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miRNA dependent gene silencing involving Ago2 mediated cleavage of a circular antisense RNA

Thomas B. Hansen, Erik D. Wiklund, Jesper B. Bramsen, Sune B. Villadsen, Aaron L. Statham, Susan J. Clark and Jørgen Kjems

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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 08 April 2011

Thank you for submitting your manuscript for consideration by The EMBO Journal. It has been now been evaluated by three referees and I enclose their reports below. As you will see the referees find the description of the miRNA mediated regulation by targeting of circular antisense transcripts to be potentially interesting and important, however, they do require further experimental analysis to make the study suitable for The EMBO Journal. All three referees are in good agreement that while the study is interesting it requires further insight into the mechanism by which antisense transcript regulates CDR1 mRNA levels and to provide some evidence that this is biologically relevant mechanism. Several scenarios are outlined in the manuscript but are not tested. These two main issue need to be addressed and would suggest that you focus on these points, which are outlined in most detail by referee #1. Given the interest in the study, should you be able to address the concerns we would be happy to consider a revised version of the manuscript for publication in The EMBO Journal.

I should remind you that it is EMBO Journal policy to allow a single round of revision only and that, therefore, acceptance or rejection of the manuscript will depend on the completeness of your responses included in the next, final version of the manuscript. When you submit a revised version to the EMBO Journal, please make sure you upload a letter of response to the referees' comments. Please note that when preparing your letter of response to the referees' comments 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 initiative, please visit our website: http://www.nature.com/emboj/about/process.html

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

Yours sincerely,

Editor
The EMBO Journal

REFEREE COMMENTS

Referee #1

In "miRNA dependent gene silencing involving Ago2 mediated cleavage of a circular antisense RNA", Hansen et al. report a series of interesting observations pertaining post-transcriptional regulation of gene expression. Particularly novel is the finding that miR-671 targets a circular antisense transcript via Ago2-mediated cleavage. Decrease in antisense levels lead to a parallel decrease of the sense transcript (a phenomenon previously reported by Burd et al.) pointing to a potential unidirectional stabilization, from antisense to sense transcripts.

While novel in some aspects, this manuscript faces two problems:

1) The authors make no attempts to position their findings in a biological context.

2) At the same time, the report fails in dissecting the reported phenomena at the molecular level. As such, the current version of the manuscript is only descriptive. The authors should test some of the hypotheses they enumerate along the text (see below) and resubmit a more comprehensive analysis of this potentially interesting regulatory system.

Specific points to be addressed by the authors:

Please attempt a Northern blot to show variations in CDR1 antisense RNA levels upon miR-671 induction and anti-miR-671 treatment in Fig. 1.

In Supp. Fig. 1, levels of miR-671 and miR-769 are very modest in the context of over-expression. In addition, the signal for miR-15b is clearly higher in miR-671 expressing cells treated with Tetracycline. Please comment on this.

Targeting the antisense transcript with siRNAs leads to a decrease in the levels of the sense transcript, however the antisense is unaffected by a decrease in the sense transcript (Figs. 3B and 3C). I encourage the authors to discuss a couple of possible scenarios and test them experimentally. In principle, if RNA 1 stabilizes RNA 2 via duplex formation (?), RNA 2 should equally stabilize RNA 1. So, why is regulation unidirectional as reported? Has this anything to do with RNAs being circular or linear? Does polyadenylation (sense transcript) play any role? Do sense and antisense interact at all? I'm afraid the circular nature of the antisense transcript also deserves further attention in the manuscript.

After Fig. 4B (the antisense transcript is cleaved by miR-671), the authors seem to avoid those key issues outlined at the beginning of this review - to study the mechanism of stabilization of sense CDR1 by the antisense transcript and to find a biological meaning for this regulatory mechanism. Unfortunately, they rather concentrate on accessory and trivial experiments, such as the impact of over-expressing wild type and mutant Ago2 and mapping miRNA-directed cleavage sites. These experiments are unnecessary and should be either removed or moved into supplemental information.

At the Discussion, the authors outline potential scenarios to explain their findings, but do not attempt any further. In some cases they admit having no clues, in others they seem to postpone key experiments for future manuscripts.

Some examples:

271-277: ... with natural antisense transcripts shielding functional sequences, protecting unstable mRNAs or changing the fate of a particular mRNA.

289-290: stating that a link between cleavage of the antisense circular transcript and repression of the sense molecule is unclear.

299-300: regarding the effect of circularization towards sense regulation. In this respect, a circular antisense transcript has already been reported by Burd et al. Therefore a reader would expect Hansen et al. to go a bit deeper in their analysis.

325: the authors claim that the conservation between human and mouse suggests biological relevance - a general statement. However, they provide no experiments in this direction. What is the function of CDR1? In which conditions, miR-671 becomes important to down-regulate both AS and S transcript levels?

326: the authors propose that miRNA-dependent regulation of antisense transcripts is a "widespread gene regulatory mechanism". What are the evidences for such a claim? The authors rather stress the novelty of their finding.

Minor corrections that the authors should implement when resubmitting:

Indicate the precise 5' and 3' termini of both transcripts in Fig. 1A.
Please enlarge Supp. Fig. 4; it is not readable at the moment.
Please add (-) in the title as indicated: ... miRNA(-)dependent gene silencing involving Ago2(-)mediated cleavage...
Figures are referred as 1a, b, c, d in the text but labeled A, B, C, D. Please correct.
Line 52: ... that promoter(-)targeting siRNAs...
Line 94: remove "endogenous"
Lane 586: from instead of form.

Referee #2

This study investigates the potential regulatory role played by miR 671 in expression of the CDR1 mRNA. The authors found that one of the strands of the miRNA has extensive complementary to a non-coding antisense RNA that is transcribed through the NDR1 locus. They investigated what effects knocking down the antisense RNA had on CDR1 expression and they observed that knockdown of the antisense destabilized the CDR1 mRNA, suggesting a positive regulatory role for the antisense. Moreover, they provide evidence that the antisense RNA is circular due to a non-linear splicing reaction. Overall, the results are potentially interesting but some parts of this study are confusing and need clarification.

1. It is not clear from the results presented what role the "passenger" strand of miR671 has on CDR1. Since it appears from the figure that the target in the AS overlaps with the CDR1 sense, does the passenger strand mediate CDR1 downregulation? Perhaps the way to address this is to carry out RACE PCR on the CDR1 mRNA when the miRNA 671 is induced. The experiments with the si-siRNA suggest that down-regulating only the antisense destabilizes the sense, but this does not rule out a combined effect by the native miRNA in downregulating both S and AS RNAs.

2. The authors should use an siRNA that targets the AS intron to determine whether or not this prevents formation of the circular mature RNA and what impact this has on the CDR1 levels.

3. The authors provide some data to suggest that the antisense is circular but this is a very confusing part of this manuscript since it is not clear to this reviewer what event led to this. Does the circular RNA have a 2'-5' linkage? If so it can be linearized by a debranching enzyme.

4. Since the miRNA can putatively downregulate both the unspliced and spliced versions of the AS,

the evidence that it is the circular version of the transcript which functionally regulates the levels of CDR1 is speculative. Clearly the proposed regulatory mechanism of a circular antisense stabilizing a linear sense mRNA from destabilization is interesting, but the data do not support this model. In short, this study needs additional data to validate the model.

Referee #3

Review of "miRNA dependent gene silencing involving Ago2 mediated cleavage of a circular antisense RNA" by Jorgen Kjems, Thomas Hansen, Erik Wiklund, Jesper Bramsen, Sune Villadsen, Aaron Statham, and Susan Clark for publication in The EMBO Journal.

Summary: This is a very interesting article that should be published in EMBO. The authors document a circular natural antisense transcript (NAT) that is the target of a miRNA, miR-671. The suppression of this NAT results in the concomitant reduction in the sense mRNA counterpart. There are however several key points that need to be clarified prior to publication. Most importantly mechanism is missing and a few key experiments might clarify this issue.

Major point:

(1) First and foremost it is unclear whether miR-671 can also target the sense counterpart and thus when the NAT is removed does miR-671 then target the CDR1 sense, i.e. is the NAT working as a sponge. This is not clear from the current manuscript.

(2) It is unclear what the control miR-769 is targeting and why it was selected?

(3) The data for miR-769 +/- Tet is not shown? Is there an effect here that limits the findings? This should also be shown especially if this control will be used throughout the manuscript.

(4) It is unclear what the effect is when Tet is not added constitutively, i.e. does the effect persist? This would be indicative of either a transcriptional or post-transcriptional method of regulation.

(5) In figure 1d the authors depict activation of expression. Is this a run-on or only mRNA analysis? A run on would indicate a transcriptional vs. post-transcriptional effect. Suggest a run on here.

(6) In figure 2a the directional RT primer doesn't look right in the figure, please check that using this primer would result in a directional RT priming of the NAT.

(7) On page 7 the authors discuss NAT levels several orders of magnitude above CDR1 mRNA and cite figure 2b. It was not clear that this figure demonstrated the change that is described. Please check that this is or is not correct.

(8) The CHIP data presented in Figure S3a-3c. Is the no antibody control subtracted from these samples, i.e. what is the background control.

Minor points:

(1) On page 4, the introduction the authors go into great detail discussing TGS, etc. There is a discussion on the variation in DNA methylation. It does appear that DNA methylation occurs but target duration is a key aspect, refer to (1-3) and consider these papers.

(2) On page 4 the authors discuss TGS and RNA/RNA interactions. This is pretty well established in several other bodies of work, please refer to (4-6).

(3) Also on page 4 the authors discuss the various modes of NAT based regulation but leave out some of the most interesting insights involving NATs in epigenetic regulation of gene expression (7, 8) and reviewed in (9).

Literature cited:

1. Hawkins PG, Santoso S, Adams C, Anest V, Morris KV. Promoter targeted small RNAs induce long-term transcriptional gene silencing in human cells. Nucleic Acids Res. 2009;37(9):2984-95. PMCID: 2685082.
2. Suzuki K, T. Shijuuku, T. Fukamachi, J. Zaunders, G. Guillemin, D. Cooper, and A. Kelleher. Prolonged transcriptional silencing and CpG methylation induced by siRNAs targeted to the HIV-1 promoter region. Journal of RNAi and Gene Silencing. 2005;1(2):66-78.
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6. Ross JP, Suetake I, Tajima S, Molloy PL. Recombinant mammalian DNA methyltransferase activity on model transcriptional gene silencing short RNA-DNA heteroduplex substrates. Biochem J. 2010;432(2):323-32.
7. Morris KV, Santoso S, Turner AM, Pastori C, Hawkins PG. Bidirectional transcription directs both transcriptional gene activation and suppression in human cells. PLoS Genet. 2008;4(11):e1000258.
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9. Morris KV. Long antisense non-coding RNAs function to direct epigenetic complexes that regulate transcription in human cells. Epigenetics. 2009;4(5).

1st Revision - authors' response 06 July 2011

Referee #1

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2) At the same time, the report fails in dissecting the reported phenomena at the molecular level.
As such, the current version of the manuscript is only descriptive. The authors should test some of the hypotheses they enumerate along the text (see below) and resubmit a more comprehensive analysis of this potentially interesting regulatory system.

We believe that the regulation by CDR1 involves a number of novel biological concepts that by itself provide novel mechanistic information. With the additional data now provided we clearly substantiated the circular nature of the antisense and the miRNA targeting of it. The exact function of the CDR1 gene product is unfortunately unknown but we have now strengthened the impact of the story by showing that the circular antisense RNA is one of the highest expressed genes in human brain and that this observation can be extended to mice. These data are available as supplementary

fig. 9. The importance of the CDR1 protein is also suggested by the notion that this protein constitutes the target for the immune response observed in patients suffering from cerebellum degeneration. Hence our investigation may also be of clinical interest in the long-term.

Specific points to be addressed by the authors:

Please attempt a Northern blot to show variations in CDR1 antisense RNA levels upon miR-671 induction and anti-miR-671 treatment in Fig. 1.

We successfully performed northern blotting using both RNA from 671-induced and 671-inhibited cells and observed in accordance with qPCR data AS reduction and increase, respectively (Fig 3C and D).

In Supp. Fig. 1, levels of miR-671 and miR-769 are very modest in the context of over-expression. In addition, the signal for miR-15b is clearly higher in miR-671 expressing cells treated with Tetracycline. Please comment on this.

We agree with the reviewer in this point. In our hands, miR-671 is poorly processed/maturated or quickly turned over, resulting in low relative levels even with high pri-miRNA expression. Therefore, low mature miR-671 is reproducibly obtained even with transient overexpression. The miR-15b, serving as loading control, suggests a slightly uneven loading, however, we argue that this does not affect the conclusion that miR-671 levels are highly increased upon tet-induction. In addition, qPCR on miR-671 levels was also performed in the initial manuscript verifying close to background levels of miR-671 without tetracycline and more than 10-fold increase upon tet-induction (Supplementary Fig. 1B).

Targeting the antisense transcript with siRNAs leads to a decrease in the levels of the sense transcript, however the antisense is unaffected by a decrease in the sense transcript (Figs. 3B and 3C).

We do not claim a reciprocal relationship between sense antisense transcript. At the contrary – the antisense transcript is clearly enhancing the sense transcript expression but not vice versa. Rather, AS levels increase upon KD of CDR1 mRNA. This we validated by CDR1 mRNA expression, which accordingly resulted in AS reduction (fig 4F and G). We speculate that this is an example of a feedback regulatory network, where AS stimulates mRNA, whereas mRNA represses AS. This is now written more clearly on page 11.

I encourage the authors to discuss a couple of possible scenarios and test them experimentally. In principle, if RNA 1 stabilizes RNA 2 via duplex formation (?), RNA 2 should equally stabilize RNA 1. So, why is regulation unidirectional as reported?

CDR1 regulation is clearly very complicated and we admit that despite trying hard (eg by investigating the DNA and histone modifications as described in the text) we have not been able to pinpoint the exact mechanism explaining the observed relationship between CDR1 mRNA and AS, however, we strongly feel that a circular AS RNA being regulated by a miRNA (with clear mechanistic details shown) and an observed correlation with CDR1 mRNA is an important observation. We do not agree that stabilization is necessarily biconditional and one could easily envision that an intrinsically unstable structure could be stabilized by another structure without having an impact on the stability of the latter. It may for instance be of importance that the antisense contains introns whereas the CDR1 gene does not.

Has this anything to do with RNAs being circular or linear? Does polyadenylation (sense transcript) play any role? Do sense and antisense interact at all?

We are able to manipulate CDR1 mRNA levels by overexpression of an AS mimic; however, whether the two RNAs interact in vivo is difficult to show experimentally. Due to the sense/antisense nature of the transcripts, we propose that direct interaction is most likely occurring but this is intrinsically

difficult to prove experimentally. Validating RNA interactions in vitro is limited by obvious technical artifacts and to our knowledge sense/antisense interactions within cells have so far not been shown convincingly. Regarding poly-adenylation of CDR1 mRNA, we obtained preliminary data suggesting that the CDR1 mRNA is in fact not polyadenylated. However, this observation was not affected by miR-671 overexpression, and as such miRNA nor AS levels seem not to play a role here. We are currently taking a more thorough look at the CDR1 3' end formation, and would prefer to keep these observations out of an already complicated story.

I'm afraid the circular nature of the antisense transcript also deserves further attention in the manuscript.

We have implemented new data to support this notion. Conducting northern blot analysis of CDR1 AS reveals, in accordance with EST sequences, that the circular RNA is a distinct species, even in brain where expression-levels are very high (Fig 2E). As an additional experiment we have now performed knockdown of the circular RNA by specifically targeting the site of circularization (Supplementary Fig. 6). This combined with circular specific PCR, TAP/EXO treatment, gel-trapping and the distinct and expected migration in agarose northern, we believe that we have proven beyond any reasonable doubt that the AS RNA indeed is circular. Furthermore, the northern blots demonstrate that the circular RNA is the predominant species and in our hands the only detectable antisense transcript.

After Fig. 4B (the antisense transcript is cleaved by miR-671), the authors seem to avoid those key issues outlined at the beginning of this review – to study the mechanism of stabilization of sense CDR1 by the antisense transcript and to find a biological meaning for this regulatory mechanism.
Unfortunately, they rather concentrate on accessory and trivial experiments, such as the impact of over-expressing wild type and mutant Ago2 and mapping miRNA-directed cleavage sites. These experiments are unnecessary and should be either removed or moved into supplemental information.

We firmly believe that AGO2-mediated cleavage of the target AS RNA is of important relevance, and certainly not a trivial observation. Only a few instances of slicer activity have been shown in mammals and this is the first instance of antisense RNA being targeted and cleaved by AGO2. Furthermore, it explains the mechanism by which miR-671 downregulates the circular RNA; the primary focus point in this manuscript. Therefore, we prefer to keep the data in fig 4 (now fig 5).

At the Discussion, the authors outline potential scenarios to explain their findings, but do not attempt any further. In some cases they admit having no clues, in others they seem to postpone key experiments for future manuscripts.

Some examples:

271-277: ... with natural antisense transcripts shielding functional sequences, protecting unstable mRNAs or changing the fate of a particular mRNA.

This is based on previous speculation in the literature, and we thus speculate that a similar scenario could be the case for CDR1.

289-290: stating that a link between cleavage of the antisense circular transcript and repression of the sense molecule is unclear.

As noted above, we have yet to reveal the mechanistic relationship between antisense and sense. As described already in the paper, we have tested some of the most obvious explanations including histone and DNA modifications which proved negative, and based on the effects of transient AS

overexpression we believe it to be a post-transcriptional mechanism. Accordingly, cleaving the CDR1 AS would lead directly to CDR1 mRNA decrease.

299-300: regarding the effect of circularization towards sense regulation. In this respect, a circular antisense transcript has already been reported by Burd et al. Therefore a reader would expect Hansen et al. to go a bit deeper in their analysis.

In contrast to Burd et al, we characterize a regulation of the circular RNA by natural RNAi mediated mechanism. Also, CDR1 AS is exclusively circular and highly expressed whereas the circular RNA in Burd et al is a low-abundance species compared to its linear cousin. Moreover, the circular RNA reported by Burd et al appears to be a by-product of alternative splicing and thus emanate from re-splicing of a lariat structure, which does not seem to be the case for the circular CDR1 AS RNA.

325: the authors claim that the conservation between human and mouse suggests biological relevance - a general statement. However, they provide no experiments in this direction. What is the function of CDR1? In which conditions, miR-671 becomes important to down-regulate both AS and S transcript levels?

The function of CDR1 remains elusive and to resolve its biological function as well as the biological relevance of regulating CDR1 mRNA by antisense RNA and miRNA is not within the scope of this manuscript. We only aim to underline possibility of biological relevance based on the fact that important sequence motifs are evolutionary conserved. Furthermore, in the revision we have shown that the CDR1 antisense is expressed at high levels in human and mouse brain tissue further strengthening the case for biological relevance.

326: the authors propose that miRNA-dependent regulation of antisense transcripts is a "widespread gene regulatory mechanism". What are the evidences for such a claim? The authors rather stress the novelty of their finding.

We agree, this proposal is not substantiated by any evidence besides CDR1 and we consequently removed this suggestion.

Minor corrections that the authors should implement when resubmitting:

Indicate the precise 5' and 3' termini of both transcripts in Fig. 1A.
Please enlarge Supp. Fig. 4; it is not readable at the moment.
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Line 94: remove "endogenous"
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All minor points have now been implemented as suggested.

Referee #2

This study investigates the potential regulatory role played by miR 671 in expression of the CDR1 mRNA. The authors found that one of the strands of the miRNA has extensive complementary to a non-coding antisense RNA that is

transcribed through the NDR1 locus. They investigated what effects knocking down the antisense RNA had on CDR1 expression and they observed that knockdown of the antisense destabilized the CDR1 mRNA, suggesting a positive regulatory role for the antisense. Moreover, they provide evidence that the antisense RNA is circular due to a non-linear splicing reaction. Overall, the results are potentially interesting but some parts of this study are confusing and need clarification.

1. It is not clear from the results presented what role the "passenger" strand of miR671 has on CDR1. Since it appears from the figure that the target in the AS overlaps with the CDR1 sense, does the passenger strand mediate CDR1 downregulation? Perhaps the way to address this is to carry out RACE PCR on the CDR1 mRNA when the miRNA 671 is induced. The experiments with the si-siRNA suggest that down-regulating only the antisense destabilizes the sense, but this does not rule out a combined effect by the native miRNA in downregulating both S and AS RNAs.

We agree with the referee on this issue. We initially discarded this idea based on the fact that the effect on CDR1 is reproduced using sisiRNA targeting the antisense; however that necessarily does not rule out that miR-671-3p could be functional to some extent. To further strengthen that miR-671-3p is not targeting CDR1 mRNA, we designed a sisi-671-3p and as shown in supplementary fig 6, no changes in CDR1 mRNA or AS levels were observed. Therefore, as expected, miR-671-3p does not seem to regulate the CDR1 locus.

2. The authors should use an siRNA that targets the AS intron to determine whether or not this prevents formation of the circular mature RNA and what impact this has on the CDR1 levels.

To address this issue we have now designed a sisiRNA targeting the canonical AS intron II (sisiUnsliced AS; Supplementary Fig. 6) Here, we observed an effect similar to other AS targeting sisRNAs. This, we argue, reflects the fact that the unspliced circular AS is the predominant endogenous species (2.7 fold higher than spliced based on northern quantification), and that the functional AS is not solely restricted to the spliced circular isoform (see also our response to point 4 below).

3. The authors provide some data to suggest that the antisense is circular but this is a very confusing part of this manuscript since it is not clear to this reviewer what event led to this. Does the circular RNA have a 2'-5' linkage? If so it can be linearized by a debranching enzyme.

The circular RNA is not expected the have 2'-5' linkage, being that the circle is exonic and not derived from an intronic lariat structure. Circle formation is expected to follow canonical splicing path except that SD and SA sites are positioned in a reversed order. We have now added a sentence at line 143 in the result clearly stating this to avoid any confusion.

4. Since the miRNA can putatively downregulate both the unspliced and spliced versions of the AS, the evidence that it is the circular version of the transcript which functionally regulates the levels of CDR1 is speculative. Clearly the proposed regulatory mechanism of a circular antisense stabilizing a linear sense mRNA from destabilization is interesting, but the data do not support this model. In short, this study needs additional data to validate the model.

Unfortunately, this point may rely on a misunderstanding and we apologize for not drawing the distinction between unspliced and linear RNA more explicitly in the manuscript. All the antisense

RNA is circular. We have not been able to detect any linear species. Regarding the unspliced AS, this only refers to a typical, canonical intron (intron II, cf. supplementary fig 3) within the circle and therefore not the splicing event giving rise to the circle. Thus, it can only be the circular AS affecting CDR1 mRNA being that there is no detectable alternative. However, we performed an experiment where we knockdown the intron II-retained circular antisense specifically (supplementary fig 6) with potent regulatory effects.

Referee #3

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Major point:

(1) First and foremost it is unclear whether miR-671 can also target the sense counterpart and thus when the NAT is removed does miR-671 then target the CDR1 sense, i.e. is the NAT working as a sponge. This is not clear from the current manuscript.

We agree that this could be a potential mechanism, however unlikely since we have not been able to identify any miR-671-5p targetsites within the CDR1 mRNA sequence. To address this issue we have now taken a sisiRNA approach where different regions within the antisense RNA was targeted (Supplementary Fig. 6). Here we see a similar effect on CDR1 mRNA, and we would argue that it is very unlikely that the antisense by chance could function as a sponge for several independent sequences.

(2) It is unclear what the control miR-769 is targeting and why it was selected?

miR-769 was initially chosen due to identified promoter-proximal target site candidates in other genes. However, no effects were observed upon miR-769 induction. Since a stable cell line expressing miR-769 had already been established, it conveniently served as a control in this study.

(3) The data for miR-769 ± Tet is not shown? Is there an effect here that limits the findings? This should also be shown especially if this control will be used throughout the manuscript.

Actually, in supplementary fig 2b and 2c, we do show the array data for miR-769 induced cells as well as qPCR without any notable effect on CDR1 levels.

(4) It is unclear what the effect is when Tet is not added constitutively, i.e. does the effect persist? This would be indicative of either a transcriptional or post-transcriptional method of regulation.

This is an interesting point and we have performed several tet-removal experiments to address the question. We observed little or no restoration of AS or CDR1 mRNA levels after 3-days off-tet. However, considering the effectiveness of tet-removal and the stability of mature miRNA, we decided to employ an anti-miR approach after tet-induction. We observe that AS and CDR1 levels increased after 48 hrs anti-miR treatment, confirming a non-epigenetic, post-transcriptional effect. We have now added these results in a new supplementary fig 5.

(5) In figure 1d the authors depict activation of expression. Is this a run-on or only mRNA analysis? A run on would indicate a transcriptional vs. post-transcriptional effect. Suggest a run on here.

Fig 1d depicts qRT-PCR analysis. Nuclear run-on is an excellent idea, which we have repeatedly attempted, but in our hands CDR1 mRNA expression in HEK293 cells is simply below detection, and we are thus unable to comply with this request.

(6) In figure 2a the directional RT primer doesn't look right in the figure, please check that using this primer would result in a directional RT priming of the NAT.

We are only using directional priming when quantifying mRNA levels. In case of AS we deploy the fact that it is circular which allows specific PCR using 'outward facing' primers (also depicted in Fig 2A)

(7) On page 7 the authors discuss NAT levels several orders of magnitude above CDR1 mRNA and cite figure 2b. It was not clear that this figure demonstrated the change that is described. Please check that this is or is not correct.

Fig 3B visualizes the steady state levels of CDR1 mRNA as well as CDR1 AS upon miR-671 expression. Axis values to the left denote relative mRNA levels whereas the right axis reflects AS levels. The figure legend has been edited to make this clearer.

(8) The CHIP data presented in Figure S3a-3c. Is the no antibody control subtracted from these samples, i.e. what is the background control.

The no antibody control was performed and was several orders below the obtained signal. Therefore, this insignificant background was ignored. (This is now Supplementary Fig 4)

Minor points:

(1) On page 4, the introduction the authors go into great detail discussing TGS, etc. There is a discussion on the variation in DNA methylation. It does appear that DNA methylation occurs but target duration is a key aspect, refer to (1-3) and consider these papers.

We now refer to the suggested paper 1 and 2 obviously missing in the initial manuscript. TGS is primarily discussed because this was what we expected to find, but so far nothing indicates that any change in DNA methylation occurs even with persistent miRNA expression for up to 40 days.

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(3) Also on page 4 the authors discuss the various modes of NAT based

regulation but leave out some of the most interesting insights involving NATs in epigenetic regulation of gene expression (7, 8) and reviewed in (9).

The relevant references have been included.

Literature cited:

1. Hawkins PG, Santoso S, Adams C, Anest V, Morris KV. Promoter targeted small RNAs induce long-term transcriptional gene silencing in human cells. Nucleic Acids Res. 2009;37(9):2984-95. PMCID: 2685082.
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4. Han J, Kim D, Morris KV. Promoter-associated RNA is required for RNA-directed transcriptional gene silencing in human cells. Proc Natl Acad Sci U S A. 2007;104(30):12422-7.
5. Napoli S, Pastori C, Magistri M, Carbone GM, Catapano CV. Promoter-specific transcriptional interference and c-myc gene silencing by siRNAs in human cells. EMBO J. 2009.{{933 Hawkins,P.G. 2009; 787 Suzuki,K. 2005}}
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2nd Editorial Decision 03 August 2011

Thank you for submitting your revised manuscript for consideration by the EMBO Journal. It has now been seen by the three original referees whose comments are enclosed. As you will see, the referees find that the manuscript has been strengthened and there are several issues that still need to be addressed, especially by referee #2 who would like an alternative scenario regarding the role of the antisense transcript be looked into.

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://www.nature.com/emboj/about/process.html

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

Yours sincerely,

Editor

The EMBO Journal

REFEREE COMMENTS

Referee #1

Second review of the manuscript by Hansen et al.

The authors have addressed most of the comments and criticisms raised by the reviewers. I appreciate their honesty in recognizing that the manuscript runs short in dissecting the molecular mechanism behind the CDR1 antisense-sense interplay. This is, at least in my opinion, the nº1 message of the paper, closely followed by the finding of a miRNA that targets a circular antisense transcript.

I would like to encourage the authors to quickly fix some errors and pay attention to the comments below to further improve their manuscript.

Main comments towards the author's response:
The authors claim that levels of miR-671 are highly increased upon Tet addition in Figure S1. However, miR-671 is hardly visible and, if normalized against miR-15, lanes + and - Tet would look pretty similar.

In my first review, when commenting about the content of the manuscript after Fig. 4B, I stated that the reader wouldn't be interested in Ago2 cleaving or not, but rather in the physical and/or physiological interaction between sense and antisense CDR1 RNA. Actually the authors make this claim at the end of the Abstract. Keeping with Figure 4B, I wonder why sisiRNAs are more efficient towards AS than Sense, being the AS much more abundant. Couldn't the authors design a more efficient siRNA or sisiRNA?

Obviously the manuscript suffers from studying very lowly expressed molecules, such as miR-671 and CDR1 mRNA. For example, in line 299, the authors mention that DCR1 mRNA was readily observed in human brain tissue, but the Northern blot shows a shadow. I'm afraid the authors somehow over-emphasize their claims. In this context, and in the presence of a pair of molecules that seem to relate to each other (directly or indirectly) but one of them is ~3 orders of magnitude more abundant than the other, I would ask the authors to at least hypothesize on a suitable mechanism.

Minor corrections:

In the Abstract, I suggest removing the sentence in line 23: Characterization... and add "circular" in line 22: ...directs cleavage of an antisense, circular transcript...

Line 75: inclusion(Beltran) into inclusion (Beltran).
Line 187: do the authors mean (Fig. 2B) or Fig. 3B? Reviewer 3 showed the same concern in (7).
Line 201: I would turn "suggests" into "suggesting".
Line 295: lanes 1, 11 and 20 or 1, 12 and 19?
Line 303: I'm afraid TBP has not been defined.

Referee #2

This revised version of the manuscript "miRNA-dependent gene silencing involving Ago2-mediated cleavage of a circular antisense RNA" describes what looks to be a novel regulatory mechanism for post-transcriptional controlling of the expression of the CDR1 mRNA. The authors have done an adquate job in responding to the previous reviews and for the most part the new experimental data adds further support to the proposed mechanism. There is however one last concern that must be

addressed either in the manuscript or in a rebuttal. This addresses the mechanism by which the circular antisense stabilizes the CDR1 mRNA. Although the authors stronly support a model in which the two RNAs interact directly, there is little experimental support for such a model. However, another possibility is that the antisense circular RNA is acting as a miRNA sponge and titrating other miRNAs that target CDR1 leading to its destablization. To this end, there are no analyses of the putative miRNA binding sites for other than 671 in either the CDR1 mRNA or the antisense. This can be carried out using bioinformatic analyses to find potential sites which exist in both transcripts. The circular molecule may be protected from miRNA mediated destabilization as opposed to the linear sense transcript. Thus, this reviewer strongly recommends that this potential mechanism of action by the antisense be thoroughly addressed.

Referee #3

I am satisfied with the responses and the added data. The authors should be commended on a tough body of work that is going to prove paradigm shifting.

2nd Revision - authors' response 19 August 2011

Referee #1

Second review of the manuscript by Hansen et al.

The authors have addressed most of the comments and criticisms raised by the reviewers. I appreciate their honesty in recognizing that the manuscript runs short in dissecting the molecular mechanism behind the CDR1 antisense-sense interplay. This is, at least in my opinion, the nº 1 message of the paper, closely followed by the finding of a miRNA that targets a circular antisense transcript. I would like to encourage the authors to quickly fix some errors and pay attention to the comments below to further improve their manuscript.

Main comments towards the author's response:

The authors claim that levels of miR-671 are highly increased upon Tet addition in Figure S1. However, miR-671 is hardly visible and, if normalized against miR-15, lanes + and - Tet would look pretty similar.

We quantified the band intensities in fig S1A. This validates an approximate 4-fold enrichment of miR-671 levels normalized to miR-15 in agreement with qPCR data in fig S1B.

In my first review, when commenting about the content of the manuscript after Fig. 4B, I stated that the reader wouldn't be interested in Ago2 cleaving or not, but rather in the physical and/or physiological interaction between sense and antisense CDR1 RNA. Actually the authors make this claim at the end of the Abstract. Keeping with Figure 4B, I wonder why sisiRNAs are more efficient towards AS than Sense, being the AS much more abundant. Couldn't the authors design a more efficient siRNA or sisiRNA?

We are indeed interested in both the physiological interaction between mRNA and antisense, and in the interaction between miRNA and antisense (also stated in the abstract) and we are fascinated by miRNA-mediated endo-cleavage of circular RNA, which we propose could serve a more general and widespread phenomenon.

Regarding sisiRNA efficiency, we suggest that the sisiRNA effect relies on many known and unknown parameters and therefore the effectiveness varies quite notably between different designs. In this particular scenario, we speculate that the abundant antisense could also be blocking the accessibility of the mRNA-specific sisiRNA, whereas the antisense is more readily exposed to sisiRNA-mediated repression by outnumbering mRNA levels.

Obviously the manuscript suffers from studying very lowly expressed molecules, such as miR-671 and CDR1 mRNA. For example, in line 299, the authors mention that DCR1 mRNA was readily observed in human brain tissue, but the Northern blot shows a shadow. I'm afraid the authors somehow over-emphasize their claims. In this context, and in the presence of a pair of molecules that seem to relate to each other (directly or indirectly) but one of them is ~3 orders of magnitude more abundant than the other, I would ask the authors to at least hypothesize on a suitable mechanism.

We agree that CDR1 is not highly expressed, however it is detectable on northern. We have corrected the manuscript accordingly to avoid over-emphasizing the mRNA expression level. In the discussion, we favor a simple model in which a direct interaction between mRNA and antisense RNA confers stability upon mRNA that otherwise alone may be intrinsically unstable. See also response to reviewer#2 below.

Minor corrections:

In the Abstract, I suggest removing the sentence in line 23:
Characterization... and add "circular" in line 22: ...directs cleavage of an antisense, circular transcript...

The abstract has been changed to accommodate the above suggestion

Line 75: inclusion(Beltran) into inclusion (Beltran).
Line 187: do the authors mean (Fig. 2B) or Fig. 3B? Reviewer 3 showed the same concern in (7).
Line 201: I would turn "suggests" into "suggesting".
Line 295: lanes 1, 11 and 20 or 1, 12 and 19?
Line 303: I'm afraid TBP has not been defined.

All minor points have been implemented.

Referee #2

This revised version of the manuscript "miRNA-dependent gene silencing involving Ago2-mediated cleavage of a circular antisense RNA" describes what looks to be a novel regulatory mechanism for post-transcriptional controlling of the expression of the CDR1 mRNA. The authors have done an adquate job in responding to the previous reviews and for the most part the new experimental data adds further support to the proposed mechanism. There is however one last concern that must be addressed either in the manuscript or in a rebuttal. This addresses the mechanism by which the circular antisense stabilizes the CDR1 mRNA. Although the authors stronly support a model in which the two RNAs interact directly, there is little experimental support for such a model. However, another possibility is that the antisense circular RNA is acting as a miRNA sponge and titrating other miRNAs that target CDR1 leading to its destablization. To this end, there are no analyses of the putative miRNA binding sites for other than 671 in either the CDR1 mRNA or the antisense. This can be carried out using bioinformatic analyses to find potential sites which exist in both transcripts. The circular molecule may be protected from miRNA mediated destabilization as opposed to the linear sense transcript. Thus, this reviewer strongly recommends that this potential mechanism of action by the antisense be thoroughly addressed.

We are very pleased that the reviewer appreciate our response and agree that at this point the current mechanism responsible for CDR1 mRNA and antisense correlation is somewhat speculative. We now discuss the possibility of a sponge model on page 16: "However, the mechanistic link between miR-671 cleavage of the circular NAT and repression of the sense mRNA is unclear. One hypothesis could be that the abundance of CDR1 NAS antisense may titrate a miRNA from acting on the CDR1 mRNA, that is a sponge-model (Poliseno et al, 2010). However, a search for single 7mer putative target seeds sequences, shared between CDR1 3'UTR and the antisense, was negative (based on miRBase ver 17) suggesting that CDR1 NAS antisense is not acting as a decoy".

Referee #3

I am satisfied with the responses and the added data. The authors should be commended on a tough body of work that is going to prove paradigm shifting.

Thank you.

