanding of RNA-targeting prokaryotic Argonautes. However, the proposed dynamic mechanism is inferred solely from static structural snapshots, without supporting kinetic or solution-based evidence. The main strength of the study lies in its structural elucidation; the mechanistic interpretation remains hypothetical at this stage.

Major Concerns
1. Dynamic mechanism not experimentally demonstrated
The central “functional cycle” model proposes reversible monomer–dimer transitions, yet cryo-EM captures only static conformations. No kinetic or solution-based data demonstrate that these transitions occur dynamically or correlate with catalysis. Complementary experiments—such as SEC-MALS, or single-molecule FRET—would be essential to determine whether the observed oligomeric states interconvert under physiological conditions and are functionally relevant.

2. Structural findings versus mechanistic claims
The cryo-EM structures convincingly define the architectures of MbpAgo in apo, binary, and ternary states. These data firmly establish conformational differences but, by themselves, do not prove a regulatory role for dimerization. The manuscript would benefit from a clearer distinction between observations directly supported by the data and mechanistic hypotheses. Statements in the Abstract and Discussion, such as “establishes the dynamic monomer–dimer transition as the regulatory mechanism,” appear stronger than warranted by the current evidence.

3. Interpretation of auxiliary nucleic acid density
The additional density bridging the PAZ and MID lobes is intriguing but remains ambiguous in identity. Since only single-stranded DNA was supplied, assignment of this density as a duplex-like nucleic acid should be made cautiously (e.g., described as “consistent with nucleic-acid–like density”). Validation through biochemical approaches—such as nuclease treatment or use of designed duplex controls—would substantiate this interpretation.

4. Functional assays remain qualitative
The cleavage assays presented throughout the study are largely qualitative, relying on gel images without quantification. Quantitative analysis, such as measuring band intensities or determining kinetic parameters, would provide a firmer basis for comparing catalytic efficiency and for guiding dependence.

5. Timescale and dynamics of the functional cycle
The proposed model implies a sequential transition between oligomeric states, but no data address the timescale, coexistence, or order of these states. Even a simplified kinetic framework or heterogeneity analysis of particle populations would substantially increase the credibility of the proposed dynamic cycle.

Minor Concerns
• The Introduction could better articulate how MbpAgo differs mechanistically from other RNA-targeting prokaryotic Argonautes, such as PliAgo or RslAgo.
• A schematic summarizing the conformational transitions among the apo, binary, and ternary states would improve figure clarity.
• The recurring phrase “fine-tune catalytic activity” could be replaced by more precise wording, such as “modulate substrate accommodation” or “stabilize the catalytic conformation.”
• The discussion of SARS-CoV-2 and HIV RNA cleavage should be framed as a proof-of-concept demonstration rather than implying biological relevance.
• The discussion should close with an explicit statement acknowledging that the proposed monomer–dimer functional model remains a working hypothesis pending dynamic validation.

This is a technically strong and carefully executed structural study that provides essential insight into the architecture and potential regulation of a DNA-guided, RNA-targeting Argonaute. The primary conclusions regarding structural features and guide–target recognition are well supported. The secondary conclusion proposing a dynamic monomer–dimer functional cycle is intriguing and potentially significant, but remains speculative in the absence of supporting kinetic or biophysical data. Recommend major revision.

Reviewer #4

(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.

Version 1:

Reviewer comments:

Reviewer #1

(Remarks to the Author)
The revisions are thorough, the new supplementary figures are high-quality and directly relevant, and the mechanistic claims are now better supported and contextualized. The manuscript has been significantly strengthened and, in my view, is now suitable for publication in Nature Communications without further revision.

Reviewer #2

(Remarks to the Author)
The authors have addressed all of my concerns.

Reviewer #3

(Remarks to the Author)
This revised submission has successfully addressed the concerns raised in the previous review.

REVIEWER COMMENTS

Reviewer #1 (Remarks to the Author):

The manuscript entitled "Dynamic monomer-dimer transition regulates DNA-guided RNA cleavage by MbpAgo" presents a series of high-resolution cryo-EM structures of MbpAgo in its apo, binary, and ternary states, together with comprehensive biochemical validation. The work provides novel and convincing mechanistic insight into how a prokaryotic Argonaute achieves DNA-guided RNA cleavage through a dynamic monomer-dimer transition, a mechanism that expands our current understanding of the functional versatility of pAgos. The work is well executed, and the overall quality of the structural and biochemical data is impressive. I believe the manuscript is suitable for publication in Nature Communications after the authors address several important issues detailed below to strengthen the conclusions and clarify mechanistic details.

1. Dimeric states have also been reported for PfAgo and CpAgo (Wang et al., Mol Cell, 2024; Liu et al., Nat Commun, 2025). The authors are encouraged to provide a systematic comparison between MbpAgo and these Argonautes, highlighting similarities and differences in their dimerization interfaces to better emphasize the unique features of MbpAgo.

Response: We thank the reviewer for this helpful suggestion. To address this point, we performed a systematic structural comparison of the dimerization interfaces in MbpAgo, PfAgo, and CpAgo and presented the detailed interaction architectures in revised Supplementary Fig. 14.

As shown in revised Supplementary Fig. 14a-f, both PfAgo and CpAgo form dimers through a similar and relatively conserved set of interfaces. In these two Argonautes, dimerization is mediated primarily by three types of interaction surfaces: (i) two N-PAZ interfaces, (ii) one PIWI-PIWI interface, and (iii) two MID-PIWI interfaces. These contacts are dominated by hydrogen bonding and electrostatic interactions between residues, consistent with previous reports. In contrast, MbpAgo exhibits a distinct and more diversified dimerization architecture. As described in Fig. 2c-g, MbpAgo dimerization involves a different set of six discrete interfaces, including two N-PIWI interfaces, one L1-MID interface, two L2-L2 interfaces, and one PIWI-PIWI interface. Notably, the N-PIWI, L1-MID, and L2-L2 interfaces are absent in PfAgo and CpAgo and therefore represent MbpAgo-specific features. These additional interfaces substantially expand the dimer contact surface and involve linker regions that are not engaged in dimerization in PfAgo or CpAgo.

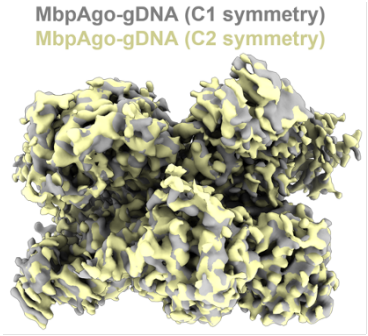
Together, this comparison highlights that although dimerization is a shared property among these pAgos, MbpAgo employs a unique combination of interfaces that differ both in domain composition and topology. This distinct dimerization mode likely reflects an adaptation of MbpAgo to its DNA-guided, RNA-targeting function and supports the notion that MbpAgo represents a mechanistically divergent member within the pAgo family. We have added the above information in our discussion section of the revised manuscript (Page 10).

2. Dimerization in PfAgo and CpAgo has been shown to stabilize catalytic loops (Wang et al., 2024; Liu et al., 2025). Does MbpAgo exhibit similar conformational rearrangements associated with activation?

Response: We thank the reviewer for this insightful question. To address this point, we performed a direct structural comparison of the catalytic loops in PfAgo, CpAgo, and MbpAgo across their inactive and active states (revised Supplementary Fig. 14g-i). In PfAgo and CpAgo, dimerization is accompanied by pronounced conformational rearrangements of the catalytic loops, repositioning key acidic residues into catalytically competent conformations upon transition from inactive to active states. In contrast, MbpAgo displays a distinct behavior. Comparison of the gDNA-bound dimeric state and the gDNA-tgRNA ternary state reveals only a subtle positional adjustment of the catalytic loops. Thus, unlike PfAgo and CpAgo, MbpAgo activation does not involve large-scale catalytic loop stabilization during dimer-to-monomer transition. These observations indicate that MbpAgo employs a mechanistically distinct activation strategy, in which dimerization primarily facilitates guide handling rather than direct catalytic loop activation. We have added the above information in our discussion section of the revised manuscript (Page 10).

3. The apo, binary, and ternary structures are all determined at high resolution, but did the authors attempt to apply symmetry constraints (such as C2) to further improve the binary complex resolution? Please clarify why the final reconstruction was performed with C1 symmetry.

Response: We thank the reviewer for this question. We tested C2 symmetry for the MbpAgo-gDNA dimer reconstruction and observed a high similarity between the C1 and C2 maps (cross-correlation coefficient = 0.9408; Response Fig. 1). However, imposing C2 symmetry did not improve the resolution. Instead, it partially obscured asymmetric features between the two protomers, including the auxiliary nucleic acid density bridging the PAZ-MID regions and subtle inter-subunit conformational differences. To preserve these biologically relevant asymmetric features, we therefore adopted C1 symmetry for the final reconstruction.



Response Fig. 1: Comparison of C1- and C2-symmetrized reconstructions of the MbpAgo-gDNA complex.

4. Is the ternary structure completely monomeric? Were any minor dimeric subpopulations observed during 2D or 3D classification?

Response: We thank the reviewer for this question. During extensive 2D and 3D classification of the MbpAgo-gDNA-tgRNA dataset, we did not observe any discernible dimeric subpopulations. All well-resolved classes corresponded to a monomeric ternary complex, and no stable or reproducible dimeric reconstructions could be identified. This indicates that the MbpAgo-gDNA-tgRNA complex is predominantly monomeric.

5. The Excess mutant shows reduced activity even with single-stranded DNA guides. Does this suggest that these residues are not only involved in stabilizing the "auxiliary density" but may also influence gDNA binding? The authors could perform EMSA or similar assays to further distinguish between effects on binding and catalysis.

Response: We thank the reviewer for this suggestion. We performed EMSA to directly compare gDNA binding by MbpAgo-WT and the Excess mutant (revised Supplementary Fig. 6d). The Excess mutant shows weaker gDNA binding than WT, with an increased dissociation constant (WT Kd = 0.1806 ± 0.0259 μM; Excess mutant Kd = 0.2711 ± 0.0218 μM). These results indicate that the residues mutated in the Excess construct contribute not only to stabilizing the auxiliary nucleic acid-like density and the dimeric binary complex, but also to gDNA binding itself. This provides a straightforward explanation for why the Excess mutant reduces cleavage activity even under ss-gDNA-guided conditions. We have added the new results in our revised manuscript (Page 6).

6. Please provide local-resolution information for the auxiliary density to support the reliability of its interpretation in that region.

Response: We thank the reviewer for this suggestion. The local-resolution map of the auxiliary nucleic acid density is now provided in the revised Supplementary Fig. 5f, supporting the reliability of its interpretation.

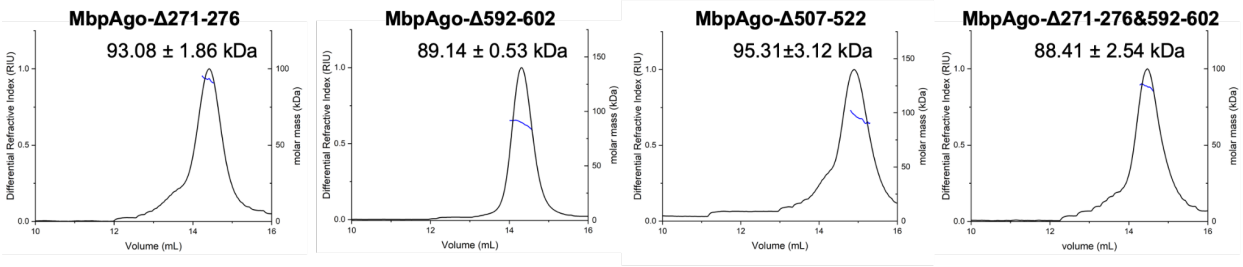
Reviewer #2 (Remarks to the Author):

Prokaryotic Argonaute (pAgo) proteins are nucleic acid–guided endonucleases. Unlike most other pAgos, MbpAgo is unique in that it uses DNA guides to specifically cleave RNA. In this study, the authors present cryo-EM structures of MbpAgo in three functional states: the apo form (without nucleic acids), the binary complex (with guide DNA), and the ternary complex (with guide DNA and target RNA). Structural comparisons reveal that upon binding guide DNA, MbpAgo forms a dimer through multiple protein–protein and protein–DNA interfaces. Binding the target RNA induces dimer dissociation, and the resulting DNA–RNA duplex adopts an A-form like structure suited for RNA recognition and cleavage.

Overall, this work is interesting, and both the cryo-EM and functional data appear solid. However, I have two comments that the authors should address:

1. The authors claim that loop truncations do not alter the structure of MbpAgo based on AlphaFold3 predictions. This argument is not convincing. The authors should express and purify these truncation mutants and use biochemical methods (such as mass photometry, analytical ultracentrifugation (AUC), or SEC-MALS) to confirm that the truncations do not affect the folding or biochemical behavior of MbpAgo.

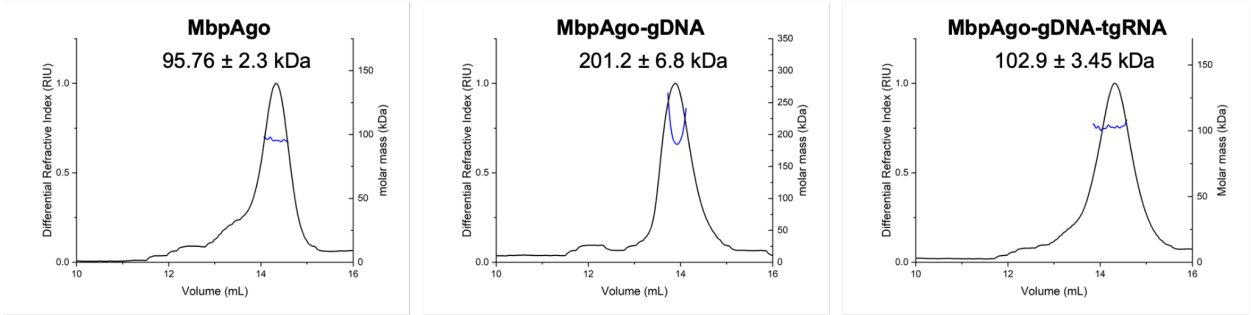
Response: We thank the reviewer for this suggestion. All loop truncation mutants were expressed, purified, and analyzed by SEC-MALS. As shown in revised Supplementary Fig. 4d (Response Fig. 2), all variants are monodisperse and exhibit molecular weights consistent with properly folded MbpAgo, comparable to the wild type. These data confirm that the truncations do not affect overall folding or biochemical behavior.



Response Fig. 2: SEC-MALS analysis of MbpAgo-Δ271-276, MbpAgo-Δ592-602, MbpAgo-Δ507-522, and MbpAgo-Δ271-276&592-602. Respective calculated molecular weight is marked on top of the peak.

2. Similarly, the authors should perform biochemical or biophysical experiments (e.g., mass photometry, AUC, or SEC-MALS) to demonstrate that guide DNA binding indeed induces MbpAgo dimerization. Cryo-EM is not an ideal technique for characterizing dimerization, as particle selection may be biased and the air–water interface can potentially disrupt protein dimers.

Response: We thank the reviewer for this important point. We performed SEC-MALS to directly assess the oligomeric state of MbpAgo in solution. As shown in revised Supplementary Fig. 4e (Response Fig. 3), apo MbpAgo has a molecular mass of ~95 kDa, consistent with a monomer, whereas binding of gDNA shifts the apparent molecular mass to ~201 kDa, indicating dimer formation. In contrast, the MbpAgo-gDNA-tgRNA complex reverts to ~103 kDa, consistent with a monomeric state. Therefore, these SEC-MALS data, in agreement with our cryo-EM analyses, demonstrate that gDNA binding indeed induces MbpAgo dimerization in solution.



Response Fig. 3: SEC-MALS analysis of MbpAgo, MbpAgo-gDNA and MbpAgo-gDNA-tgRNA complexes.

Reviewer #3 (Remarks to the Author):

This manuscript reports a series of high-resolution cryo-EM structures of Mucilaginibacter paludis Argonaute (MbpAgo) in its apo, guide-bound, and guide–target states. The structures reveal distinct monomeric and dimeric conformations, suggesting a "functional cycle" in which MbpAgo transitions between oligomeric states during DNA-guided RNA cleavage.

The structural work is technically rigorous and significantly expands understanding of RNA-targeting prokaryotic Argonautes. However, the proposed dynamic mechanism is inferred solely from static structural snapshots, without supporting kinetic or solution-based evidence. The main strength of the study lies in its structural elucidation; the mechanistic interpretation remains hypothetical at this stage.

Major Concerns

1. Dynamic mechanism not experimentally demonstrated

The central "functional cycle" model proposes reversible monomer–dimer transitions, yet cryo-EM captures only static conformations. No kinetic or solution-based data demonstrate that these transitions occur dynamically or correlate with catalysis. Complementary experiments—such as SEC-MALS, or single-molecule FRET—would be essential to determine whether the observed oligomeric states interconvert under physiological conditions and are functionally relevant.

Response: We thank the reviewer for raising this important point. Since cryo-EM captures static conformations, we complemented these structures with solution-based SEC-MALS measurements to probe MbpAgo oligomeric states under near-physiological conditions (20 mM Tris, pH 7.5, 200 mM NaCl) that is commonly used in *in vitro* studies of Ago proteins to maintain solubility. As shown in revised Supplementary Fig. 4e (Response Fig. 3), MbpAgo is monomeric in the apo state (~95 kDa), undergoes dimerization upon gDNA binding (~201 kDa), and reverts to a monomeric state upon formation of the gDNA-tgRNA ternary complex (~103 kDa). These results demonstrate that the monomer-dimer-monomer transition is reversible and coupled to guide and substrate binding. Therefore, the consistency between SEC-MALS and cryo-EM across distinct functional states, together with our biochemical validation of distinct functional roles for the monomeric and dimeric forms (Figs. 1-4), provides reliable experimental support that these oligomeric transitions occur in solution and are directly coupled to the catalytic cycle.

2. Structural findings versus mechanistic claims

The cryo-EM structures convincingly define the architectures of MbpAgo in apo, binary, and ternary states. These data firmly establish conformational differences but, by themselves, do not prove a regulatory role for dimerization. The manuscript would benefit from a clearer distinction

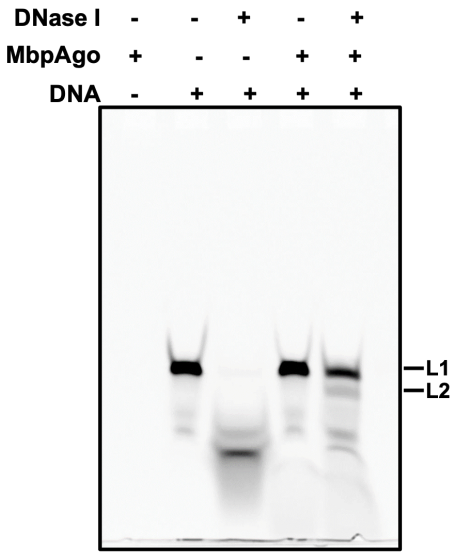
between observations directly supported by the data and mechanistic hypotheses. Statements in the Abstract and Discussion, such as "establishes the dynamic monomer–dimer transition as the regulatory mechanism," appear stronger than warranted by the current evidence.

Response: We thank the reviewer for this important comment. Accordingly, we have revised the wording in the Abstract and Discussion to replace "establishes" with "suggests". We now describe the monomer-dimer cycle as a working model that integrates cryo-EM snapshots with complementary biochemical data, including SEC-MALS, kinetic assays, mutational analysis, and functional cleavage assays. These revisions ensure that mechanistic conclusions are presented as hypotheses supported by multiple lines of evidence rather than as definitive proof.

3. Interpretation of auxiliary nucleic acid density

The additional density bridging the PAZ and MID lobes is intriguing but remains ambiguous in identity. Since only single-stranded DNA was supplied, assignment of this density as a duplex-like nucleic acid should be made cautiously (e.g., described as "consistent with nucleic-acid–like density"). Validation through biochemical approaches—such as nuclease treatment or use of designed duplex controls—would substantiate this interpretation.

Response: We thank the reviewer for the insightful suggestion to biochemically validate the ambiguous density. As advised, we performed DNase I footprinting assays on the MbpAgo-gDNA complex using an 18-nt 3′-FAM-labeled ssDNA (revised Fig. 3c; Response Fig. 4). In the absence of MbpAgo, DNase I treatment results in extensive digestion of the DNA, yielding only short fragments. In contrast, when MbpAgo is present, DNase I digestion produces two predominant protected DNA fragments (L1 and L2), indicating that MbpAgo shields defined regions of the supplied ssDNA from nuclease access. The appearance of two discrete protected species suggests that the bound ssDNA can adopt alternative protected conformations within the complex rather than a single uniform binding mode. We interpret the shorter protected fragment (L2) as consistent with the auxiliary nucleic acid-like density, in which partial cleavage of the 5′ region occurs while the 3′-FAM label is retained. By contrast, the 5′ region of the guide engaged in the canonical binding mode is tightly stabilized by interactions with MbpAgo and is therefore largely resistant to cleavage. Therefore, the DNase I protection pattern suggests that the auxiliary density corresponds to bound DNA. To remain cautious in light of the experimental conditions, we have revised the manuscript to describe this feature as "consistent with nucleic-acid-like density" (Page 6).



Response Fig. 4: DNase I footprinting assays on the MbpAgo-gDNA complex using an 18-nt 3′-FAM labeled single-stranded DNA. DNase I digestion produces two predominant protected DNA fragments (L1 and L2).

4. Functional assays remain qualitative

The cleavage assays presented throughout the study are largely qualitative, relying on gel images without quantification. Quantitative analysis, such as measuring band intensities or determining kinetic parameters, would provide a firmer basis for comparing catalytic efficiency and for guiding dependence.

Response: We thank the reviewer for this helpful suggestion. The quantitative data have now been added to the revised figures.

5. Timescale and dynamics of the functional cycle

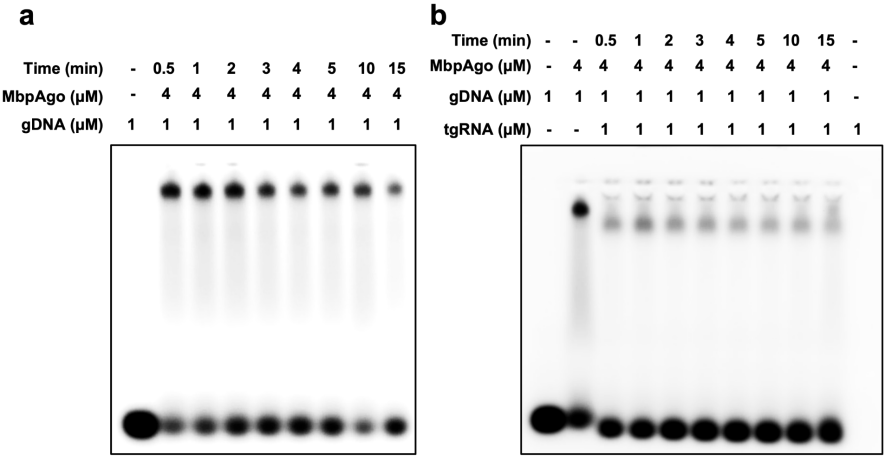
The proposed model implies a sequential transition between oligomeric states, but no data address the timescale, coexistence, or order of these states. Even a simplified kinetic framework or heterogeneity analysis of particle populations would substantially increase the credibility of the proposed dynamic cycle.

Response: We thank the reviewer for raising this important point regarding the timescale and dynamics of the proposed functional cycle. To experimentally probe the kinetics of MbpAgo oligomerization in solution, we performed time-resolved native agarose gel assays monitoring MbpAgo-gDNA and MbpAgo-gDNA-tgRNA complexes over a 0.5-15 min time window (revised Supplementary Fig. 12; Response Fig. 5).

In the presence of gDNA, the mobility of the MbpAgo–gDNA complex reaches a stable distribution within the earliest measurable time point (≤ 0.5 min) and remains unchanged thereafter (revised Supplementary Fig. 12a; Response Fig. 5a), indicating that gDNA-induced assembly occurs rapidly on the minute timescale. Importantly, no progressive band shift or emergence of additional discrete species is observed over time, suggesting that the dominant oligomeric state is established quickly rather than through a slow sequential conversion.

When both gDNA and tgRNA are present, we note that native agarose gel electrophoresis also reports on the shift of nucleic-acid-associated species (revised Supplementary Fig. 12b; Response Fig. 5b). Therefore, although these data are not interpreted as directly visualizing monomer-dimer interconversion, they provide a qualitative kinetic constraint showing that guide loading, target binding, and associated assembly equilibrate rapidly and do not involve slow, time-dependent redistribution among multiple long-lived oligomeric populations.

Taken together with SEC-MALS measurements defining the oligomeric states under apo, gDNA-binding and gDNA-tgRNA-binding conditions (revised Supplementary Fig. 4e; Response Fig. 3), as well as cryo-EM classifications showing distinct, non-coexisting assemblies (Supplementary Figs. 2, 5, 8), these results support a model in which MbpAgo transitions between oligomeric states in a substrate-dependent manner on a fast timescale, rather than through slow or heterogeneous interconversion. We have clarified this point in the revised manuscript (Page 9).



Response Fig. 5: Time-resolved native gel analysis of MbpAgo-nucleic-acid complexes.

a, MbpAgo-WT (4 μM) was incubated with 3′-FAM-labeled 18-nt gDNA (1 μM), and samples were analyzed by 0.5% native agarose gel at the indicated time points.

b, As in (a), with addition of tgRNA (1 μM) to preformed MbpAgo-gDNA complexes, revealing time-dependent mobility changes consistent with RNA-induced complex remodeling.

Minor Concerns

• The Introduction could better articulate how MbpAgo differs mechanistically from other RNA-targeting prokaryotic Argonautes, such as PliAgo or RslAgo.

Response: We thank the reviewer for this helpful suggestion. We have revised the Introduction to better distinguish MbpAgo from other RNA-targeting pAgos, such as PliAgo and RslAgo. While PliAgo and RslAgo preferentially mediate DNA-guided RNA cleavage, they show more restrictive guide requirements, including a stronger dependence on 5′-phosphorylated DNA guides and higher sensitivity to guide-target mismatches. In contrast, MbpAgo was reported to efficiently use both 5′-phosphorylated and 5′-hydroxylated DNA guides, tolerate mismatches across the guide-target duplex, and cleave structured RNA substrates. These differences suggest that MbpAgo engages DNA guides and RNA targets through distinct recognition principles within this subgroup of RNA-targeting pAgos, thereby motivating the need for structural insight into its mechanism. We have incorporated this comparison into the revised Introduction.

• A schematic summarizing the conformational transitions among the apo, binary, and ternary states would improve figure clarity.

Response: We thank the reviewer for this helpful suggestion. We have added a schematic summarizing the conformational transitions of MbpAgo across the apo, binary (gDNA-bound dimer), and ternary (gDNA-tgRNA-bound monomer) states (Fig. 5c; revised Supplementary Fig. 11). These schematics highlight the key oligomeric changes and associated domain rearrangements. In addition, we provide a supplementary movie (revised Supplementary Video 1) illustrating these transitions as a visual aid. We have added these new information in our revised manuscript (Pages 8,9)

• The recurring phrase “fine-tune catalytic activity” could be replaced by more precise wording, such as “modulate substrate accommodation” or “stabilize the catalytic conformation.”

Response: We have replaced “fine-tune catalytic activity” with “stabilize the catalytic conformation” as suggested.

• The discussion of SARS-CoV-2 and HIV RNA cleavage should be framed as a proof-of-concept demonstration rather than implying biological relevance.

Response: We thank the reviewer for this clarification and agree that the cleavage of SARS-CoV-2 and HIV-1 RNAs should be interpreted as a proof-of-concept demonstration. We have revised the Discussion accordingly.

• The discussion should close with an explicit statement acknowledging that the proposed monomer–dimer functional model remains a working hypothesis pending dynamic validation.

Response: We thank the reviewer for this constructive suggestion. We agree that fully resolving the real-time dynamics of the proposed monomer-dimer cycle will ultimately benefit from higher time-resolution kinetic and/or single-molecule approaches. To address this point, we have revised

the Discussion to state that the monomer-dimer functional cycle is a working model. At the same time, we emphasize that it is supported by multiple independent lines of evidence: (i) cryo-EM snapshots capturing distinct apo, binary, and ternary states, (ii) SEC-MALS measurements showing substrate-dependent oligomeric changes in solution (revised Supplementary Fig. 4e; Response Fig. 3), (iii) time-resolved native agarose gel assays providing minute-timescale kinetic constraints on MbpAgo-gDNA complex formation and RNA-triggered remodeling (revised Supplementary Fig. 12; Response Fig. 5), and (iv) mutational and biochemical assays linking dimerization-related features to cleavage activity. We now close the Discussion by acknowledging that further dynamic validation is still needed, while noting that the current data together support a consistent, experimentally supported model for MbpAgo function (Page 11).

This is a technically strong and carefully executed structural study that provides essential insight into the architecture and potential regulation of a DNA-guided, RNA-targeting Argonaute. The primary conclusions regarding structural features and guide–target recognition are well supported. The secondary conclusion proposing a dynamic monomer–dimer functional cycle is intriguing and potentially significant, but remains speculative in the absence of supporting kinetic or biophysical data. Recommend major revision.

Reviewer #4 (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.