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Editing independent effects of ADARs on the miRNA/siRNA pathways

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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 22 April 2009

Thank you very much for submitting your research manuscript for consideration to The EMBO Journal editorial office.

I did receive three rather encouraging reports from scientists working in the field that you will find attached below. All three referees indicate a strong interest in your study, but they also request modifications that will have to involve further experimentation. As the reports are rather self-explanatory, there is not much reason for me to repeat the details here. Most importantly, ref#2 remarks more generally on the experimental setup and suggests repeating at least some of the crucial experiments in cells that do express the miRNA-376 cluster. To improve the broader appeal, this ref also recommends to extend to a more global picture indicating other miRNAs that might potentially be subjected to this type of processing. Ref#3 seems particularly concerned with data quantification, but further suggests valuable experiments that could help, at least genetically, to dissect the step that is blocked by ADAR binding.

Overall, we kindly ask you to respond to the referee comments and experimentally address as far as you can during the limited time of one round of major amendments.
I also have to remind you that the final decision on acceptance or rejection will depend on the content of the final version of your manuscript and that it is EMBO Journal policy to usually allow a single round of revisions only.

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

REFEREE REPORTS

Referee #1 (Remarks to the Author):

This study has shown that ADARs can specifically affect processing of pri-miRNAs independent of editing activity. ADAR2 modulates processing of miR-376a2 while ADAR1 affects RNAi via the siRNA pathway. In the case of miR-376a2 the authors show that drosha processing is inhibited by ADAR2.
The authors describe an interesting and thorough study that reveals a novel mechanism for how ADARs can antagonize the miRNA and RNAi pathways. While previous studies have also shown antagonism between these pathways, this study is unique in showing antagonism that does not rely on editing. This observation reveals a new role for ADARs, and demonstrates the importance of interplay between important biological pathways in cells.

My minor comments are as follows:

1. Supplemental Figure S1 - the text (p9) states that "previous work has shown that this protein, expressed.....". If this experiment was done previously, a reference should be given. If it was carried out for this study, the text should be adjusted to reflect this.
2. It would be helpful to refer to Supplementary Figure 2B when referring to the binding sites within the luciferase reporters for edited or unedited miRNAs.
3. In the text (p11) it says "This five-fold discrimination by the mature unedited microRNAs between the unedited and the edited sites is strong, considering....". Can the authors clarify what is meant by "strong".
4. It would be helpful in the description of Figure 2 to refer to the specific mutants in the text, rather than just (for example) "deaminase mutant ADARs".
5. Figure 3A - is it possible to show data for quantitation of mature miR-376a2 (as shown for pre-miR 376a2)? Also, ADAR1 also appears to cause a reduction in RNA levels - can the authors comment?
6. Figure 4D should be described more explicitly in the text.
7. Can the authors comment on the amount of editing of the white hairpin seen by endogenous dADAR or by ADAR1 p150?

Referee #2 (Remarks to the Author):

The manuscript by Heale et al. reveals unexpected properties from ADARs, enzymes that edit mRNAs and microRNAs. The authors carried out a structural/functional analysis to dissect the effect of ADARs on a particular miRNA cluster. The highlight of the manuscript relies in finding that catalytically inactive ADARs can alter miRNA processing by impairing cleavage by Drosha in the nucleus.

This is a suitable paper for EMBO Journal provided the authors address the following queries:

Regarding the general experimental setup, the authors use HEK 293T cells as an assay tube where they add plasmids encoding flag-tagged-ADARs (which supplement endogenous ADAR) and the miRNA-376-cluster (not expressed in HEK 293T). Wouldn't be worth to carry out some of these experiments in a more natural environment, i.e. on cells that do express the miRNA-376 cluster, which are then treated with siRNAs against endogenous ADARs and rescued with wild type or mutant ADARs insensitive to the siRNAs? The authors will be able to monitor levels of mature miRNA-376a2, evaluating the inhibitory effect of each ADAR enzyme.

In page 9 the authors reason that editing of a pri-miRNA by ADAR 2 - a form excluded from the nucleus - must be due to leakage of the primary miRNA into the cytoplasm. To support this statement, I would ask the authors to perform a Northern Blot or qPCR analysis potentially adding a supplementary figure to the manuscript.

In Fig. 3A the authors should design a probe against the precursor miRNA. The current Northern Blot is rather unclear, and such a probe could identify with more certainty the band corresponding to the pre-miRNA.

In Fig. 3C, the authors may want to show a shorter exposure of the gel to better judge the potential

accumulation of the pri-miR-376a2 upon addition of ADAR2.

It is said that this regulatory mechanism could affect the expression of a large number of miRNAs. In this regard, why not providing a rather global picture by looking at other miRNAs potentially subjected to this processing?

Minor points and typos:

Fig.1A differs from Fig. 1A in Kawahara et al. Science 2007. Please double-check.
Page 10, second paragraph, it should read Processing of pre-miR-376a2...
Page 10, last paragraph, Supplementary Fig. S2B.
Page 14, at the end of second paragraph, adjust Fig. 4A to 4B.
Page 18, second line from the bottom, substitute pre-miRNA by pri-miRNA.

Referee #3 (Remarks to the Author):

The role of ADARs in antagonizing and modulating RNA silencing is one of the newest areas of small RNA research. This manuscript uses a combination of in vitro (mammals) and in vivo (flies) studies to test ideas about how ADARs can respecify microRNA targets. In the process, they discover a new role for ADAR in antagonizing RNAi, presumably by binding dsRNA or siRNAs, rather than by editing.

The quality of the science is quite high, and only a few additional experiments are required to ready the work for publication. With these, the manuscript will be entirely suitable for publication in EMBO J.

(1) Binding data (Figure S1A) should be displayed on a semi-log plot and fit to the Hill equation or the Langmuir isotherm. A comparison of the apparent KD values determined by fitting the data is a good start, but when comparing two different proteins it can be quite misleading, as not all purified proteins have the same percent activity. Thus, a stoichiometric titration experiment is needed to determine the actual percent active protein for each protein to be compared. See, for example, Figure 2 in Batey, R. T., and Williamson, J. R. (1996). Interaction of the Bacillus stearothermophilus ribosomal protein S15 with 16 S rRNA: I. Defining the minimal RNA site. J Mol Biol 261, 536-549.

(2) The authors should show quantitative eye pigment assays for the experiments in Figure 5 (the mean of multiple biologically independent readings {plus minus} standard deviation). Visual assessment of changes in this assay is difficult as the human eye records color in a highly non-linear way. For example, compare the images of fly eyes with the quantitative data in Figure 8 of Fˆrstemann et al. (Fˆrstemann, K., Tomari, Y., Du, T., Vagin, V. V., Denli, A. M., Bratu, D. P., Klattenhoff, C., Theurkauf, W. E., and Zamore, P. D. (2005). Normal microRNA maturation and germ-line stem cell maintenance requires Loquacious, a double-stranded RNA-binding domain protein. PLoS Biol 3, e236.)

(3) Is w-hairpin RNAi increased in a fly bearing the adar mutation?

(4) The authors can use the genetic system to dissect the step in RNAi that is blocked by ADAR binding. If ADAR binds dsRNA then increasing the expression of Dicer-2 (construct available from Barry Dickson) should decrease the inhibition; conversely decreasing the dcr-2 dose should increase inhibition. If ADAR binds siRNAs, then increasing R2D2 should decrease inhibition, whereas decreasing the r2d2 dose should increase inhibition.

Minor Points

The w hairpin containing an intron was reported by Carthew and colleagues in Lee, Y. S., and Carthew, R. W. (2003; Making a better RNAi vector for Drosophila: use of intron spacers. Methods 30, 322-329), not Lee et al. (2004).

"residual luciferase units": if the authors mean the unit RLU (relative light unit), this is simply the

number given to you by the luminometer. As the authors report relative changes in light output (normalized to an internal control), the quantities they report are dimensionless and therefore aren't really units.

On page 18, at the bottom, why is it "surprising" that Drosha is unaffected by a G:U wobble several bases away from the cleavage site? Doesn't Drosha cope with such discontinuities in most pri-miRNA substrates normally?

What chromosome(s) and chromosome arms are the transgenic insertions on (page 24 at bottom).

1st Revision - Authors' Response 15 July 2009

Referee #1.

Minor comments

1. We have modified the text on page 9 and remove the statement 'previous work'. This was a mistake as the work was carried out in this study and not previously published. We now state 'This protein, expressed in and purified from the yeast *Pichia pastoris* binds long dsRNA in a filter-binding assay similarly to wild-type ADAR1 but does not deaminate long dsRNA (Supplemental Fig. S1).'
2. On page 10 we now refer to Supplementary Figure 2B when referring to the binding sites within the luciferase reporters for edited and unedited miRNAs.
   On page11 we have substituted the word 'strong' for 'highly specific'.
   We state 'This five-fold discrimination by the mature unedited microRNAs between the unedited and the edited target sites is highly specific, considering that the edited target sites in each case differ by only one nucleotide in the 22 base sequence.'
3. We now refer to the specific ADAR1 deaminase mutants in Figure 2 as (ADAR p150 E912A and ADAR p110 E912A) and the Z-DNA binding domains of ADAR1 (ADAR1 1-442).
4. We have included the data on the quantification of mature mir-376a2 (Figure 3). The reviewer is correct in that we also see a reduction in pre-miRNA when ADAR1 binds (Figure 3A and B). However this result is very variable whereas the reduction observed with ADAR2 is much stronger and highly reproducible. This can also be seen with the mutants, ADAR2 and ADAR2E/A give very similar results (Fig. 4) whereas there is variability between ADAR1 and the ADAR1 mutants. As we are not as confident of the affect on pre-miRNA processing by ADAR1 we do not want to emphasise it in the manuscript. However we have modified the manuscript in different places in the Discussion to reflect that there is an affect by ADAR1 an example is 'What is really surprising is that ADAR1 binds to and edits the +4 position in mir-376a2 to between 40-50% yet its affect on Drosha processing is not as strong as ADAR2 (Figure 3).'
5. We now describe Figure 4D in the text; 'Similar to pri-mir-376a2, expression of pri-mir-376a1 alone showed that the activity of pri-mir-376a1 was inhibited by ADAR2 E319A (Fig. 4C). In addition a northern analysis was performed to determine the processing of pri-mir-376a1 (Fig. 4D). It was affected by the binding of either active or inactive ADAR2 but not by ADAR1 (Fig. 4D).'
6. Only the *white* hairpin is highly edited by ADAR1p150 in the transgenic fly lines. We have included a figure for supplementary; (S5A) and mention it in the text.

Referee #2.

1. The reviewer suggested a very good experiment in that we should knockdown endogenous ADAR2 with siRNAs and look at the effect on processing of the endogenous mir376 cluster. We have put considerable effort into performing this experiment but are unable to

do it. The major problem is that there is no cell line that has high endogenous levels of ADAR2 and pri-mir-376a2. We analysed different neuronal cell lines but as you can see (Figure 1 for the Reviewer), the level of both ADAR2 and mir376 are very low in comparison to human brain as measured by qPCR. We also analysed editing of the pre-mir376a2 in the neuronal cell lines of different origins namely: SH-SY5Y, LA1-55n and NB69, in addition to PANC-1 and Hela cell lines. However the level of editing at the +4 site in pri-mir-376a2 is negligible, indicating a lack of functional activity of ADAR2 on the pri-miRNA. Our sample of human brain, on the other hand, has a high degree of editing with a G peak at the same intensity as the A peak (Our sample of human brain RNA is not from the cerebellum, thus we did not expect to have >90% editing as found in human cerebellum). Only in transfected 293T is editing at a similar level as is found in human cerebellum which is approximately 80%. In retrospect we realise that no cell line has ever been reported that has endogenous ADAR2 activity that is comparable to that found in the brain.

2. To verify that pri-miRNA leaks into the cytoplasm we have performed qPCR on RNA from transfected and non-transfected 293T (Supplementary Figure S3A). As can be seen in the figure the qPCR was performed with primers and their positions are indicated (Supplementary Figure S3 C, D). In addition endpoint PCR was performed to demonstrate the presence of pri-mir-376a2 in both cytoplasmic and nuclear fractions from 4 separate transfections.
3. We have performed a Northern Blot with a different probe as requested by the reviewer (Supplementary Figure S4). The new probe is complementary to the loop of pre-miR-376a2.
4. Figure 3C is not a Northern Blot, the transcript is internally labelled with α-^{32} P so therefore it is not possible to get a shorter exposure to see if there is accumulation of pri-miR-376a2.
5. We began to perform experiments in cell lines to find other miRNAs that were also affected in processing by ADARs. However when we realised how low ADAR activity was in every cell line we tested versus brain, we decided this was not a good experimental approach and abandoned it. We are in the process of obtaining the ADAR null mice from Professor Seeburg and in the future we plan to address this question in mice.

Minor points and typos

1. The numbering we used in Figure 1 is slightly different to Kawahara *et al*. Science 2007. The nomenclature we used is from miRBASE http://microrna.sanger.ac.uk/sequences/ and we mention this in the figure legend. The distance between the pri-miRNAs we obtained from UCSC genome browser. In Figure 1 we only indicate the adenosines that are highly edited within the miRNAs.
2. We have changed the text to pre-miR-376a2 (page 10).
3. We have changed the text to Supplementary Fig. S2B (page 10).
4. I am confused, as at the end of page 14 second paragraph we are discussing Figure 4A not 4B so we have not changed the text. I hope we have not misunderstood the reviewer .
5. We have changed the text from pre-miRNA to pri-miRNA to (page 18).

Referee #3

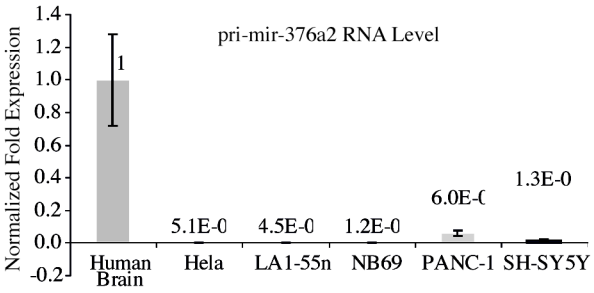
1. We now display the binding data on a semi-log plot as requested and fit to the Langmuir equation (Supplementary Figure S1) and reproduced the graphical analysis of stochiocomentry performed in (Batey, R. T., and Williamson, J. R. (1996). Interaction of the Bacillus stearothermophilus ribosomal protein S15 with 16 S rRNA: I. Defining the minimal RNA site. J Mol Biol 261, 536-549). We also state the Kd as 0.016 nM for ADAR1 p110 and 0.015 nM for ADAR1 G1007R. As the substrate we use has multiple binding site we did a stoichiometric titration on pre-miR376a2 that is shorter and has one site (+44) that is highly edited by ADAR1. However pre-miR376a2 is not suitable for filter binding as the maximum retention we achieved with ADAR1 or ADAR1 G1007R was 30%. It has previously been reported that some RNAs are not suitable for filter binding and give a maximum of 30% (K. B Hall and J. K Kranz, 1999 Nitrocellulose Filter Binding for Determination of Dissociation Constants. RNA-Protein

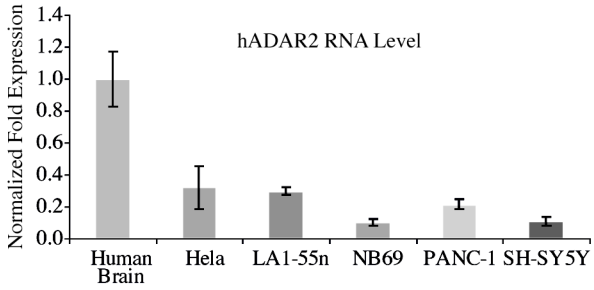
Interaction Protocols Edited by Susan Haynes). Our data suggests that ADAR1 and ADAR1 G1007R have similar binding affinities for RNA.

2. We absolutely agree with the reviewer that this cannot be done with human eyes and hence we determined the eye pigmentation by 2 different methods which gave us essentially the same result. First we beheaded 50 flies and ethanol extracted the pigment and measured the absorption at 480 nm as described by (Pal-Bhadra et al., 2004). As we were not satisfied with the numbers we obtained because the eye colours are in the low part of the measurable range, we then photographed both eyes from 10 different flies from each genotype and analysis were performed with in-house scripts written for IPLab Spectrum, in which the pixel intensity was measured in the red, blue and green spectrums. We have added a figure to the supplementary data (S5B) with the quantification we obtained from IPLab. We now have a new Figure 5 as we perform additional crosses with *Dicer-2* as requested and we photographed all the fly heads in one session with the same settings. We used a different imaging system for this and we give the details in Material and Methods. We were unable to re-quantify the eye color as we did not have enough flies of the appropriate classes.
3. W-hairpin RNAi is not increased in the *Adar* mutant flies and we now mention this on page 17.
4. ADAR1p150 has been shown to bind to both long dsRNA and to the Dicer 22-mers products so it is not easy to predict how ADAR1 antagonism would be affected by changing the dosage levels of *Dicer-2*, *R2D2*, or *Ago-2*. The only relevant experiment we could complete within the time available was to see the affect on hADAR1p150 antagonism by crossing it with *UAS-Dicer-2*. In the additional panel to Figure 5, we show that increasing DICER-2 enhance RNA interference due to *GMR>wIR* and decreases the antagonistic effects of ADAR1p150. This suggests that part of the effect of ADAR1p150 is due to the binding and editing of long dsRNA as it competes with DICER-2. We have added a paragraph with this result to the Results section.

Minor points

1. We have changed the reference as requested.
2. We have substituted ‘residual luciferase units’ for ‘Fold luciferase over mir376 alone’.
3. What we are endeavouring to convey that it is surprising that ADAR1, that is a larger protein than ADAR2, has very little affect on miRNA processing when it binds the +4 position that is close to the Drosha cleavage site. We have now modified this section as requested by reviewer 1.
4. The different insertion of UAS-ADARs and their chromosomal locations have been added to text in the legend of Fig. 5.





Sequence Chromatograms of endogenous pri-mir-376a2:
Editing only in Human Brain RNA