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Helix-7 in Argonaute2 shapes the microRNA seed region for rapid target recognition

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(Note: With the exception of the correction of typographical or spelling errors that could be a source of ambiguity, letters and reports are not edited. The original formatting of letters and referee reports may not be reflected in this compilation.)

1st Editorial Decision 23 February 2017

Thank you for submitting your manuscript for consideration by the EMBO Journal. It has now been seen by three referees whose comments are shown below.

As you will see from the reports, all referees express interest in the findings reported in your manuscript, although they also list a number of points that will have be addressed before they can support publication of your manuscript in The EMBO Journal.

Given the referees' overall positive recommendations, I would like to invite you to submit a revised version of the manuscript, addressing the comments of all three reviewers. I should add that it is EMBO Journal policy to allow only a single round of revision, and acceptance of your manuscript will therefore depend on the completeness of your responses in this revised version.

For the revised manuscript I would particularly ask you to focus your efforts on the following points:
-> Include data on the on rates for target binding as requested by referees #1 and #2
-> Test/clarify any discrepancy between the full and partial helix 7 deletion (ref #2)
-> Please expand the methods section as outlined by ref #1 and discuss the implications for lateral diffusion
-> Finally, you will see that ref #3 finds that the manuscript would be significantly strengthened if the FRET measurements conducted could be extended to a broader range of target vs non-target sequences, in order to shed more light on the target discrimination mechanism itself. In my view, this is a relevant point and I'd encourage you to address it.

Thank you for the opportunity to consider your work for publication. I look forward to your revision.

REFEREE REPORTS

Referee #1:

In the manuscript by Klum et al., the authors test how helix-7 of the Ago2 protein contributes to the binding properties of the miRNA guide to target RNA. The authors build on previous observations that Ago proteins modulate the RNA-RNA interactions between the miRNA guide and target by increasing association rates and decreasing dissociation rates relative to RNA-RNA interactions in solution, and propose that helix-7 is a principle element in modulating these rates. Through bulk biochemical and single-molecule measurements the authors demonstrate that mutation of helix-7 does not affect overall binding affinity but does decrease both association and dissociation rates ~10 fold. They infer from these measurements, as well as from crystal structures of WT and helix-7 mutant Ago2, that helix-7 mediates this behavior by facilitating two opposing preorganized conformations of the seed 3′ end. They reason that the effect of helix-7 to promote target binding at positions 6 and 7 while also stabilizing a conformation incapable of pairing beyond position 5 causes the decreased rate constants obtained with Δhelix-7 Ago2, as well its decreased specificity for complete seed pairing over a partial match to nucleotides 2-5. Finally, the authors show that the effects of helix-7 on targeting binding also influence lateral diffusion of Ago2. These observations are novel and noteworthy, as they provide a compelling physical mechanism for how Ago proteins reshape the RNA-binding properties of the RNA guide.

Major Concerns:
1. Because the authors have a single-molecule setup suitable for determining on rates, it is very surprising that they do not report any on rates determined using this setup and instead only show rates from bulk biochemistry experiments. What were the on rates determined with the single-molecule set up, and how did these compare to those determined by bulk biochemistry?

2. Figure 4 / Methods. These data don't necessarily demonstrate a perturbation of lateral diffusion. If instead of lateral diffusion, the molecule dissociated and rapidly re-bound the RNA target at the proximal site, the 10-fold reduction in both koff and kon with the Δhelix-7 mutant could account for the 50-fold change in kshutting(obs). In addition, methods for how kshuttling(obs) is determined should be described, and most importantly, the dwell times in each state should be shown. The trace depicting the shuttling for WT Ago2 exhibits very transient occupancy in the low-FRET site in comparison to (Chandradoss et al., 2015). Do the authors have an explanation for this, and the low-FRET dwell time substantially increase for the Δhelix-7 mutant? Additionally, this figure should be annotated to provide labels for which regions of the trace are being designated as low or high FRET (as done in Figure 3 of Chandradoss et al., 2015).

3. Methods. The methods for the single-molecule experiments and analysis are not described, with only a citation to Chandradoss et al., 2015, where methods of the analysis were not described. For example, were the traces fit with an HMM?

Minor Concerns:
1. Is there any concern that the Δhelix-7 or MI-AA Ago2 mutants were not as specifically purified with the capture oligo, since their extended kon and koff rates predispose them to be less specific? Since the proteins were quantified using RNA blot and Bradford, perhaps the comparison of these two numbers could be used to demonstrate that the purified protein contains mostly miR-122.

2. Figure 3F,G. The main text indicates that dwell times of targets pairing to g2-g6 with the Δhelix-7 Ago2 were fit significantly better with a double exponential. The statistical test for this difference should be shown, as well as the corresponding values for a single- and double-exponential fit.

3. Figure 3F,G / Methods. Because the average dwell times are not an weighted average of their two

component dwell times, the authors should say more about how their averages were derived.

4. Figure 5D. The Kd in the text is 30 μM which does not match the number in the figure.

5. Figure 6 C,D. The kobs for WT Ago2 cannot be confidently estimated and should therefore be presented as > 6.4 {plus minus} 0.2 min-1. This limit should be propagated to the calculated kon (> 4.6 x 109 M-1 min-1) as well.

6. Figure 6 C,D / Methods, Target association assays. The formula provided is incorrect in two ways: 1.) [ET] should be used instead of [ST], and the kernel to the exponential function should include time: e-(kon*[Et] + koff)*t.

7. Figure 6 D. The legend describes the kobs as being plotted with standard mean error indicated. However, error bars are missing and should be added.

Referee #2:

In this manuscript, Klum et al. report that helix-7 in human Ago2 protein acts to facilitate rapid and accurate search for cognate seed-matched target RNAs. Although conceptual advance might be somewhat limited because the importance of helix-7 has already been proposed in previous studies, the beautiful combination of biochemical, single-molecule, and structural analyses in this study provided an empirical, solid evidence for the molecular role of helix-7. I only have a couple of comments to be considered before the manuscript is ready for publication in The EMBO Journal.

Major points:
1. The fact that sufficient x-ray diffraction quality was not obtained for crystals of delta-helix-7 implies a possibility that the deletion reduced the structural stability (even though the overall protein expression level was unaffected). The authors instead used a weaker helix-7 mutation (MI-AA) for structural analysis but characterization of this MI-AA mutant is currently very limited (Fig. 7A and B only). At least some of the key analyses should be additionally performed to confirm that the MI-AA and delta-helix-7 mutants show the similar biochemical and single-molecule behaviors.
2. Related to the above point, can the authors rule out the possibility that the observed heterogeneity in binding events for delta-helix-7 (Fig. 3F) is caused by its structural instability?

Minor points:
1. Why don't the authors obtain Kobs values from their single-molecule analysis data?
2. The lateral diffusion data in Fig. 4 is interesting but appears rather auxiliary in this study, and perhaps can be moved to Supplemental Data. Also, FRET efficiency should be added in Fig. 4B; temporal change in FRET efficiency is hard to be seen in the current graph.
3. Fig. 6D and Fig. 7B seem to be derived from the same data, and if so, that should be clearly indicated.
4. Material and methods for single-molecule analyses should be added.

Referee #3:

The manuscript of Klum et al. focuses of helix 7 of Argonaute 2, exploring its effect on the interaction of miRNA-loaded Ago2 with the target (a more accurate title would reveal that the study is about helix 7 of Ago2). Although the study seems to be well executed, I find it difficult to see how it helps understanding how Ago2 discriminates between targets and non-targets. The authors show that the function of helix 7 is to accelerate both the association and dissociation of Ago2 with seed-matched targets. I would then have expected the authors to study these properties for a spectrum of targets and non-targets to show that non-targets are sufficiently different in these properties in their interaction with the wildtype Ago2. It is not clear to me that this is the case. For example, the dwell time on the g2-g4 target was ~0.25s, increasing up to 1.96s on g2-g7. This difference seems relatively large, but it is between a 3 nucleotide seed match and a 6 nucleotide seed match. There are presumably a lot of non-functional sites that have more than 3

nucleotide matching the miRNA seed. How do the authors envision that these are discriminated against? Can the authors improve miRNA target prediction based on their results?

1st Revision - authors' response 13 July 2017

We thank all three Referees for considering our work and suggesting ways to improve upon it. A major request was to obtain binding rates using single-molecule data. This presented a technical challenge as it required us to develop new protocols for purifying and accurately quantifying fluorescently labelled Ago2-miR complexes. We apologize for extended time it took develop these techniques and submit a revised manuscript. We believe the manuscript has benefited substantially from these and other efforts to address the Referees comments, and hope it is now acceptable for publication. In response to the Referee's comments, revised manuscript includes:

• Single molecule measurements of on-rates for target binding, as requested by Referees #1 and #2
• Additional characterization of the partial helix 7 deletion (MI-AA mutant), as requested by Referee #2
• Additional assessment of the stability/folding of the Δhelix-7 mutant, as requested by Referee #2.
• Expanded methods describing single molecule experiments and clarification of lateral diffusion data, as requested by Referee #1
• Single molecule experiments for a spectrum of off-target sequences to shed more light on the target discrimination mechanism, as requested by Referee #3.

Point-by-point responses to the Referee's comments are highlighted in blue coloured text below.

Referee #1:
In the manuscript by Klum et al., the authors test how helix-7 of the Ago2 protein contributes to the binding properties of the miRNA guide to target RNA. The authors build on previous observations that Ago proteins modulate the RNA-RNA interactions between the miRNA guide and target by increasing association rates and decreasing dissociation rates relative to RNA-RNA interactions in solution, and propose that helix-7 is a principle element in modulating these rates. Through bulk biochemical and single-molecule measurements the authors demonstrate that mutation of helix-7 does not affect overall binding affinity but does decrease both association and dissociation rates ~10 fold. They infer from these measurements, as well as from crystal structures of WT and helix-7 mutant Ago2, that helix-7 mediates this behavior by facilitating two opposing preorganized conformations of the seed 3′ end. They reason that the effect of helix-7 to promote target binding at positions 6 and 7 while also stabilizing a conformation incapable of pairing beyond position 5 causes the decreased rate constants obtained with Δhelix-7 Ago2, as well its decreased specificity for complete seed pairing over a partial match to nucleotides 2-5. Finally, the authors show that the effects of helix-7 on targeting binding also influence lateral diffusion of Ago2. **These observations are novel and noteworthy, as they provide a compelling physical mechanism for how Ago proteins reshape the RNA-binding properties of the RNA guide.**

We thank the reviewer for the enthusiastic response.

1. Because the authors have a single-molecule setup suitable for determining on rates, it is very surprising that they do not report any on rates determined using this setup and instead only show rates from bulk biochemistry experiments. What were the on rates determined with the single-molecule set up, and how did these compare to those determined by bulk biochemistry?

To obtain accurate binding rates using single-molecule analysis, we have purified Ago2 pre-loaded with dye-labelled guide RNA and have conducted the requested single-molecule experiments. The results are included in the revised manuscript (Figure 6c).

The single-molecule data further support the conclusion that the elimination of the helix-7 decelerates the binding of Ago2 to target RNAs. Using this experimental setup, the binding rate (k_{on}) of WT Ago2 was 0.33 /nM/sec while the k_{on} for Δhelix-7 was 5-fold lower, 0.067 /nM/sec.

2. Figure 4 / Methods. These data don't necessarily demonstrate a perturbation of lateral diffusion. If instead of lateral diffusion, the molecule dissociated and rapidly re-bound the RNA target at the proximal site, the 10-fold reduction in both koff and kon with the Δhelix-7 mutant could account for the 50-fold change in kshutting(obs).

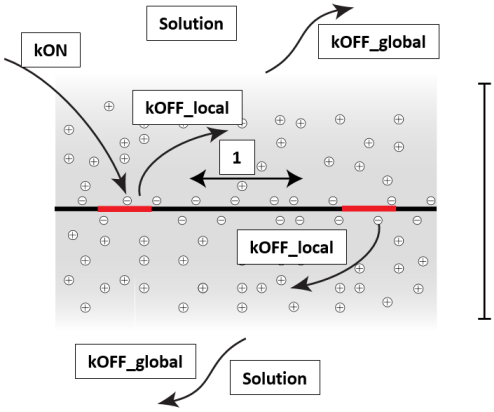

Figure R1. Binding and dissociation within electrostatic cloud formed by negatively charged RNA

We agree with Referee #1 that the 10-fold reduction in both k_{off} and k_{on} would account for the 50-fold change in $k_{shutting}$. Lateral diffusion consists of a series of local dissociation and re-binding within an electrostatically confined volume (Figure R1). (Any departure away from this volume will be observed as the loss of fluorescence signals, which we do not register as lateral diffusion.) We speculate that this local dissociation and re-binding is likely to be facilitated by helix-7 just as the global dissociation and re-binding is, and thus the lateral diffusion is influenced by helix-7. However, we acknowledge that, as suggested by Referee #2 (below), this result is somewhat auxiliary in this study and thus have moved the lateral diffusion figure and text to supplemental information.

In addition, methods for how kshuttling(obs) is determined should be described, and most importantly, the dwell times in each state should be shown.

A detailed description has been added to the Methods section. The dwell-time of each state is included in Figure S3.

The trace depicting the shuttling for WT Ago2 exhibits very transient occupancy in the low-FRET site in comparison to (Chandradoss et al., 2015).

We thank Referee 1 for identifying this point of confusion. We replaced the trace with a time trace that shows clear transitions between two FRET states and is a better representative of the overall data.

Do the authors have an explanation for this, and the low-FRET dwell time substantially increase for the Δhelix-7 mutant?

We have added a supplementary figure showing dwell time histograms of low-FRET and high-FRET states. In both cases the dwell-times are greater for the Δhelix-7 mutant.

Additionally, this figure should be annotated to provide labels for which regions of the trace are being designated as low or high FRET (as done in Figure 3 of Chandradoss et al., 2015).

We thank Referee 1 for this suggestion. FRET efficiency traces have been added to indicate two distinct FRET states.

3. Methods. The methods for the single-molecule experiments and analysis are not described, with only a citation to Chandradoss et al., 2015, where methods of the analysis were not described. For example, were the traces fit with an HMM?

We thank Referee 1 for pointing this out. We have added a detailed procedure in the Methods section, including a description on trace analysis, to the revised manuscript.

Minor Concerns:
1. Is there any concern that the Δhelix-7 or MI-AA Ago2 mutants were not as specifically purified with the capture oligo, since their extended kon and koff rates predispose them to be less specific?

Since the proteins were quantified using RNA blot and Bradford, perhaps the comparison of these two numbers could be used to demonstrate that the purified protein contains mostly miR-122.

This study used two independently prepared samples of wild type Ago2-miR122 and three preparations of the Δhelix-7 mutant bound to miR-122. The average amount of miR-122 loaded into the mutant Ago2 (per total protein) was indeed ~15% lower in the mutant (Figure R2). However, this difference is not large enough to impact any major conclusions of the study.

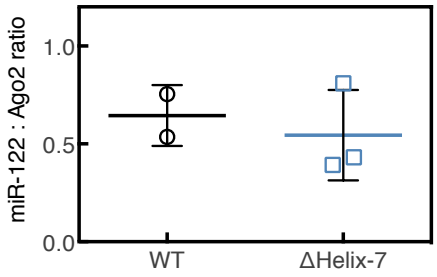

Figure R2. Quantification of miR-122 in preparations of wild type (WT) and Δhelix-7 Ago2. Ratio of miR-122 (determined by Northern blot, using unbound miR-122 as a standard) to Ago2 (determined by Bradford assay, using BSA as a standard) is plotted for each preparation used in this study.

2. Figure 3F,G. The main text indicates that dwell times of targets pairing to g2-g6 with the Δhelix-7 Ago2 were fit significantly better with a double exponential. The statistical test for this difference should be shown, as well as the corresponding values for a single- and double-exponential fit.

We have conducted t-test by comparing single-exponential fits with double-exponential fits, using four sets of data per construct. The single and double exponential fits were significantly different. We have obtained 0.01566 for g2-g6 and 0.000389 for g2-g7. Additionally we have indicated R^2 values of each fit in figure legends.

We have added the dwell times from the single exponential fit to the figure legends.

3. Figure 3F,G / Methods. Because the average dwell times are not an weighted average of their two component dwell times, the authors should say more about how their averages were derived.

A description is added in the Methods section

4. Figure 5D. The Kd in the text is 30 μM which does not match the number in the figure.

We thank Referee 1 for identifying this discrepancy. The Kd value for these data can only be approximated because binding was not strong enough to determine Bmax. However, if we assume a Bmax value of 0.8, which is the average of related target RNAs in this experiment, a Kd value of 30 μM fits the data best. We have ensured the figure and main text all use this value in the revised manuscript.

5. Figure 6 C,D. The kobs for WT Ago2 cannot be confidently estimated and should therefore be presented as > 6.4 {plus minus} 0.2 min-1. This limit should be propagated to the calculated kon (> 4.6 x 109 M-1 min-1) as well.

We thank Referee 1 for this suggestion. We changed the "=" signs to "≥", as suggested.

6. Figure 6 C,D / Methods, Target association assays. The formula provided is incorrect in two ways: 1.) [ET] should be used instead of [ST], and the kernel to the exponential function should include time: e-(kon*[Et] + koff)*t.

We thank Referee 1 for spotting these typos. The errors have been corrected in the revised manuscript.

7. Figure 6 D. The legend describes the kobs as being plotted with standard mean error indicated. However, error bars are missing and should be added. *We have added the requested error bars.*

Referee #2:
In this manuscript, Klum et al. report that helix-7 in human Ago2 protein acts to facilitate rapid and accurate search for cognate seed-matched target RNAs. Although conceptual advance might be somewhat limited because the importance of helix-7 has already been proposed in previous studies, **the beautiful combination of biochemical, single-molecule, and structural analyses** in this study provided an empirical, solid evidence for the molecular role of helix-7. I only have a couple of comments to be considered before the manuscript is ready for publication in The EMBO Journal.

Major points:
1. The fact that sufficient x-ray diffraction quality was not obtained for crystals of delta-helix-7 implies a possibility that the deletion reduced the structural stability (even though the overall protein expression level was unaffected). The authors instead used a weaker helix-7 mutation (MI-AA) for structural analysis but characterization of this MI-AA mutant is currently very limited (Fig. 7A and B only). At least some of the key analyses should be additionally performed to confirm that the MI-AA and delta-helix-7 mutants show the similar biochemical and single-molecule behaviors.

We thank Referee 2 for raising this important point. Accordingly, we carried out single-molecule measurements of the MI-AA mutant binding to RNAs with increasing complementarity to the seed region. These new data can be found in revised Figure 3. As seen for the full helix-7 deletion, the MI-AA mutant Ago2 displays extended dwell-times on RNAs imperfectly matching the miRNA seed. These results add further support to the hypothesis that helix-7 modulates the rate of guide:target pairing in the 3′ end of the seed.

2. Related to the above point, can the authors rule out the possibility that the observed heterogeneity in binding events for delta-helix-7 (Fig. 3F) is caused by its structural instability?

We thank Referee 2 for raising this second major point. We have addressed the question of structural instability in two ways. First, we compared the stability of wild type and Dhelix-7 Ago2 by assessing susceptibility to heat inactivation. We found that Dhelix-7 Ago2 is indeed moderately more susceptible than wild type to heat, indicative of a decreased structural stability. However, temperatures required to observe any inactivation (>56 ˚C) well exceed our assay conditions (~25 ˚C), indicating that the Dhelix-7 Ago2 was structurally stable in all of our experiments. Further supporting this notion, we measured the ultraviolet circular dichroism (UV CD) spectra of wild type and Dhelix-7 Ago2 samples at 25 ˚C and found extensive overlap, indicative of similar folding and overall conformation. These results can be found in a new supplementary figure (Figure S1). Taken with the new single-molecule data demonstrating that the MI-AA mutant behaves similarly to the Dhelix-7 Ago2 (Figure 3), and the structural similarities between wild-type Ago2 and the MI-AA mutant (Figure 7), we believe the functional differences between wild type Ago2 and the helix-7 mutants are not likely due to differences in structural instability.

Minor points:

1. Why don't the authors obtain Kobs values from their single-molecule analysis data?

To obtain accurate binding rates using single-molecule analysis, we have purified Ago pre-loaded with dye-labelled guide. Single-molecule experiment data are included in the revised version (Figure 3). The result agrees with bulk experiments.

2. The lateral diffusion data in Fig. 4 is interesting but appears rather auxiliary in this study, and perhaps can be moved to Supplemental Data.

We agree with Referee 2. As suggested, we have relocated Figure 4 to Supplemental Data.

Also, FRET efficiency should be added in Fig. 4B; temporal change in FRET efficiency is hard to be seen in the current graph.

We have included traces showing FRET values. We have also replaced the fluorescence trace with a more representative example that better demonstrates the clear transition between FRET states.

3. Fig. 6D and Fig. 7B seem to be derived from the same data, and if so, that should be clearly indicated.

Referee 2 is correct. In the revised manuscript these data are presented only in Figure 6D.

4. Material and methods for single-molecule analyses should be added.

We thank Referee 2 for pointing this out. We have added a detailed procedure in the Methods section.

Referee #3:
The manuscript of Klum et al. focuses of helix 7 of Argonaute 2, exploring its effect on the interaction of miRNA-loaded Ago2 with the target (a more accurate title would reveal that the study is about helix 7 of Ago2).

We thank Referee 3 for this suggestion and accordingly have retitled the manuscript, "*Helix-7 in Argonaute2 shapes the microRNA seed region for rapid target searches*"

Although the study seems to be well executed, I find it difficult to see how it helps understanding how Ago2 discriminates between targets and non-targets.

Upon reading this comment we realized that the original manuscript contained a major point of confusion—we used the word 'target' in two different ways: 1) in some contexts we used 'target' to refer to actual miRNA targets, seed-matched mRNAs that are bound and repressed by Ago2 under physiological conditions; 2) in other places we used 'target' to refer to RNAs with any degree of seed complementarity. Thus, many of the 'targets' used in our experiments would be considered 'off-targets' in a physiological context. To clarify this issue we have rewritten the manuscript such that the word 'target' is only used to describe seed-matched RNAs, and the term 'off-target' is used for RNAs with partial seed matches. We have also changed the section titled "Helix-7 controls guide:target interactions in the 3' end of the seed" to "Helix-7 minimizes dwell time on off-targets" and rewritten the section to emphasize this point.

The authors show that the function of helix 7 is to accelerate both the association and dissociation of Ago2 with seed-matched targets. I would then have expected the authors to study these properties for a spectrum of targets and non-targets to show that non-targets are sufficiently different in these properties in their interaction with the wildtype Ago2. It is not clear to me that this is the case.

We apologize for this confusion and respectfully request Referee 3 to examine the revised Figure 3, which contains dwell time data for a let-7 target (with full seed complementarity) and four related let-7 off-targets (with partial seed complementarity). The data show that wild type Ago2 effectively discriminates the target RNA (Figure 3E, right most column) from the spectrum of off-targets. The helix-7 mutants (Fig 3G and GI) spend significantly more time on the off-targets than wild type Ago2 does. The revised manuscript also contains additional experiments comparing the dwell times of Δhelix-7 and wild type Ago2 on 6 more off-targets (Figure S2). The new data further indicate that the Δhelix-7 mutant is particularly inefficient at recognizing mismatches at the 3' end of the miRNA seed region.

For example, the dwell time on the g2-g4 target was ~0.25s, ncreasing up to 1.96s on g2-g7. This difference seems relatively large, but it is between a 3 nucleotide seed match and a 6 nucleotide seed match. There are presumably a lot of non-functional sites that have more than 3 nucleotide matching the miRNA seed. How do the authors envision that these are discriminated against?

The g2-g4 and g2-g7 RNAs are both released from Ago2 relatively quickly and thus we suggest these should be considered off-targets. We envision that Ago2 dismisses related non-functional sites in a similar way. For comparison, Ago2 binds to the g2-g8 (fully seed-matched) target ≥150–1200 times longer (see Figure 3E).

Can the authors improve miRNA target prediction based on their results?

We do not believe that our results can be directly used to improve miRNA target prediction. The goal of this study was to understand how the structure of Argonaute shapes the binding properties of the miRNA seed, and our results are well aligned with criteria currently used to predict miRNA target sites. However, one unexplained feature of targeting that potentially relates to this work is variability found in the functional length miRNA seed. That is, some seed sequences bind g2–g7 and g2–g8 matched targets equivalently well, while other seed sequences display a substantial difference in affinity for g2–g7 vs. g2–g8 matched RNAs (see Figures 3E and 3F of Schirle, et al., Science 2014, PMID: 25359968). Our model for a two-part seed, with 3' pairing modulated by helix-7, provides a mechanistic framework to further explore this phenomenon, which, if understood could improve miRNA target prediction.

2nd Editorial Decision 27 July 2017

Thank you for submitting your manuscript for consideration by the EMBO Journal. Your handling editor Anne is away at the moment, and I am thus stepping in.
Your work has now been seen by two referees again whose comments are enclosed. As you will see, both referees express interest in your manuscript and are broadly in favour of publication, pending satisfactory minor revision.

I would thus like to ask you to address the remaining concerns/requests and to provide a final version of your manuscript.

REFEREE REPORTS

Referee #1:

The authors have satisfactorily addressed all of my concerns.

Typo: On page 17, in the methods describing the fitting of the double-exponential curve, there is a missing delta in the formula for the average dwell time.

Referee #2:

The authors have appropriately addressed my previous concerns and the manuscript is ready for publication. One very minor comment: it would be nice if the authors can add an illustration (supplementary figure) to explain the following statement in page 10.

Additionally, residues adjacent to helix-7 in the L1 stalk (S189-G194) and loop connecting helix-7 to the PAZ domain (K355-T361) are disordered. These observations indicate that the MI-AA mutations increase the mobility of helix-7, likely by uncoupling its motions from the PAZ domain and L2 stalk.

2nd Revision - authors' response 08 August 2017

Please find our revised manuscript "*Helix-7 in Argonaute2 shapes the microRNA seed region for rapid target recognition*."

We hope that the revised manuscript is acceptable for publication.

3rd Editorial Decision 10 August 2017

Thanks for sending the final revision of your manuscript, I am pleased to inform you that your study has now been accepted for publication in The EMBO Journal.

Please note that it is EMBO Journal policy for the transcript of the editorial process (containing referee reports and your response letter) to be published as an online supplement to each paper. If you do NOT want this, you will need to inform the Editorial Office via email immediately. More information is available here: http://emboj.embopress.org/about#Transparent_Process.

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If you have any questions, please feel free to contact me. Thank you for your contribution to The EMBO Journal and congratulations on this nicely executed work!

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Corresponding Author Name:	Ian J. MacRae, Ph.D. ; Chirlmin Joo, Ph.D.
Journal Submitted to:	The EMBO Journal
Manuscript Number:	EMBOJ-2017-96474

Reporting Checklist For Life Sciences Articles (Rev. July 2015)

This checklist is used to ensure good reporting standards and to improve the reproducibility of published results. These guidelines are consistent with the Principles and Guidelines for Reporting Preclinical Research issued by the NIH in 2014. Please follow the journal's authorship guidelines in preparing your manuscript.

A- Figures

1. Data

The data shown in figures should satisfy the following conditions:

- the data were obtained and processed according to the field's best practice and are presented to reflect the results of the experiments in an accurate and unbiased manner.
- figure panels include only data points, measurements or observations that can be compared to each other in a scientifically meaningful way.
- graphs include clearly labeled error bars for independent experiments and sample sizes. Unless justified, error bars should not be shown for technical replicates.
- If n< 5, the individual data points from each experiment should be plotted and any statistical test employed should be justified
- Source Data should be included to report the data underlying graphs. Please follow the guidelines set out in the author ship guidelines on Data Presentation.

2. Captions

Each figure caption should contain the following information, for each panel where they are relevant:

- a specification of the experimental system investigated (eg cell line, species name).
- the assay(s) and method(s) used to carry out the reported observations and measurements
- an explicit mention of the biological and chemical entity(ies) that are being measured.
- an explicit mention of the biological and chemical entity(ies) that are altered/varied/perturbed in a controlled manner.

- the exact sample size (n) for each experimental group/condition, given as a number, not a range;
- a description of the sample collection allowing the reader to understand whether the samples represent technical or biological replicates (including how many animals, litters, cultures, etc.).
- a statement of how many times the experiment shown was independently replicated in the laboratory.
- definitions of statistical methods and measures:
  - common tests, such as t-test (please specify whether paired vs. unpaired), simple χ2 tests, Wilcoxon and Mann-Whitney tests, can be unambiguously identified by name only, but more complex techniques should be described in the methods section;
  - are tests one-sided or two-sided?
  - are there adjustments for multiple comparisons?
  - exact statistical test results, e.g., P values = x but not P values < x;
  - definition of 'center values' as median or average;
  - definition of error bars as s.d. or s.e.m.

Any descriptions too long for the figure legend should be included in the methods section and/or with the source data.

In the pink boxes below, please ensure that the answers to the following questions are reported in the manuscript itself. Every question should be answered. If the question is not relevant to your research, please write NA (non applicable). We encourage you to include a specific subsection in the methods section for statistics, reagents, animal models and human subjects.

USEFUL LINKS FOR COMPLETING THIS FORM

http://www.antibodypedia.com	Antibodypedia
http://1degreebio.org	1DegreeBio
http://www.equator-network.org/reporting-guidelines/improving-bioscience-research-repo	ARRIVE Guidelines
http://grants.nih.gov/grants/olaw/olaw.htm	NIH Guidelines in animal use
http://www.mrc.ac.uk/Ourresearch/Ethicsresearchguidance/Useofanimals/index.htm	MRC Guidelines on animal use
http://ClinicalTrials.gov	Clinical Trial registration
http://www.consort-statement.org	CONSORT Flow Diagram
http://www.consort-statement.org/checklists/view/32-consort/66-title	CONSORT Check List
http://www.equator-network.org/reporting-guidelines/reporting-recommendations-for-tum	REMARK Reporting Guidelines (marker prognostic studies)
http://datadryad.org	Dryad
http://figshare.com	Figshare
http://www.ncbi.nlm.nih.gov/gap	dbGAP
http://www.ebi.ac.uk/ega	EGA
http://biomodels.net/	Biomodels Database
http://biomodels.net/miriam/	MIRIAM Guidelines
http://jjj.biochem.sun.ac.za	JWS Online
http://oba.od.nih.gov/biosecurity/biosecurity_documents.html	Biosecurity Documents from NIH
http://www.selectagents.gov/	List of Select Agents

B- Statistics and general methods

Please fill out these boxes ↓ (Do not worry if you cannot see all your text once you press return)

1.a. How was the sample size chosen to ensure adequate power to detect a pre-specified effect size?	No statistical comparisons are presented. Experiment replicates were performed on separate days, with at least three replicates performed and the values of the standard deviation provided, shown as error bars. The minimum replicate number (3) was chosen to establish that effects seen were reproducible.
1.b. For animal studies, include a statement about sample size estimate even if no statistical methods were used.	NA
2. Describe inclusion/exclusion criteria if samples or animals were excluded from the analysis. Were the criteria pre-established?	NA
3. Were any steps taken to minimize the effects of subjective bias when allocating animals/samples to treatment (e.g. randomization procedure)? If yes, please describe.	NA
For animal studies, include a statement about randomization even if no randomization was used.	NA
4.a. Were any steps taken to minimize the effects of subjective bias during group allocation or/and when assessing results (e.g. blinding of the investigator)? If yes please describe.	NA
4.b. For animal studies, include a statement about blinding even if no blinding was done	NA
5. For every figure, are statistical tests justified as appropriate?	No statistical tests were performed. However, means were compared between wild type and mutant samples, and standard deviation was plotted.
Do the data meet the assumptions of the tests (e.g., normal distribution)? Describe any methods used to assess it.	NA
Is there an estimate of variation within each group of data?	Standard deviations are provided for data.
Is the variance similar between the groups that are being statistically compared?	No statistical tests were performed.

C- Reagents

6. To show that antibodies were profiled for use in the system under study (assay and species), provide a citation, catalog number and/or clone number, supplementary information or reference to an antibody validation profile. e.g., Antibodypedia (see link list at top right), 1DegreeBio (see link list at top right).	NA
7. Identify the source of cell lines and report if they were recently authenticated (e.g., by STR profiling) and tested for mycoplasma contamination.	NA

* for all hyperlinks, please see the table at the top right of the document

D- Animal Models

8. Report species, strain, gender, age of animals and genetic modification status where applicable. Please detail housing and husbandry conditions and the source of animals.	NA
9. For experiments involving live vertebrates, include a statement of compliance with ethical regulations and identify the committee(s) approving the experiments.	NA
10. We recommend consulting the ARRIVE guidelines (see link list at top right) (PLoS Biol. 8(6), e1000412, 2010) to ensure that other relevant aspects of animal studies are adequately reported. See author guidelines, under 'Reporting Guidelines'. See also: NIH (see link list at top right) and MRC (see link list at top right) recommendations. Please confirm compliance.	NA

E- Human Subjects

11. Identify the committee(s) approving the study protocol.	NA
12. Include a statement confirming that informed consent was obtained from all subjects and that the experiments conformed to the principles set out in the WMA Declaration of Helsinki and the Department of Health and Human Services Belmont Report.	NA
13. For publication of patient photos, include a statement confirming that consent to publish was obtained.	NA

14. Report any restrictions on the availability (and/or on the use) of human data or samples.	NA
15. Report the clinical trial registration number (at ClinicalTrials.gov or equivalent), where applicable.	NA
16. For phase II and III randomized controlled trials, please refer to the CONSORT flow diagram (see link list at top right) and submit the CONSORT checklist (see link list at top right) with your submission. See author guidelines, under 'Reporting Guidelines'. Please confirm you have submitted this list.	NA
17. For tumor marker prognostic studies, we recommend that you follow the REMARK reporting guidelines (see link list at top right). See author guidelines, under 'Reporting Guidelines'. Please confirm you have followed these guidelines.	NA

F- Data Accessibility

18: Provide a "Data Availability" section at the end of the Materials & Methods, listing the accession codes for data generated in this study and deposited in a public database (e.g. RNA-Seq data: Gene Expression Omnibus GSE39462, Proteomics data: PRIDE PXD000208 etc.) Please refer to our author guidelines for 'Data Deposition'. Data deposition in a public repository is mandatory for: a. Protein, DNA and RNA sequences b. Macromolecular structures c. Crystallographic data for small molecules d. Functional genomics data e. Proteomics and molecular interactions	The PDB accession code for the Ago2 MI-AA structure (SWEA) has been provided in both the Acknowledgements section at the end of the main text and in the Data Availability section at the end of Materials and Methods.
19. Deposition is strongly recommended for any datasets that are central and integral to the study; please consider the journal's data policy. If no structured public repository exists for a given data type, we encourage the provision of datasets in the manuscript as a Supplementary Document (see author guidelines under 'Expanded View' or in unstructured repositories such as Dryad (see link list at top right) or Figshare (see link list at top right).	NA
20. Access to human clinical and genomic datasets should be provided with as few restrictions as possible while respecting ethical obligations to the patients and relevant medical and legal issues. If practically possible and compatible with the individual consent agreement used in the study, such data should be deposited in one of the major public access-controlled repositories such as dbGAP (see link list at top right) or EGA (see link list at top right).	NA
21. Computational models that are central and integral to a study should be shared without restrictions and provided in a machine-readable form. The relevant accession numbers or links should be provided. When possible, standardized format (SBML, CellML) should be used instead of scripts (e.g. MATLAB). Authors are strongly encouraged to follow the MIRIAM guidelines (see link list at top right) and deposit their model in a public database such as Biomodels (see link list at top right) or JWS Online (see link list at top right). If computer source code is provided with the paper, it should be deposited in a public repository or included in supplementary information.	NA

G- Dual use research of concern

22. Could your study fall under dual use research restrictions? Please check biosecurity documents (see link list at top right) and list of select agents and toxins (APHIS/CDC) (see link list at top right). According to our biosecurity guidelines, provide a statement only if it could.	NA