THE EMBO JOURNAL

Beyond the seed: structural basis for supplementary microRNA targeting by human Argonaute2

Jessica Sheu-Gruttadauria, Yao Xiao, Luca F. R. Gebert, and Ian J. MacRae

Review timeline:

Submission date:	21st Nov 2018
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Editor: Ieva Gailite

Transaction Report:

(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 17th Dec 2018

Thank you for submitting your manuscript for consideration by the EMBO Journal. We have now received three referee reports on your manuscript, which are included below for your information.

As you will see from the comments, all reviewers appreciate the proposed mechanism and the high quality of presented data. Given these positive recommendations, I would like to invite you to submit a revised manuscript in which you address the comments of the reviewers.

When preparing your letter of response to the referees' comments, please bear in mind that this will form part of the Review Process File, and will therefore be available online to the community. For more details on our Transparent Editorial Process, please visit our website: http://emboj.embopress.org/about#Transparent_Process

We generally allow three months as standard revision time. Please contact us in advance if you would need an additional extension. As a matter of policy, competing manuscripts published during this period will not negatively impact on our assessment of the conceptual advance presented by your study. However, please contact me as soon as possible upon publication of any related work in order to discuss how to proceed.

REFEREE REPORTS:

Referee #1:

Here, Sheu-Gruttadauria and colleagues provide a beautifully-written manuscript that provides interesting structural and functional insights into microRNA-mRNA hybridization in the context of human Argonaute 2 protein. Specifically, their crystal structure demonstrates that Ago2 creates a supplementary chamber that houses up to five microRNA-target base pairs. They go on to show that the seed and supplementary chambers can be bridged by an unstructured target loop of up to 15

nucleotides, but that the seed and supplementary chambers reside next to each other. In addition, they carry out miRNA-target binding assays, as well as a microRNA silencing assay in cells, which support the additional base pairing plays a role in target binding and microRNA repression. The data presented in this manuscript is of high quality, and I'm sure that it will be of interest to the scientific community. I support its publication in EMBO J.

One question that I do have is regarding functional data (Figure EV3). The authors posit that "increased target affinity associated with supplementary interactions may translate into enhanced repression in mammalian cells". Indeed, they do see an increase in repression with supplementary interactions, but can they rule out that the correlation between supplementary interaction and repression isn't just due to endonuclease cleavage by AGO2 in vivo (RNAi) rather than cleavage-independent (miRNA) silencing? To test this, the authors could always knockdown GW182 proteins in cells to test whether the silencing they are observing is GW182-dependent or not.

Referee #2:

This manuscript reports a structural and biochemical analysis of miRNA-target interaction that focuses on the contribution of base pairing at the 3' end of the miRNA. The seed region at the 5' end of the miRNA is the primary determinant for target binding and repression. And while a contribution of the so-called 3' supplementary binding to target recognition had also been noticed, sequence conservation, as well as biochemical analyses suggested that this was modest. Here, the authors provide the first structural model of an Argonaute protein (human Ago2) loaded with a miRNA (mir-122) and bound to a target forming base-pairing interactions with the seed sequence as well as with the 3' region of the miRNA. This model allows the authors to make a number of new observations and predictions about the contribution of the 3' supplementary interactions, but also about the mechanism of miRNA target recognition in general. The authors test some of these predictions using biochemical assays to uncover novel insight into miRNA-target interactions.

Specifically, the authors find that:
1. The miRNA-target duplex is discontinuous: positions 2-8 of the miRNA bind as reported in other seed-paired structures, positions 13-16 form a duplex within a separate pocket of Ago2, which they call the supplementary chamber.
2. Ago2 must undergo a conformational change to "open" the supplementary chamber and provide the space needed for supplementary pairing. The authors identify a "hinge" region that would enable such conformational change while still maintaining seed pairing.
3. The position of the seed and supplementary duplexes in their model predicts that the mRNA target could include a loop/bridge between the nucleotides that pair to the seed and those that pair to positions 13-16. The authors measure the affinity of targets with different bridge lengths that are predicted to maintain a similar seed and supplementary pairing, and report two surprising findings: i) the increase in affinity provided by 3' supplementary binding can be up to more than an order of magnitude higher than that of seed-only pairing (previously, more modest contributions had been reported), ii) the length of the loop between the two mRNA pairing regions can be up to 15-nt long, if the 3' pairing sequence is CG-rich. In addition, the authors measure the level of miRNA-mediated repression of a reporter with different degrees of 3' supplementary pairing and suggest that this has a measurable effect on the level of repression.
4. In order to form the 3' supplementary duplex, the 3' half of the miRNA adopts an extended conformation while still maintaining interaction of the 3' terminal nucleotide within the PAZ domain of Ago2. This leads the authors to hypothesize that a slightly longer miRNA might relieve the tension imposed on the miRNA and stabilize 3' supplementary binding. The authors provide in vitro biochemical support for this hypothesis and therefore conclude that isomiRs with one or two nucleotide length difference at the 3' end are likely to have different effects on targets that allow 3' supplementary pairing.

Overall, the work is very clearly presented, insightful and, in my opinion, the author's conclusions are generally well substantiated. I would however, like to raise one concern regarding the experiments with bridging loops shown in Figure 4 (and EV1): while the design of the miRNA and RNA target sequences indeed would predict that these base pair as shown in Fig EV1, we don't really have any experimental indication that these are the structures whose affinities are being

measured. Because the implications of the existence of such bipartite target sites are very important for target prediction, I think this would need further support (to exclude an alternative structure where stabilization comes from the longer target rather than 3' pairing). A relatively simple experiment that would strengthen the point that the 3' supplementary pairing is indeed occurring with the target containing the 15-nt bridge, would be to measure the Kd for two additional miRNA-target pairs: one where they introduce one or two mismatches in the 3' region (affinity should go back to seed-only level) and another in which they make the compensatory mutations on the other RNA strand (affinity should be restored).

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MacRae and colleagues determined the crystal structure of human Ago2-RISC recognizing a target RNA through the seed and 3' supplementary regions while avoiding central base pairing. This new structure strongly supports a recently proposed, revised model of how human Ago2 establishes small RNA-target RNA recognition beyond the seed region (seed -> 3' supplementary -> central). Moreover, the authors show that the guide small RNA length greatly affects the contribution of 3' supplementary pairing to target affinity, opening a door for the biological significance of 3' isomiRs. Overall, the study is carefully conducted and the manuscript is well written. I support the publication of this manuscript after a minor revision as follows.

1. In Figure 4D, the authors demonstrate that a bridge length as short as 1 nt can strongly enhance the target affinity. However, the gap between the seed and supplementary chambers in the current structure appears to be too big for 1 nt RNA to bridge. Presumably, this suggests that the gap size itself is variable through overall conformational changes of Ago2 (between the N-PAZ and MIDI-PIWI lobes).
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3. Page 14, the bottom line: "grove" should be "groove."

1st Revision - authors' response 19th Feb 2019

Please see next page.

We are grateful to the referees for their encouraging comments and thoughts for improving our manuscript. We have provided detailed responses below:

Referee #1:

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We thank Referee #1 for raising this issue. We believe we can rule out the possibility that cleavage is due to endonuclease activity of AGO2 via RNAi because all targets examined contained mismatches to the miRNA central region (nucleotides 9-12). Base pairing in this region is required for AGO cleavage activity (Elbashir, ***et al.***, The EMBO Journal (2001) 20, 6877-6888). This effect has been well established by other labs and has been observed in our hands as well (Fig. R1):

Figure for referees removed.

Data in Fig. R1 are part of a separate study, so we would prefer to not include these in this manuscript, but are happy to show the image for the purpose of addressing the concern raised by Referee #1. To clarify the issue in the manuscript we added the following to the main text:

"All targets contained mismatches to the miRNA central region (g9–g12), which inhibit Argonaute nuclease activity (Elbashir, Martinez et al., 2001)".

Referee #2:

This manuscript reports a structural and biochemical analysis of miRNA-target interaction that focuses on the contribution of base pairing at the 3' end of the miRNA. The seed region at the 5' end of the miRNA is the primary determinant for target binding and repression. And while a contribution of the so-called 3' supplementary binding to target recognition had also been noticed, sequence conservation, as well as biochemical analyses suggested that this was modest. Here, the authors provide the first structural model of an Argonaute protein (human Ago2) loaded with a miRNA (mir-122) and bound to a target forming base-pairing interactions with the seed sequence as well as with the 3' region of the miRNA. This model allows the authors to make a number of new observations and predictions about the contribution of the 3' supplementary interactions, but also about the mechanism of miRNA target recognition in general. The authors test some of these predictions using biochemical assays to uncover novel insight into miRNA-target interactions.

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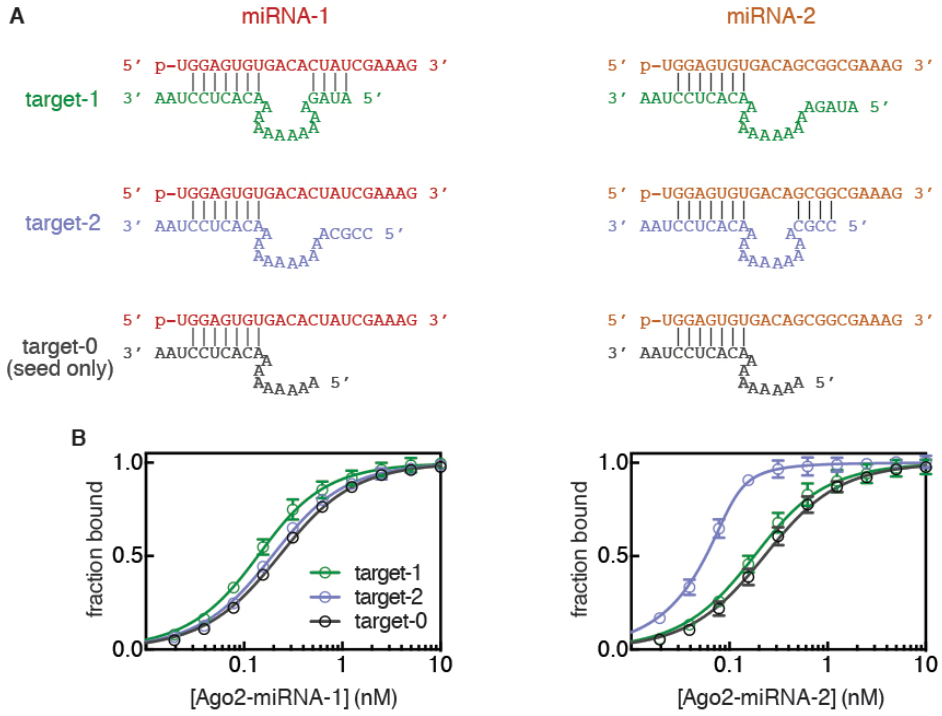
an alternative structure where stabilization comes from the longer target rather than 3' pairing). A relatively simple experiment that would strengthen the point that the 3' supplementary pairing is indeed occurring with the target containing the 15-nt bridge, would be to measure the Kd for two additional miRNA-target pairs: one where they introduce one or two mismatches in the 3' region (affinity should go back to seed-only level) and another in which they make the compensatory mutations on the other RNA strand (affinity should be restored).

We thank Referee #2 for highlighting this concern. In fact, over the course of the work we had the same thought and had already conducted a cross comparison experiment similar to that proposed above (the main difference being we used a 10 nt. bridging loop, which is more affordable to synthesize, instead of the suggested 15 nt. bridge). We used our two miR-122 variant system, where Ago2 was loaded with a miRNA-122 variant containing either an AU-rich (miRNA-1) or GC-rich (miRNA-2) supplementary sequence. We measured the affinity of both Ago2 variant complexes for targets with complementary to the common seed sequence separated from either miRNA-1 or miRNA-2 supplementary sequences by a 10 nt. bridging loop (Fig. R2).



C

	miRNA-1 K_D (pM)	miRNA-2 K_D (pM)
target-1	88±6	129±9
target-2	143±10	7±1
target-0	177±6	177±9

Figure R2. Cross comparison of miRNA-122 and supplementary target variants. A. Predicted base pairing interactions between two variants of miR-122 (miRNA-1 and miRNA-2) and three target RNAs (targets 1, 2 and 0). **B.** Fraction target bound plotted as a function of Ago2-miRNA concentration. **C.** Calculated dissociation constants (K_D) from data shown in B. Values represent means ± SEM from three independent experiments.

For both targets not matching the supplementary region of the tested miRNA we observed a small (1.2–1.4 fold) increase in affinity compared to the seed-only target control. This difference may be associated with increased target length. In contrast, target affinity increased 25-fold over the seed-only control when nucleotides complementary to the GC-rich supplementary region of miRNA-2 were included. The difference in affinities of the Ago2-miRNA-2 complex for target-1 and target-2 indicates that supplementary interactions are occurring and contributing substantially to binding (as opposed to target length effects). Additionally, target affinity increased 2-fold over the seed-only control with addition of interactions with the AU-rich supplementary region of miRNA-2, indicating the even AU-rich supplementary interactions can be contribute (although only modestly in this case) to target affinity across a 10 nt. bridge. The revised manuscript includes Fig. R2 as Fig. EV3 and the following text to clarify this point:

“Affinity differences do not appear to be related to differences in target RNA length, as a length matched target with a 10 nt bridging loop but lacking a GC-rich supplementary sequence bound with an affinity close to the seed-only control (Fig. EV3).”

Referee #3:

MacRae and colleagues determined the crystal structure of human Ago2-RISC recognizing a target RNA through the seed and 3' supplementary regions while avoiding central base pairing. This new structure strongly supports a recently proposed, revised model of how human Ago2 establishes small RNA-target RNA recognition beyond the seed region (seed -> 3' supplementary -> central). Moreover, the authors show that the guide small RNA length greatly affects the contribution of 3' supplementary pairing to target affinity, opening a door for the biological significance of 3' isomiRs. Overall, the study is carefully conducted and the manuscript is well written. I support the publication of this manuscript after a minor revision as follows.

1. In Figure 4D, the authors demonstrate that a bridge length as short as 1 nt can strongly enhance the target affinity. However, the gap between the seed and supplementary chambers in the current structure appears to be too big for 1 nt RNA to bridge. Presumably, this suggests that the gap size itself is variable through overall conformational changes of Ago2 (between the N-PAZ and MIDI-PIWI lobes).

We thank Referee #3 for raising this intriguing issue. We agree that the observation of a functional 1 nt bridge raises the possibility that the miRNA central region may be able to compact 3–4 Å more than observed in the crystallized conformation, bringing the seed and supplementary regions close enough to be bridged by a single nucleotide. This would likely require further shifts in the central gate, which is plausible but may begin to introduce strain on the PAZ domain and influence 3′-end retention. Alternatively, it is possible that one or two of the target nucleotides presumed to be paired to the supplementary region actually serve instead as part of the bridge. This notion is consistent with the observed increase in affinity upon increasing bridge length by a single nucleotide and the observation that even two well-positioned GC supplementary pairs can measurably enhance target affinity (Fig. EV2). We expanded the Discussion section to include these thoughts in the revised manuscript.

2. The authors speculate that the modest effects of 3' supplementary pairing reported previously may be attributed to differences in the small RNA lengths and sequences, but do not provide any direct evidence. It is recommended that the authors (re-)measure the affinity of (at least

some of) the previously reported small RNA-target RNA pairs (with and without 3' supplementary pairing) in their hands and directly compare them with others in the current study.

We are grateful to Referee #3 for this idea and performed the recommended experiment. The most widely referenced paper reporting affinity contributions of supplementary interactions is Wee, *et al.*, Cell 2012, which reported the affinity of the mouse Ago2-let7a complex for various target RNAs. We therefore repeated the binding experiments described in this manuscript. Care was taken to use guide and target RNAs identical in both sequence and length to those described by Wee, *et al*. As seen previously, we observed only a modest increase (1.8-fold) in affinity with the introduction of supplementary interactions (Fig. R3).

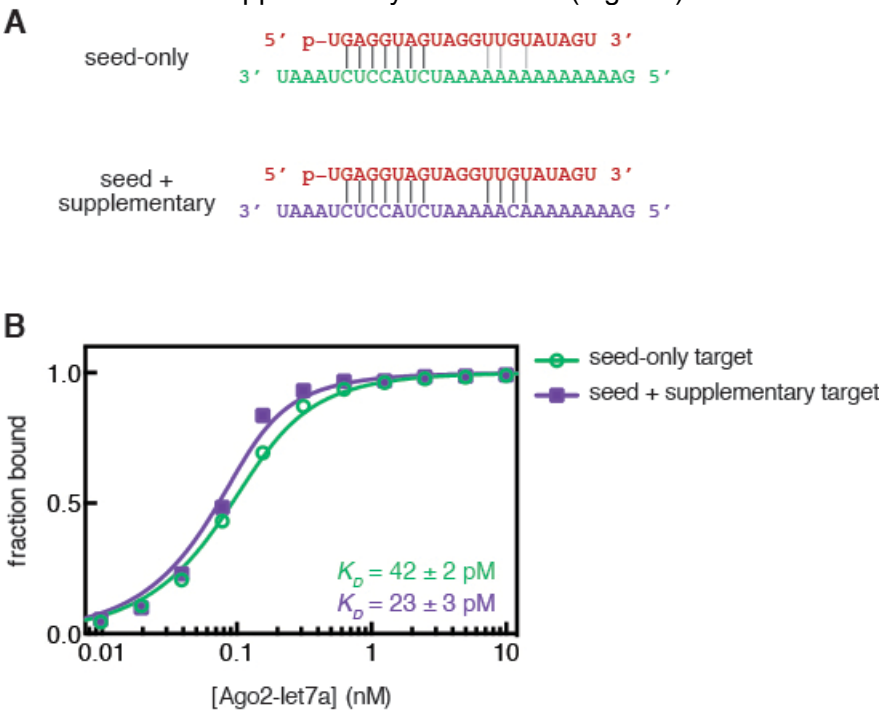


Fig. R3. Re-measurement of the affinity of a previously reported small RNA-target RNA pair. A. let-7a (red) shown paired to seed-only (green) and seed plus supplementary (purple) target RNAs. Vertical black lines indicate predicted base pairing interactions. Vertical gray lines indicate potential (previously unnoticed) supplementary base pairs. **B.** Fraction target RNA bound versus Ago2-let7a concentration. Dissociation constant (K_D) values for the two targets are indicated. Plotted data are the average of three replicate experiments. Error bars (which are too short to be seen) indicate SEM.

Notably, the dissociation constant we measured for the seed-only target closely matches the K_D reported previously (42 pM versus 26 pM). We therefore suspect that the conclusions reached in our study differ from those in Wee *et al*., not because of differences in experimental setup or technique, but simply because we examined a wider range of miRNA sequences and lengths.

We do not know with certainty why supplementary interactions appear to make an especially small contribution to target affinity when using the let-7a guide/target combination of Wee, *et al.* However, several possibilities come to mind: 1) the let-7a seed-only target binds with notably high affinity (~5x higher than seed-only miR-122 target, which has the same seed GC content), potentially making the addition of supplementary interactions less important or more difficult to measure; 2) close examination of the seed-only target sequence reveals potential base pairs to U nucleotides at positions g13, g14 and g16 of let-7a (note vertical gray lines in Fig. R3A), raising the possibility that affinity measurements of the seed-only control were skewed by the

presence of unnoticed supplementary interactions with the A-rich 5' end of the target RNA used. 3) The let7a isomiR used by Wee *et al.* was 21 nt. long, which we have found leads to weaker supplementary interactions than longer isomiRs. We suspect that one or more of these factors may be at play in this case.

To include these results in the manuscript we have added the following to the main text:

“To rule out the possibility of differences in experimental setup or technique, we measured the affinity of Ago2-let7 for targets of identical sequence to those reported previously (Wee et al., 2012) and obtained very similar results (Fig. EV5). This finding supports the notion that differences in the miRNA seed and supplementary sequences confer differences in target affinity (Salomon et al., 2015).”

We have also included Fig. R3 as Fig. EV5 in the revised manuscript.

3. Page 14, the bottom line: "grove" should be "groove."

Thank you—we made the change.

2nd Editorial Decision 15th Mar 2019

Thank you for submitting a revised version of your manuscript. The manuscript has now been seen by all original referees, who find that their main concerns have been addressed and they now support publication of the manuscript. There remain only a few mainly editorial issues that have to be addressed before I can extend formal acceptance of the manuscript:
1. Please provide final textual clarifications as requested by reviewer #3.

REFEREE REPORTS:

Referee #1:

I am satisfied with the authors' responses to my review and support the publication of their manuscript in EMBO J.

Referee #2:

The work is interesting to a broad audience, has important implications for how we think about miRNA targeting and the conclusions presented are well supported by the data.
The authors have addressed the concern I had raised in my original review with an appropriate and well-explained experiment, and I don't have any further suggestions. I fully support publication of this work.

Referee #3:

The authors have revised the manuscript appropriately and is virtually ready for publication. However, I could not fully understand the authors' statement that "In addition, the central region of the guide RNA may compact more than in the crystallized conformation, bringing the seed and supplementary regions closer together. To accommodate this compaction, however, the central gate would have to open further to widen the seed or supplementary chambers." Why does the compaction in the central region require "opening" of the central gate and "widening" of the seed/supplementary chambers?

2nd Revision - authors' response 27th Mar 2019

We are delighted that the Referees support publication. Here we address the final comment of Referee #3:

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We changed the sentence to read:
“To accommodate this compaction, however, the central gate would have to open further to provide space of the compacted central region and allow seed and supplementary duplexes to move closer to each other.”

We hope that this change clarifies the issue and thank the reviewers again for their time and insight.

The authors performed all requested editorial changes.

3rd Editorial Decision 8th Apr 2019

Thanks very much for approving the final changes in your manuscript. I am now happy to inform you that your manuscript has been accepted for publication in the EMBO Journal.

EMBO PRESS

YOU MUST COMPLETE ALL CELLS WITH A PINK BACKGROUND ↓

PLEASE NOTE THAT THIS CHECKLIST WILL BE PUBLISHED ALONGSIDE YOUR PAPER

Corresponding Author Name: Ian J. MacRae
Journal Submitted to: The EMBO Journal
Manuscript Number: EMBOJ-2018-101153R

Reporting Checklist For Life Sciences Articles (Rev. June 2017)

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.

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?	Sample size was chosen to by a combination of experience with the experimental systems used, pre-liminary experiments to establish estimated effect sizes, and standard practice in the field.
1.b. For animal studies, include a statement about sample size estimate even if no statistical methods were used.	N/A
2. Describe inclusion/exclusion criteria if samples or animals were excluded from the analysis. Were the criteria pre-established?	N/A
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.	N/A
For animal studies, include a statement about randomization even if no randomization was used.	N/A
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.	N/A
4.b. For animal studies, include a statement about blinding even if no blinding was done	N/A
5. For every figure, are statistical tests justified as appropriate?	Yes
Do the data meet the assumptions of the tests (e.g., normal distribution)? Describe any methods used to assess it.	N/A
Is there an estimate of variation within each group of data?	Yes
Is the variance similar between the groups that are being statistically compared?	Yes

C- Reagents

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http://biomodels.net/miriam/
http://jjj.biochem.sun.ac.za
http://oba.od.nih.gov/biosecurity/biosecurity_documents.html
http://www.selectagents.gov/

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).	N/A
7. Identify the source of cell lines and report if they were recently authenticated (e.g., by STR profiling) and tested for mycoplasma contamination.	HEK 293 cells were used in some experiments. Cells used had not been recently authenticated or tested for mycoplasma contamination.

* 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.	N/A
9. For experiments involving live vertebrates, include a statement of compliance with ethical regulations and identify the committee(s) approving the experiments.	N/A
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.	N/A

E- Human Subjects

11. Identify the committee(s) approving the study protocol.	N/A
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.	N/A
13. For publication of patient photos, include a statement confirming that consent to publish was obtained.	N/A
14. Report any restrictions on the availability (and/or on the use) of human data or samples.	N/A
15. Report the clinical trial registration number (at ClinicalTrials.gov or equivalent), where applicable.	N/A
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.	N/A
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.	N/A

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	X-ray diffraction data and coordinates of the seed plus supplementary-paired Ago2-miRNA-target complex have been deposited in the Protein Data Bank (PDB) (6N4O).
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).	Relevant data has been depositied in publically available database (see above).
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).	N/A
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.	N/A

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.	No