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Genome-scale, single cell-type resolution of microRNA activities within a whole plant organ

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Previous referee reports and authors' response on an earlier version of this manuscript

Referee #1:

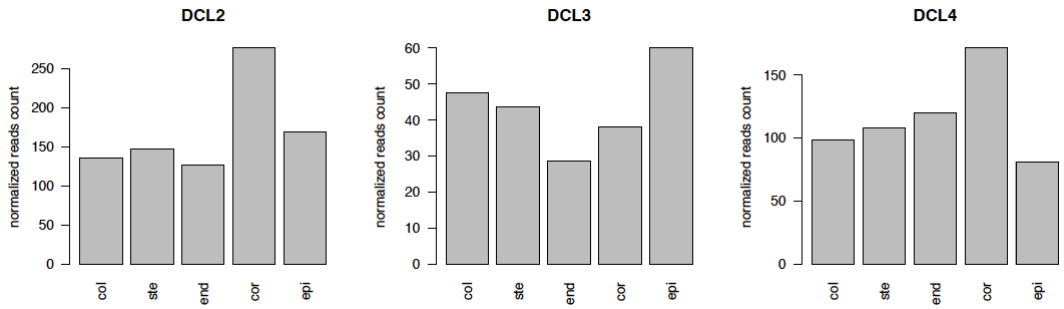
The manuscript presents an approach to explore the miRNA signatures in five cell types in the Arabidopsis root. By expressing a FLAGs fused to AGO under cell type-specific promoters in mutant enriching miRNA contents, the authors have shown differential miRNA loading patterns in the different cell types. In a complementary set of experiments, the authors IP'ed FLAGs fused to RPL18 expressed under the cell-specific promoters and compared the expression levels of miRNA targets in WT and in miRNA-deficient mutants in each cell type. They demonstrate cell type-specific effects of selected individual miRNA on their targets. Thus, the study provides a comprehensive analysis of miRNA signatures in resolved sub-populations, exploring both loading and activity and effect on target transcripts. While the overall approach is exciting, the paper is hard to follow and must be re-written to clarify notations and systems to make it more accessible to a broad readership. The following are specific points that should be addressed.
The view that the paper is hard to follow was not shared by reviewer 3 ("*The paper is very well written*") **but we concede that he/she is likely more accustomed to the plant root system and plant miRNA-target analysis given his/her comments. The goal here is obviously to reach the largest audience possible because the method and concepts of gene regulations uncovered through its use are relevant well beyond plant systems. One aspect we felt was missing was a clear depiction of miRoot and of its readouts, because they are used all throughout. This has now been added (in purple in the pdf file). We have also re-written various parts including by articulating the content of the first part around the key challenges posed by the analysis in order to guide the reader through, and provide a clear rationale, to the various steps. We used subheadings to qualify our findings in terms of their broad significance in the second part which we also endeavoured to re-write for enhanced clarity. We have also added small schemes in our figures that encapsulate the discoveries made regarding spatial genetic regulations, thereby signifying their broad significance. The insight of referee 1 was very much appreciated in building up what we hope is now a more approachable manuscript.**

1.1. In the title and abstract, as well as in several places in the manuscript, the method described is wrongly addressed as "single cell resolution". The method applied in this study is single cell-type resolution, as differences between different cells of the same type could not be captured in this setting. Thus claims of single cell resolution or single cell experimentation should be removed and replaced by single cell-type or cell sub-populations.
This is a completely valid point also raised by referees 2 and 3: these are cell files representing a single TYPE, nut not individual, single cells. Accordingly, all references to "single cell" have been changed to "single cell-type" in the manuscript and supplementary material.

1.2. To avoid inherent suppression of AGO, the authors carried on the FLAG::AGO IP in triple mutants. The deduction from mutants to WT requires exploring the differential effects of the mutants on the five cell types. For example, the dcl genes could have an enhanced function in the Stele layers, causing a broader range of miRNA

loading when knocked down. The authors should therefore validate equal levels of the dcl proteins in the WT cell types.
This is a valid point from the referee, which we did not consider as potentially contributing to the substantial miRNA loading into AGO1 observed in the stele compared to other root layers. The referee's argument is that DCL2, 3 or 4 (or combinations thereof) being potentially more abundant (and hence more functional) in the stele, their genetic ablation would manifest as increased DCL1 activity because of the known competition of DCLs for substrates (here miRNA precursors).

Using the replicate polysome sequencing data, we have now verified that the difference in translated DCL2, DCL3 and DCL4 mRNA levels (as a proxy of their protein levels since we do not have good antibodies for DCL2 and DCL4) never exceeds 2 folds between one layer and the others. The slightly over-accumulating layer varies depending on the DCL considered but never includes the stele. We know that DCL genes are haplo-sufficient (heterozygous mutants accumulate WT levels of DCL-specific sRNAs and have no overt phenotypes); therefore, the observed maximum variations (two folds) are unlikely to have strong biological significance, if any:



At a direct molecular level, a potentially enhanced function for DCL1 in the *dcl234* background would lead to a global increase in miRNA reads. Yet Figure 1E shows both enrichment and depletion in distinct miRNAs in a cell layer-intrinsic manner. Even in the stele for which more than 50% of miRNA seem to over-accumulate compared with other cell layers, we can still identify miRNAs that are depleted.

We believe the translatome analysis and explanation above argue against the stele-specific DCL1 over-accumulation hypothesis in *dcl234*. This is nonetheless a valid point, which we have now highlighted and discussed in line 120-123 of the revised manuscript.

1.3. In hyl1 mutants, there is a global gene expression difference, of which miRNA targets are unexpectedly often a minority. This is particularly apparent in Fig. 2d, where the miRNA targets are a small minority out of the genes that are up-regulated in the hyl1 mutant. Specifically, the sentence: "As expected, the bulk of miRNA targets was upregulated in hyl1-vs-WT cells", while non-miRNA target accumulation remained largely unchanged (Fig. 2D)." is not supported by the results shown in Fig. 2d. The small effect of the hyl1 mutants on the miRNA targets render this system questionable.
The text from line 171-176 has now been added to clarify what appears to be a mere misunderstanding. Key to this point is that NOT ALL miRNA targets are upregulated in each cell-layer, which actually demonstrates the importance of using a cell-type specific approach. Nonetheless, on the whole (all layers together), changes affect most known miRNA targets. In several instances, a modest (approx. 2-folds) increase in specific miRNA target levels may be seen in *hyl1* vs WT whole roots. However, looking at specific cell-types independently, differences of much higher amplitudes are revealed.
When the steady-state levels of a miRNA target are changed, the bulk of that change is an increase in translatome signal in the *hyl1* background demonstrating the validity of the approach. As many miRNA targets are transcription factors it is entirely expected that many changes in non-miRNA targets will, and in fact did, occur explaining the comparatively low proportion represented by direct miRNA targets in each individual cell type. We also draw the attention of the reviewer to the fact that every single cell-type specific analysis conducted with fluorescent target reporters in the second part of the study effectively validated the *hyl1* results inferred from miRoot.

1.4. The authors should validate some of the deduced functional miRNA-target interactions by silencing individual miRNAs using si and exploring the effect on the target transcripts. Comparing the effects of the silencing to those observed in hyl1 mutants can justify using the hyl1 mutants.
The use of target mimics to KO individual families of miRNAs would itself represent up to a year of work to be completed. The use of *hyl1* was mandatory, as there is simply no other way to gain a genome wide assessment of miRNA-mediated gene regulation. The use of individual mimics would indeed allow the detailed analysis of a single miRNA-regulation gene network but, in our opinion, is out of the scope of our work. This is, in fact, typically the type of refined studies our browser would likely prompt from laboratories interested in specific miRNA:target interactions. We further note that comparisons with several previously published examples (e.g. miR396 and its targets, the GRF proteins) (Rodriguez *et al*., 2015) also demonstrate the validity of our approach.

1.5. The data shown in Fig. d and its relation to Fig. 2c are not clear. Please clarify what are these "validated miRNA targets" and how many are shown in the panel
These were specified in the material and methods section in which the key reference is given (Arribas-Hernandez *et al*., 2016). The authors collated all the validated examples in Arabidopsis since the discovery of the first plant miRNAs and their targets in 2002. The list represents a set of firmly established

targets due to the perfect or near-perfect base-pairing complementarity of target:miRNA sites in plants as opposed to the limited pairing seen in metazoans, and hence the relatively poor definition of targets in these organisms. As disclosed in the compilation of Arribas-Hernandez *et al*., 2016 there are 249 non-redundant target mRNAs from 660 miRNA-mRNA pairs.

Referee #2:

The authors have analysed in this manuscript the area of activities of miRNAs in Arabidopsis roots. They first identify miRNAs loaded by AGO1, with cell-type resolution, before analysing the translatome in WT and hyl1 mutant. They next illustrate several examples of original regulations among conserved miRNAs. Despite a huge amount of work of quality, several aspects are troublesome:

2.1 The title is overclaiming: this is cell-type resolution and not a single-cell resolution.
As with the first point of reviewer 1 we agree and all references to single cell resolution have been changed to single cell-type.

2.2 Whereas the first part of the manuscript is really interesting, the second part is too long and is only a catalog of miRNAs: despite a strong interest for specialists of each miRNA, a broader audience will be quickly bored.
We respectfully disagree with the reviewer but admit that the motivations for our choice of specific miRNA-target interactions in the second part of the study was not well explained. This is not a catalogue, but instead specific examples highlighting particular modes of gene regulation in space that struck us by their incredible sophistication and/or complexity as well as hitherto undescribed or fully unexpected phenomena not documented in any organism thus far. This view is actually shared by reviewer 3.

We have altered/added the text to highlight the novelty and of each example (e.g. lines 273-275) and also included schematic representations of each '*spatial mode of actio*n' (see Figures 3G, 4G, 4J, 5H and 6F) to, again, highlight the broad biological implications of each example. We have now specified that these examples are not just applicable to plants but represent broad concepts with possible equivalents in the metazoans in which this type of spatial study at such a resolution has never been documented.

2.3 The authors have not used a promoter specific for QC. Because these data are lacking for a global comprehension, is there any rationale for it?
The QC represents only 4-7 cells within the entire root and so its specific analysis with the methods used in the manuscript represented an unsurmountable technical challenge. In addition, previous studies (e.g. Breakfield et al., 2012) incorporated the QC by utilizing the SCR promoter which is expressed both in the endodermis and in the QC, so in reality our approach DOES cover the QC. Nonetheless, without using a QC-specific promoter (e.g. WOX5) to adequately isolate this small group of cells, we felt that our data would have been overreaching and, perhaps, misleading, had we claimed coverage of the QC with SCR, so we preferred to adopt the opposite stance. Our standpoint is now clearly articulated from lines 71-75 of the revised manuscript.

Note that we are trying to solve this gap, including by using mutations that expand the QC's domain albeit also activate it by switching a quiescent into an actively diving niche. Nonetheless, these are ongoing approaches far from completion and we feel the manuscript is solid even without the specification of the QC. Again, this has been acceptable in other high profile studies (e.g. Breakfield et al., 2012).

2.4 The authors have focused their analysis (in the manuscript) on conserved miRNAs (156-447). What about others: they never talk about them (except in Suppl. Tables)?
The discovery of new miRNAs was an expected outcome of the study given its depth and cell-type specific nature. Use of the *dcl234* background (although for a totally different reason i.e. to avoid AGO1 co-suppression) also undoubtedly contributed to the discovery of a staggering number of potential new miRNAs. But we emphasize that this aspect represents a minute portion of the manuscript. This was deliberate as we did not want to distract the reader from the main focus of the study: spatial gene regulations by known miRNAs resolved at a genome and cell sub populations scale. In our opinion, the sheer number of potentially new miRNAs discovered, their target validation (which is not trivial at the cell-specific level) and the functional characterization of at least some of them call for a separate study altogether, which is now explained in lines 97-99 of the amended manuscript. When the new miRNAs and targets are validated they will be added to the miRoot browser as well.

2.5 Several articles have shown that miR* are bound by AGO1. It seems that authors have found them (see Suppl. Tables) but they never discuss it.
This is true. while, again, not the primary focus of this study, we have addressed the point raised by referee 2 in lines 212-215 of the amended text referring to a new supp. Data S8 providing such examples. Note that miRoot specifies the 3p or 5p nature of the miRNA strand (a nomenclature more accurate than the original miR/miR*) so that, in reality this information is systematically embedded in the miRoot readouts. This is now better explained in a section describing the browser and its main amenabilities, which we felt was lacking in the previous version of the manuscript. We believe this addition also provides more substance to the first part of the study, which seems to be the preferred part of referee 2, unlike that of referee 3.

Referee #3:

In this manuscript, the authors use a non-invasive approach and examine the spatial patterns of miRNA loading and miRNA targeting in the root of A. thaliana. They analyze and report on each of several root cell types separately. The findings from their deep sequencing analyses were coupled to independent experimentation and lead to several intriguing discoveries. The latter include the identification of many novel Arabidopsis miRNAs, new modes of spatial regulation, transport across root layers with different cellular makeup, etc. The paper is very well written. The presentation is crisp and to the point. The experiments are well thought out and tie together. The Figures are well designed. It is clear that the authors put a tremendous amount of effort in the design and execution of the work, as well as the presentation. I review papers every few days and only rarely I come across manuscripts of such quality: the authors are to be commented for that. There are several interesting findings described in the manuscript. I highlight a few here together with some comments:

3.1 The discovery of many novel Arabidopsis miRNAs 60% of which map to TEs. It will be worth adding a paragraph in the Discussion section to address this observation and discuss its ramifications.
This is a good point; we have added a paragraph (lines 574-587) to the discussion addressing the new miRNAs.

3.2 I particularly enjoyed the findings about the miR-169 family and their layer-dependent expression. This is a very interesting result, also with important ramifications. As the authors know, recent reports have shown that human miRNAs express multiple isoforms at the same time whose profile differences permit cancer type and tissue classification. The authors report that miR-169a and miR-169d-g are expressed homogeneously across layers. Do the respective miRNA hairpins express the same or different isoforms across the layers? No matter what the answer is here it is interesting to know it.
We can only assess this by using pre-miRNAs associated to polysomes which may not be a truly accurate representation of the expression domains. That being said, the data could be shown but this would rely on the pre-miR169s being substantially present on polysomes which is the case of some, but not all pre-miRNAs; possibly because some contain mini-ORFs.

3.3 I thought that the presented evidence about cell-cell movement is very interesting as it is intriguing.
We thank the review for the comment, which somehow contradicts the view of referee 2 that our examples were a catalogue. We were, too, surprised:

a. Of how widespread the movement process appears to be;
b. The amazing nature of the evacuation-filling system where one layer seems to act as a mere reservoir of mature miRNAs that are then disseminated in to the others

Minor Comments:
3.4 In the title, abstract, and in much of the text, the authors use "single cell" as a modifier that refers to their sequencing whereas what they really mean to do is to use it as a modifier to the cell types that they examine. This should be made unambiguously clear in a revised version of the text.
As with this has been modified as per the request of all reviewers. This is certainly a very valid point.

3.5 The use of the English language is masterful but leads frequently to unnecessarily long sentences that make it difficult to read. The authors should consider breaking such long sentences into smaller ones.
This is a valid point and as a whole we have tried to identify any overly long sentences and break them up into small ones.

3.6 Also, the authors make an excessive use of prepositional phrases. This should be fixed in the revised version.
As with point 3.5 we have addressed this issue to our best in the text.

3.7 Line 489: "Fig. 5K" should be "Fig. 3K".
This has been changed.

3.8 Line 90: Since "intergenic loci" and "tRNA loci" are not disjoint categories the authors will need to recalculate the reported percentages. Two related questions: 1) were there no novel miRNAs in "intronic" regions? This should be discussed in the text. 2) I am curious: how many of the miRNAs that mapped to tRNAs were derived from mitochondrial tRNAs vs. nuclear tRNAs?
The materials and methods section was modified to address the first issue with miRNA prediction. For question 1 this was explained in the materials and methods section, the annotation process is iterative: "*Annotation of predicted miRNA was done by comparing genomic positions against TAIR10 annotations. If several overlaps were found, annotation was attributed following a priority order as follows: tRNA, snRNA, snoRNA, TE, pseudogene, intron, exon).*" For question 2 this information is present in table S3. Of the 22 predicated miRNAs annotated as tRNAs only one was located in the mitochondrial genome.

1st Editorial Decision 21st Jan 2019

Thank you for submitting your manuscript to The EMBO Journal. I would like to apologise again for the unusual delay in the evaluation of your manuscript. I have contacted two arbitrating advisors to assess the manuscript and the transferred referee reports. Since one of the advisors has now informed me that he/she will not be able to submit their report after a protracted delay, I have decided to reach the decision based on the report I have already received. I have copied the advisor's comments below.

As you can see, the advisor appreciates the study and finds that it will be of value as a resource. The advisor also points out a number of textual modifications that would be necessary to improve the accessibility of the manuscript. From the editorial side, we also find that the rationale of the experimental setup and target identification needs further clarification to serve as a resource for the more general audience of our journal. I would like to invite you to submit a revised manuscript in which you include the requested amendments to the data presentation and discussion.

When submitting your revised manuscript, please carefully review the instructions that follow below. In addition, during our routine image analysis we noted the following issues in the figures that have to be addressed before the manuscript can be published:
1) Please improve the resolution quality of the figures
2) There are empty panels in Fig 1C, CB panels in IP; Fig S2 U6 panel in IP
3) Figures 6H, S16B and S19B - please increase contrast in the CB panels, as currently the signal is difficult to discern
4) Please submit source data for all figures. We would need one file per figure (which can be a composite of source data from several panels) in jpg, gif or PDF format, uploaded as "Source data files". The gels should be labelled with the appropriate figure/panel number, and should have molecular weight markers; further annotation would clearly be useful but is not essential. These files will be published online with the article as a supplementary "Source Data".

REFEREE REPORTS:

Advisor's comments:

From my reading of the paper, I think it appropriate for an EMBO Journal resource paper. It is innovative, ambitious in scope and, with the developed browser feature, should indeed be an attractive tool for surveying the developmental impact of miRNAs. Now, I fully agree with you (and the reviewers) that the last part of results/discussion is WAY too long, not because any individual example goes too much into detail, but because too many examples are provided! It reads as if the authors desperately want to drive home the point with too many example that their approach fits published data, but also uncovers many novel, spatial and temporal miRNA regulation patterns.

I think the authors should considerably re-write this entire last section, focusing on one or two examples where they can show that their data fits some well worked-out, known examples of spatial miRNA regulation (as for example the regulation of HD-ZIPIII TFs by miRNAs165/66. Added to this should be a better discussion of how their data fits (or is at odds with) a similar data set published in Genome Research in 2012. I fully agree with the authors that the Genome Research paper uses a very different approach (protoplasting), which has its problems. But their own (in principle more elegant) approach has its own shortcomings, notably the use of a triple DCR mutant as a background and the control comparison to HYL1, which is a very pleiotropic mutant. Such a comparison would not take away anything from their ressource, but allow researchers to assess the robustness of each data set.

As to the discussion of novel targets, again one or too better worked-out examples should be enough. The discussion could also be shortened and rather be focused on relating this resource to other resources out there and pointing out how this resource could be utilised and possibly extended and improved. I think it's a really significant resource and dataset that the authors present.

1st Revision - authors' response 20th Mar 2019

Please, find first our answers to the specific points raised about the text of the manuscript:

1. *As you can see, the advisor appreciates the study and finds that it will be of value as a resource.*

The text had been initially tailored to reflect the value of the study both as (i) a resource and an enabling technology to (ii) uncover new aspects of miRNA-mediated gene regulation. Given our

previous submission, there was initially a stronger emphasis on (ii) and we have now taken into account the advisor's comments to bring more weight on the resource aspects as well by amending the text and reducing the number of examples shown in the main text, as explained in points 4-7.

2. *The advisor also points out a number of textual modifications that would be necessary to improve the accessibility of the manuscript.*

We believe we have now made those modifications where appropriate in order to improve clarity, as explained in points 4-7 below.

3. *From the editorial side, we also find that the rationale of the experimental setup and target identification needs further clarification to serve as a resource for the more general audience of our journal.*

We have now made modifications to clarify the technical aspects of the study, which was indeed necessary to the reader. As part of this, the beginning of the section "*The cell-type specific root-tip miRNA-targetome"* has been entirely re-written to provide a clear rationale for the use of *hyl1* in the targetome studies. The whole new paragraph is found between line 145 and 161 of the revised text.

4. *I think the authors should considerably re-write this entire last section, focusing on one or two examples where they can show that their data fits some well worked-out, known examples of spatial miRNA regulation (as for example the regulation of HD-ZIPIII TFs by miRNAs165/66.*

We certainly agree that the examples provided in the first version were too numerous, yet felt that restricting a smaller number of examples, in the revised manuscript, to mere confirmatory results would not do justice to the work accomplished using the method, which in many aspects is an eye opener about the versatility of miRNA-mediated gene regulation in space.
While there was definitively space for improvement on the "resource" aspect of the study, we felt no real justification for this aspect to totally supersede the other, equally important one, i.*e.* the use of the resource to discover novel aspects of plant miRNA biology. We were not desperate to fit such data to published ones since they describe, for their most, entirely unexplored and quite important facets of miRNA biology including some that readily explain previously-made, unexplained observations. As much as the resource *per se*, we believe these findings should also be of interest to the EMBO J readership. In fact, it is for this precise reason that we chose EMBO J as a venue for our revised manuscript from *Nature*, as opposed to a method/resource-oriented journal such as *Nature Methods* (which is in the *Nature* franchise, unlike EMBO J). We further note that only one of the three original referees felt there were too many examples (though without questioning the underlying results) whereas one, on the contrary, found all these examples fascinating and indeed unraveling in the context of previous findings. No reviewer, however, advocated the complete re-focus of the work as a "resource" only. For these reasons, we disagree to abandon a large part of the work to fit a "resource-only" format, yet we certainly concede the need to emphasize this aspect more and have worked on the text accordingly.
To reach a compromise and also declutter the main text, which was an issue for one referee and the advisor, we have removed the MYB33^{miR159}/MYB65^{miR159} , LAC4^{miR397} and NAC1^{miR164}/NAC6m^{iR164} examples entirely and are summarized briefly from line 255-264 in the revised manuscript. These major portions of data have now been transferred to the supplementary material since we do not intend to produce any follow ups on these analyses *i.e.* we may as well disclose them, unless the editor believes it is not needed. However, we kept the non-cell autonomous regulations by many miRNAs as an intact component of the manuscript to illustrate the value of our approach. We did this primarily because there were so far only two documented examples of miRNA cell-cell movement in plants and the method described in the paper allowed us to uncover that it is, in fact, a common feature of miRNAs, not an anecdote.
If the editor feels there is still too much emphasis on new results in the "movement" sections, we would reluctantly be prepared to remove the last part "*pri/pre-miRNA cell-to-cell movement is not exclusive to the root-tip or the root*" and, much more reluctantly "*Mature miR395 moves from cell-to-cell*", both of which could be kept for publication elsewhere. At this stage, however, and with the re-focus of the text -including the whole discussion- to emphasize better the resource aspect of the study, we would rather keep the revised manuscript as it is given that, in addition, the number of

figures is now well balanced: 3 technical, 4 analytical. We believe that the aspect of movement is truly one of the novel and in all likelihood highly cited elements of the manuscript.

5. *Added to this should be a better discussion of how their data fits (or is at odds with) a similar data set published in Genome Research in 2012.*

We have now alluded in more details to the Breakfield *et al*, 2012 study in the discussion (line 515 to line 525), especially to emphasize (i) the limitations of **our own** approach and (ii) that the two studies actually provide different answers about distinct types of small RNAs. Nonetheless, we also provided the editor what we believe are valid, factual reasons for not elaborating further on a comparison between drastically dissimilar approaches. We have, however, taken onboard the valid following points from the advisor.

6. *I fully agree with the authors that the Genome Research paper uses a very different approach (protoplasting), which has its problems. But their own (in principle more elegant) approach has its own shortcomings, notably the use of a triple DCR mutant as a background and the control comparison to HYL1, which is a very pleiotropic mutant.*

We fully concur and have completely re-written the discussion to highlight in a now dedicated technical section, our system's potential pitfalls. This technical section was created to emphasize the 'resource' aspect of the study and it encompasses line 509 to line 551 of the revised manuscript. It also now mentions a recently developed 2'-O-methylation method for miRNA isolation in metazoans (line 509 to line 515), which, we thought, was fair to mention as a recent and quite original implement to large scale miRNA studies (though the technic is not applicable to plant sRNAs for obvious reasons). As to the limitations of our method, we mention and moderate the *hyl1* pleiotropy problem, and highlight how the necessary use of *dlc234* could bias some outputs. We also elaborate on how the method could be used by others and further improved.

7. *The discussion could also be shortened and rather be focused on relating this resource to other resources out there and pointing out how this resource could be utilized and possibly extended and improved.*

See our answer to point 6 above. We believe the discussion is now more balanced between truly technical aspects, in its first part, and argumentation/contextualization of some of the major results, in its second part.

Please, find now the answers to the technical points as provided by the first author and co-corresponding author, Chris Brosnan, who assembled all the figures from the experimental data:

1) Please improve the resolution quality of the figures

The resolution of all figures has been improved to a higher quality for publication.

2) There are empty panels in Fig 1C, CB panels in IP; Fig S2 U6 panel in IP

For Figure 1C, since we are only dealing with IP'ed data we have only included the IP immunoblots and shown the Coomassie blue to prove equal loading of the samples. The U6 panel in Fig S2 has been replaced but is still weak (see source data for this) as is expected from a successful immunoprecipitation (U6 does not bind AGO).

3) Figures 6H, S16B and S19B - please increase contrast in the CB panels, as currently the signal is difficult to discern

The contrast of the CB panels have been increased for Figures 6H, S16B and S19B.

4) Please submit source data for all figures. We would need one file per figure (which can be a composite of source data from several panels) in jpg, gif or PDF format, uploaded as "Source data files". The gels should be labelled with the appropriate figure/panel number, and should have

molecular weight markers; further annotation would clearly be useful but is not essential. These files will be published online with the article as a supplementary "Source Data".

Source data have been provided for each figure in the manuscript.

2nd Editorial Decision 15th Apr 2019

Thank you for submitting a revised version of your manuscript. The manuscript has now been seen by the original arbitrating advisor, who is now broadly in favour of publication of the manuscript. There remain only a few editorial issues that have to be dealt with before I can extend formal acceptance of the manuscript:

REFEREE REPORT :

Referee #1:

I have read the authors replies and looked again over the revised manuscript. I am impressed by their thorough comparison between their dataset and that of Breakfield et al. The lack of similarities between the datasets is quite worrisome though. Yet I am inclined to follow the authors' explanations of why the two data sets are incomparable and I think they have provided sufficient data in their own work that validates their dataset and have demonstrated that it will be a very useful community resource for discovery and hypothesis generation.

2nd Revision - authors' response 3rd May 2019

The authors performed all requested editorial changes.

3rd Editorial Decision 7th May 2019

Thank you for introducing the final adjustments in the manuscript. I am now pleased to inform you that your manuscript has been accepted for publication in The EMBO Journal. Congratulations!

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: Christopher Brosnan and Olivier Voinnet
Journal Submitted to: EMBO journal
Manuscript Number: EMBO-2018-100754

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 $\chi 2$ 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?	na
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?	yes
Do the data meet the assumptions of the tests (e.g., normal distribution)? Describe any methods used to assess it.	yes. Tests are listed in Appendix Materials and methods
Is there an estimate of variation within each group of data?	na

USEFUL LINKS FOR COMPLETING THIS FORM

http://www.antibodypedia.com
http://1degreebio.org
http://www.equator-network.org/reporting-guidelines/improving-bioscience-research-repo

http://grants.nih.gov/grants/olaw/olaw.htm
http://www.mrc.ac.uk/Ourresearch/Ethicsresearchguidance/Useofanimals/index.htm
http://ClinicalTrials.gov
http://www.consort-statement.org
http://www.consort-statement.org/checklists/view/32-consort/66-title

http://www.equator-network.org/reporting-guidelines/reporting-recommendations-for-tun

http://datadryad.org

http://figshare.com

http://www.ncbi.nlm.nih.gov/gap

http://www.ebi.ac.uk/ega

http://biomodels.net/

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

Is the variance similar between the groups that are being statistically compared?	na

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).	All antibodies are listed in the Appendix Materials and methods section
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	This is shown in the Appendix materials and methods section
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