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# miRISC and the CCR4-NOT complex silence mRNA targets independently of 43S ribosomal scanning

Duygu Kuzuoğlu-Öztürk, Dipankar Bhandari, Eric Huntzinger, Maria Fauser, Sigrun Helms and Elisa Izaurralde

*Corresponding author: Elisa Izaurralde, Max Planck Institute for Developmental Biology*

**Review timeline:**

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| Submission date: | 21 August 2015 |
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(Editor Anne Nielsen)

## Transaction Report:

1st Editorial Decision 06 October 2015

Thank you for submitting your manuscript for consideration by The EMBO Journal and my apologies for the extended duration of the review process in this case. Your manuscript has now been seen by three referees whose comments are shown below.

As you will see, the referees all express interest in the findings reported in your manuscript, although ref #1 does raise a number of concerns about the conclusiveness of this work over previous reports reaching different conclusions. In addition, ref #2 encourages the inclusion of additional data to further dissect the role for DDX6 in mediating translational repression.

In light of the slight discrepancy in the ratings and recommendations from the referees, I conducted an additional round of cross-ref commenting and received further input from ref#3. This person was less concerned about the discrepancy with earlier findings and instead commended the authors of this study for their rigorous approach to testing and characterizing the constructs used as well as to the use of multiple systems to validate the findings. In addition, ref #3 found that while the role for DDX6 in translational repression is an intriguing question it would be out of the scope of the current work.

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

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For the revised manuscript I would particularly ask you to focus your efforts on the following points:
-> Please clarify the points raised by ref #1 with regard to conclusiveness over previous work.
-> If possible, please include/discuss further data that could shed light on the role for DDX6 in mediating translational repression.
-> Please provide the RACE data requested by ref#3 and discuss the consequences for conclusions derived from the TISU-reporters.

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

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Referee #1:

Despite recent progress, the mechanisms by which miRNAs function are still poorly understood at the molecular level. A particularly contentious issue has been the involvement of eIF4A and its paralog eIF4A2. In previous work (Meijer et al., Science 2013), it was suggested that eIF4A2 but not eIF4A was required to mediate repression by miRNAs. Consistent with a function of eIF4As as helicases that unwind 5' UTRs to promote ribosomal scanning, silencing required long, structured 5'UTRs, a notion also supported by in vitro work (Ricci et al., NAR 2013). Two other publications (Fukao et al. Mol Cell 2014, Fukuya et al., Mol Cell 2014) reported that miRNAs act to displace eIF4A from mRNAs as a means to inhibit their repression, with eIF4A and eIF4A2 being interchangeable. Moreover, Fukuya et al., reported that in flies GW182 and Ago1 acted through different molecular mechanisms and, at least in part, independently of one another. This finding contrasts with the prevailing hierarchical model that AGO recruits GW182/TNRC6 recruits CCR4-NOT complex recruits DDX6 helicase to achieve (translational) silencing through an unknown mechanism. Finally, the recent generation of eIF4A2 knock-out cell lines revealed no defect in miRNA-mediated silencing (Galicia-Vazquez et al., RNA 2015).

Some clean-up work for the field seems clearly in order. Izaurralde and colleagues do just that through a large set of experiments that uses mostly tethering assays to understand the hierarchy of miRNA pathway factors and the requirement for long/structured 5'UTRs. The data provide evidence for the hierarchical model with a unified function of AGO, GW182, CCR4-NOT and DDX6 in both human and fly cells, and for repression irrespective of 5'UTR length (arguing against eIF4A as the sole/major target of repression). These findings are important and publishable (some points to be addresses are listed below). However, as things stand, the current discrepancies in the field suggest that experimental set-ups strongly affect outcomes. Indeed, one general frustration in this field is that different labs cannot reproduce one anothers' results, and that little exploration has gone into why. An example of this is also seen here, where Izaurralde and colleagues use a reporter with a CAA-unstructured 5'UTR, previously reported to be eIF4A-independent, but shown here to be eIF4A-dependent. What are the differences between this and the previous experiment? What reason is there to trust this rather than the earlier publication? I wish the authors would do more to convince me that we are not merely replacing arbitrarily one set of potentially nonphysiological conditions with another.

Taken together, this is a timely piece of work that is generally well done experimentally. Its publication in EMBO could be considered although personally, for the reasons listed above, I am not overly enthusiastic about it.

Major points
1. Why is the IP efficiency so poor in the experiments? For instance, in Fig. 1A, only 1/20 of the HA-tagged Ago protein appears to be pulled down (signal of 20% immunoprecipitate equals that of 1% input). This might be expected of a co-immunoprecipitating protein, but is very unusual for an IP "bait" - can the authors be sure that this minor fraction is representative of the bulk with regard to GW182 interaction? In the same vein, these numbers are obviously important but missing for Fig. 4G and 6G.

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2. What is the subcellular localization of the MALAT1-based reporter? MALAT1 itself is nuclear, and this localization seems to be in part driven by the 3' end (Miyagawa et al., RNA 2012), which also appears to be included in the reporter used here. If a substantial fraction of this RNA is nuclear, it will not be translated and also resist miRNA activity - inferences on miRNA activity would thus be misleading. (For instance, protein will only be produced from the cytoplasmic faction; if this mRNA fraction is substantially degraded but the majority of the reporter is nuclear, an appearance of translational repression will result.) A substantial nuclear pool would also explain the reduced translational efficiency of the MALAT1-based reporters (Fig. S4) relative to their "poly(A)" counterparts.

3. Fig. 4A: treatment with the eFI4A inhibitor silvestrol suggests that all reporters require at least some level of eIF4A activity. Does this not invalidate the assumption that the different reporters can be used to test miRNA activity in a situation where eIF4A is not required?

4. Fig. 4G, H: immunoprecipitation of eIF4A2 is performed to test whether its binding to a (stable) target reporter changes upon the target's repression by miRNAs, and the answer is no. However, I wonder about the reliability of this assay - the same amounts of eIF4A2 co-immunoprecipitate very different levels of even control mRNA (e.g., compare lane 10 to 9 or 11 in Fig. 4G). It seems even control RNA levels bound can easily vary by two-fold, suggesting that the complexes are not very stable in the first place. With details on the IP efficiency missing (% loaded, see point 1 above), it becomes even harder to judge the suitability of this assay.

5. Fig. 6G-L: Whereas the authors conclude (p. 16, bottom) that the NOT1 mutant that they use does not interact with DDX6, Fig. 6G indicates a reduction, not a complete loss of interaction. This does not affect the conclusion that DDX6 interaction seems required for translational miRNA target reporter repression. However, it provides an alternative interpretation for the continued reporter destabilization, namely that low amounts of DDX6 recruitment suffice for destabilization, not translational repression.

6. Fig. S4J: Are the lanes from one autoradiograph, same exposure, or multiple ones? It would generally help if the authors could indicate for all the figures which autoradiographs are continuous, spliced, or mixed and matched, this is not always evident (e.g., for 5F I assume it is one single, unspliced autoradiograph?!)

7. P.17, second paragraph: the authors refer to Fig. S3D, E for evidence that Me31B tethering can suppress mRNAs independent of 5' UTR length - I can find no data on this in that figure.

8. Galicia-Vazquez et al., RNA 2015, recently showed that eIF4A2 is dispensable for miRNA-mediated gene silencing - this should be mentioned in the introduction rather than as a confirmatory finding, in passing, in the discussion.

Minor points

1. Fig. 2K, L: (Partial) rescue of Ago1 knockdown with Ago1 pocket mutants: It would be useful to test the abundance of the transgenic relative to the endogenous proteins. Are they partially active at endogenous-like levels or when overexpressed?

2. Fig. 2K: Neither the main text nor the figure legend mention if the miRNAs (miR-1 and miR9b, respectively) are included in the transfection mix.

3. Although mutant DCP2 impairs reporter degradation, this effect appears rather partial (Fig. 2N). This might be a consequence of the specific mutant, but it its equally possible that deadenylation- and decapping-dependent degradation is only a minor pathway that the proteins activate. The authors may wish to qualify their conclusions accordingly.

4. Fig. 6E-F: a) I do not understand how the TISU-based reporter mRNA levels were quantified, the figure shows a rather extended smear?! b) It is not clear what "8xlet7 -or +" indicates: are they comparing reporters with/without let-7 binding sites, or with mutated let-7sites? The figure legend says "R-Luc-let-7 reporters or the corresponding reporters carrying mutations in the let-7 binding sites". However, in Fig.S6I-J, this reporter is called "8xlet7mut". Consistency needed.

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5. Fig. S2I, J: blots are lacking loading controls - needed if to make the point that tethered protein levels are not changing. Asterisk in J is not explained.

6. P.8, "Hs AGO2 repressed the translation of this reporter without causing mRNA degradation at the concentration tested (Fig 2H-J)". Cannot find the concentration anywhere.

7. P. 16, l. 3 - typo, "hypuristanol"

8. P.22, ll.6-7: "Therefore, it is possible that in addition to DDX6, other factors are involved in coupling deadenylation to decapping" - what is the evidence that DDX6 has any role in this in the first place? As the authors mention previously, the available evidence chiefly support a role of DDX6 in translational repression.

Additional suggestions

The authors have nice and comprehensive data, they might try to find a better way of presenting them to do them justice. For instance:

1. The results section starts out with a full figure on validation of the importance of the tryptophane-binding pockets on Ago for interaction with GW182/TNRC6. This is an important validation, but really background information and could easily go into the supplements.

2. It is not clear how the DDX6 experiments relate to the main body of the text. I would suggest incorporating the conclusions on DDX6 involvement in scanning-independent silencing in the main tethering experiment (AGOs, CCR4/NOT and GWs).

3. More generally, the use of cells from two different species and numerous reporters makes it sometimes very difficult for the reader to penetrate the figures. Why not try to find some color/pattern code to present the data, e.g., present results for human versus Drosophila cell lines in different colors so that it is obvious at a glance what is presented. Hatched vs. crosshatched vs shaded for different reporter types?

Referee #2:

The miRNA induced silencing complex (AGO + GW182) post-transcriptionally silences gene expression by inhibiting mRNA translation and subsequently initiating mRNA deadenylation, decapping and decay. miRNA-mediated deadenylation, via recruitment of the CCR4-NOT complex by GW182, is relatively well understood. Interestingly, the CCR4-NOT complex has also been linked to deadenylation-independent translation repression. How the CCR4-NOT and its interacting partner DDX6 inhibit mRNA translation is still unclear. Several groups have recently suggested that this translational inhibition may be the result of targeting the translation initiation factor eIF4A, and interfering with ribosomal subunit scanning and translation initiation (Meijer et al., 2013; Fukaya et al., 2014; Fukao et al., 2014). Here, Kuzuoğlu-Öztürk et al. submit a manuscript tests this model. Based on their data, they conclude that miRNA-mediated translational repression does not target scanning ribosomal subunits, nor does it target eIF4A.
This paper does not provide any major new insight into how miRNAs repress translation (many groups have now reported that miRNA silencing requires AGO, GW182, CCR4-NOT and DDX6). However, it is an important body of work because it convincingly demonstrates that miRNA-mediated translational repression through DDX6 does not target eIF4A or ribosomal scanning and is not impacted by mRNA 5'UTR secondary structure. Ultimately, what would improve this paper is if the authors determined how DDX6 is repressing translation initiation. Multiple proteins can directly bind DDX6, including EDC3, LSM14, PATL1 and 4E-T. The authors could knock down these factors, either individually or in combinations and determine if any are required for DDX6-mediated translational inhibition.

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Referee #3:

MicroRNAs regulate target mRNAs through a combination of translational repression and mRNA degradation. Much effort has gone into delineating the molecular mechanism underlying miRNA-mediated translational repression. Most models of translational repression involve the recruitment of GW182 to an mRNA by Argonaute, which then recruits the CCR4-NOT complex, but the events downstream of the recruitment of the CCR4-NOT complex that lead to translational repression are unclear due to conflicting reports on the roles of DDX6 and eIF4A in causing translational repression. Additionally, in flies it has been reported that Argonaute can mediate both GW182-dependent and -independent translational repression, suggesting that there may be additional or species-specific mechanisms of translational repression. In this paper, Kuzuoglu-Ozturk et al. address several of the conflicting results in this field using molecular tethering and reporter gene assays. They determine that miRNA-mediated repression in both fly and human cells requires the W-binding pockets of Ago, with additional work consistent with a model that this repression is mediated through GW182. The authors use reporter genes that initiate translation independently of ribosome scanning and with partial independence from eIF4A to conclude that no aspect of miRNA-mediated repression requires ribosome scanning, and thus it is unlikely that regulation of eIF4A is involved in miRNA-mediated translational repression. Lastly, they show that DDX6, a translational repressor recruited by the CCR4-NOT complex, is required for the translational repression mediated by this complex, and they use sucrose gradient sedimentation to identify initiation as the step of translation regulated by DDX6 in human cells.

These results clarify several areas of confusion about the mechanism of miRNA-mediated translational repression and advance our understanding of this process. The experiments are extensive, and several lines of evidence are used to support the major conclusions. Overall, this is an excellent study, with just a few issues that should be addressed before publication.

Major points:
1. A primary focus of this manuscript is that miRNA-mediated repression is not dependent on ribosome scanning. To investigate this, the authors used reporter RNAs with a very short 5' UTR or a TISU element such that translation initiation would occur without any ribosome scanning. If transcription were initiated upstream of the assumed transcription start site, the resulting transcript could have a 5' UTR of sufficient length for scanning to occur, which would confound interpretation of these experiments. The authors mention 5' RACE data and say it confirms the 5' ends of the transcripts with a 8 nt 5' UTR or a TISU element, but don't show any data from the RACE experiments. They should show these data in a way that the reader can see the fraction of transcripts that begin at the intended 5' ends. The fact that a northern of a reporter transcript containing a TISU element seems to show two bands, one which runs at approximately the same molecular weight as the reporter with a 216 nt 5' UTR (Figure S4C, reporter d), demonstrates the need for these data.

Minor points:
2. Substantial translational repression was observed when the NOT1 mutant that does not interact with DDX6 was tethered to a reporter transcript with either a MALAT1 3' end or a poly(A) tail (Figure 6 H, J). Might this be due to the residual binding of DDX6 (Figure 6G)? Otherwise, this result would seem to conflict with the conclusion that miRNA-mediated translational repression is mediated by DDX6 (including results from this manuscript and a previous manuscript from the lab). The authors should clarify in the text.

3. The authors treat cells with silvestrol and make statements about its impact on translation at several points in the manuscript (i.e. Fig. 4A), but in all but one case they only measure changes in luciferase activity and do not seem to also look for changes in mRNA abundance. Additionally, in the case in which mRNA was measured, they found that the transcripts seemed to be stabilized by the treatment. To make conclusions about silvestrol inhibiting translation, the authors need to make mRNA abundance measurements in the corresponding samples.

4. In the experiments where a decapping mutant is overexpressed, no accumulation of deadenylated species seem to occur in the control tethering experiments despite decapping being inhibited (Fig. 2 M, N). Do the authors have an explanation for this?

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5. Can the authors confirm that in cases where comparisons are made concerning absolute RNA abundance (i.e. page 15, ...the mRNA reporters were stabilized by the silvestrol treatment in the absence of the tethered proteins...) that the samples are on the same northern blot despite the panels being separated?

1st Revision - authors' response 24 December 2015

**Response to referee 1**

The referee states that *"Despite recent progress, the mechanisms by which miRNAs function are still poorly understood at the molecular level. A particularly contentious issue has been the involvement of eIF4A and its paralog eIF4A2. In previous work (Meijer et al., Science 2013), it was suggested that eIF4A2 but not eIF4A was required to mediate repression by miRNAs. Consistent with a function of eIF4As as helicases that unwind 5′ UTRs to promote ribosomal scanning, silencing required long, structured 5'UTRs, a notion also supported by in vitro work (Ricci et al., NAR 2013)".*

We would like to note that the manuscript by Ricci et al. concluded that secondary structure in the 5′-UTR does not linearly correlate with silencing. On page 593 of their manuscript, the authors wrote: "Interestingly, the nature of the 5′-UTR considerably influenced the level of miRNA-mediated inhibition. However, we were surprised to observe that we could not draw any linear correlation between the complexity of the 5′-UTR and the level of miRNA repression." (see also Fig. 4).

*Two other publications (Fukao et al. Mol Cell 2014, Fukuya et al. Mol Cell 2014) reported that miRNAs act to displace eIF4A from mRNAs as a means to inhibit their repression, with eIF4A and eIF4A2 being interchangeable. Moreover, Fukuya et al. reported that in flies GW182 and Ago1 acted through different molecular mechanisms and, at least in part, independently of one another. This finding contrasts with the prevailing hierarchical model that AGO recruits GW182/TNRC6 recruits CCR4-NOT complex recruits DDX6 helicase to achieve (translational) silencing through an unknown mechanism. Finally, the recent generation of eIF4A2 knock-out cell lines revealed no defect in miRNA-mediated silencing (Galicia-Vazquez et al. RNA 2015).*

*Some clean-up work for the field seems clearly in order. Izaurralde and colleagues do just that through a large set of experiments that uses mostly tethering assays to understand the hierarchy of miRNA pathway factors and the requirement for long/structured 5'UTRs. The data provide evidence for the hierarchical model with a unified function of AGO, GW182, CCR4-NOT and DDX6 in both human and fly cells, and for repression irrespective of 5'UTR length (arguing against eIF4A as the sole/major target of repression). These findings are important and publishable (some points to be addresses are listed below). However, as things stand, the current discrepancies in the field suggest*

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*that experimental set-ups strongly affect outcomes. Indeed, one general frustration in this field is that different labs cannot reproduce one anothers' results, and that little exploration has gone into why.*

The miRNA field is marked by controversy; however, many controversies have been solved over time. Regarding the role of eIF4A2 in silencing, an interaction between eIF4A2 and NOT1 was not seen in four different labs (our lab, and the labs of Filipowicz, Conti and Sonenberg). Two other labs showed that eIF4A2 is not recruited to silenced mRNAs but rather dissociates from silenced targets (manuscripts by Fukao et al. and Fukaya et al.). Pelletier and coworkers reported that the eIF4A2 knockout does not suppress miRNA-mediated silencing (Galicia-Vazquez et al., RNA 2015) and Ricci et al. concluded that there is no linear correlation between 5′-UTR secondary structure and silencing. Notably, Bartel and coworkers reported no significant role for 5′-UTR features on the efficiency of silencing (Agarwal et al. eLife 2015, 4:e05005). We discuss this reference now in our manuscript. Thus, in our view, there is currently more consistency than controversy.

*An example of this is also seen here, where Izaurralde and colleagues use a reporter with a CAA-unstructured 5′-UTR, previously reported to be eIF4A-independent, but shown here to be eIF4A-dependent. What are the differences between this and the previous experiment? What reason is there to trust this rather than the earlier publication? I wish the authors would do more to convince me that we are not merely replacing arbitrarily one set of potentially nonphysiological conditions with another.*

One possible difference is that previous studies used RNA transfections instead of DNA transfection. Furthermore, as mentioned above, the Ricci et al. paper showed that there is no clear correlation between secondary structure and silencing. Finally, Bartel and coworkers reported that 5′-UTR features such as length and secondary structure do not reliably correlate with repression (Agarwal et al. eLife 2015, 4:e05005). In fact, none of the informative features found in in this study had anything to do with 5′-UTRs. Thus, 4 labs (our lab, and the labs of Bartel, Filipowicz and Ohlmann) have found no clear correlation between 5′-UTR structure and silencing using different approaches.

*Major points*

1. *Why is the IP efficiency so poor in the experiments? For instance, in Fig. 1A, only 1/20 of the HA-tagged Ago protein appears to be pulled down (signal of 20% immunoprecipitate equals that of 1% input). This might be expected of a co-immunoprecipitating protein, but is very unusual for an IP "bait" - can the authors be sure that this minor fraction is representative of the bulk with regard to GW182 interaction? In the same vein, these numbers are obviously important but missing for Fig. 4G and 6G.*

IP efficiencies of 20% are low but not poor. These lower efficiencies are observed in the IPs

performed with the anti-HA antibody. We obtained higher efficiencies when other antibodies are used (for example, anti-GFP and anti-eIF4A2). Because many antibodies are used in this manuscript, we believe that the general statement of the referee is not justified. Furthermore, the interaction between AGO and GW182 and between AGO and miRNAs has been demonstrated in numerous papers, and we have no reason to question the validity of these interactions even if the efficiency of our IP is 20%. The requested information has been included in Figure 4G and 6G.

*2. What is the subcellular localization of the MALAT1-based reporter? MALAT1 itself is nuclear, and this localization seems to be in part driven by the 3' end (Miyagawa et al., RNA 2012), which also appears to be included in the reporter used here. If a substantial fraction of this RNA is nuclear, it will not be translated and also resist miRNA activity - inferences on miRNA activity would thus be misleading. (For instance, protein will only be produced from the cytoplasmic faction; if this mRNA fraction is substantially degraded but the majority of the reporter is nuclear, an appearance of translational repression will result.) A substantial nuclear pool would also explain the reduced translational efficiency of the MALAT1-based reporters (Fig. S4) relative to their "poly(A)" counterparts.*

The MALAT sequence included in our reporter is a short 175-nt sequence present at the 3′-end of the MALAT1 RNA. It is not the complete MALAT1 3′-UTR, which contains nuclear retention signals. It triggers cleavage by RNase P, and therefore, the RNA is not polyadenylated. This sequence has been described and characterized in the manuscript by Wilusz et al. that clearly showed that the sequence promotes nuclear export. We have no reason to question these observations. Additionally, the decrease on translation efficiency of the MALAT reporters is unlikely due to nuclear retention because this reporter can be fully degraded when we increase the expression of tethered GW182 proteins, which suggest that the majority of the reporter is present in the cytoplasm. Finally, our conclusions will remain unchanged even if the pool of the reporter that is exported and translated will be degraded by the tethered proteins, because we analyze silencing, which is the combination of translational repression and mRNA destabilization.

*3. Fig. 4A: treatment with the eFI4A inhibitor silvestrol suggests that all reporters require at least some level of eIF4A activity. Does this not invalidate the assumption that the different reporters can be used to test miRNA activity in a situation where eIF4A is not required?*

First, we would like to mention that the TISU element was described and characterized by Dikstein and coworker in several manuscripts. This group has shown that translation driven by TISU is eIF4A independent. We have no reason to question their observations. Additionally, it is important to note that silvestrol was added to the cells 24 hrs after transfection, and therefore, some luciferase accumulates in the cells before translation is inhibited. Given that our R-Luc protein has a half-life of approximately 4 hrs and the silvestrol treatment is for 16 hrs, we do not expect a complete inhibition of luciferase expression. Furthermore, as explained in the response to referee 3, reporter

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mRNA levels increase in silvestrol-treated cells, indicating that we underestimated the inhibitory effect of silvestrol on translation. Finally, we use the TISU reporter to demonstrate that silencing occurs even when translation is initiated by a scanning-independent mechanism. This conclusion is based on the length of the 5′-UTR (9 nt) and is independent of the results obtained with silvestrol.

*4. Fig. 4G, H: immunoprecipitation of eIF4A2 is performed to test whether its binding to a (stable) target reporter changes upon the target's repression by miRNAs, and the answer is no. However, I wonder about the reliability of this assay - the same amounts of eIF4A2 co-immunoprecipitate very different levels of even control mRNA (e.g., compare lane 10 to 9 or 11 in Fig. 4G). It seems even control RNA levels bound can easily vary by two-fold, suggesting that the complexes are not very stable in the first place. With details on the IP efficiency missing (% loaded, see point 1 above), it becomes even harder to judge the suitability of this assay.*

The experiments are reliable, as indicated by the graph shown in Figure 4H, which represents the mean ± standard deviations of 5 independent experiments. The error bars clearly show that the assay is reliable. Figure 4G shows one representative northern blot whereas Fig. 4H shows the quantification of 5 independent experiments. In the northern blot shown in Figure 4G, there was more RNA loaded in lane 10. We did not consider this a major issue because the increase is observed for both the reporter and the transfection control that is used to normalize for these differences. We have now included an additional northern blot in Appendix Fig S3 to further support the reproducibility of the results. The details of the IP efficiency are now included in the Figure legend. For the western blot, 2% of the input and 6% of the IP were analyzed. For the northern blot, it was 2% of the input and 40% of the IP.

*5. Fig. 6G-L: Whereas the authors conclude (p. 16, bottom) that the NOT1 mutant that they use does not interact with DDX6, Fig. 6G indicates a reduction, not a complete loss of interaction. This does not affect the conclusion that DDX6 interaction seems required for translational miRNA target reporter repression. However, it provides an alternative interpretation for the continued reporter destabilization, namely that low amounts of DDX6 recruitment suffice for destabilization, not translational repression.*

We agree with the reviewer and have modified the text accordingly.

*6. Fig. S4J: Are the lanes from one autoradiograph, same exposure, or multiple ones? It would generally help if the authors could indicate for all the figures which autoradiographs are continuous, spliced, or mixed and matched, this is not always evident (e.g., for 5F I assume it is one single, unspliced autoradiograph?!)*

We confirm that the samples were analyzed on the same northern blot. This is clear in Figure 5F. In Fig EV3M (previously S4J), the panels are separated because additional samples were loaded.

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However, the source data for Fig. EV3M clearly shows that the samples were analyzed on the same northern blot. In general, the inclusion of the source data for all northern and western blots clarifies this question.

*7. P.17, second paragraph: the authors refer to Fig. S3D, E for evidence that Me31B tethering can suppress mRNAs independent of 5' UTR length - I can find no data on this in that figure.*

This was a mistake. These data are shown in Fig. 7E.

*8. Galicia-Vazquez et al., RNA 2015, recently showed that eIF4A2 is dispensable for miRNA-mediated gene silencing - this should be mentioned in the introduction rather than as a confirmatory finding, in passing, in the discussion.*

We agree with the reviewer and have modified the text accordingly.

*Minor points*

1. *Fig. 2K, L: (Partial) rescue of Ago1 knockdown with Ago1 pocket mutants: It would be useful to test the abundance of the transgenic relative to the endogenous proteins. Are they partially active at endogenous-like levels or when overexpressed?*

We agree with the reviewer and have included a western blot showing that the transgenic proteins are expressed at levels comparable to the endogenous protein (Fig. EV1I).

*2. Fig. 2K: Neither the main text nor the figure legend mention if the miRNAs (miR-1 and miR9b, respectively) are included in the transfection mix.*

We added the requested information in the Figure legends.

*3. Although mutant DCP2 impairs reporter degradation, this effect appears rather partial (Fig. 2N). This might be a consequence of the specific mutant, but it its equally possible that deadenylation- and decapping-dependent degradation is only a minor pathway that the proteins activate. The authors may wish to qualify their conclusions accordingly.*

The effect is indeed partial, but we do not expect a complete effect for this type of experiment in which the DCP2 mutant acts as dominant negative mutant but the wild-type protein is still present. There is extensive data published by our lab and other labs showing that AGO1, GW182, NOT1 and miRNAs degrade mRNA targets through deadenylation-dependent decapping. We do not understand why the referee is now questioning these previous publications.

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*4. Fig. 6E-F: a) I do not understand how the TISU-based reporter mRNA levels were quantified, the figure shows a rather extended smear?! b) It is not clear what "8xlet7 -or +" indicates: are they comparing reporters with/without let-7 binding sites, or with mutated let-7sites? The figure legend says "R-Luc-let-7 reporters or the corresponding reporters carrying mutations in the let-7 binding sites". However, in Fig.S6I-J, this reporter is called "8xlet7mut". Consistency needed.*

We apologize for the confusion–the reporter carries mutations in the let7-binding sites. We replaced the (-) with Mut to be consistent with the other figures. The northern blot in Figure 6F has now been replaced by a better quality northern blot. Because the panels are not separated it is now clear that the TISU reporter is shorter as expected.

*5. Fig. S2I, J: blots are lacking loading controls - needed if to make the point that tethered protein levels are not changing. Asterisk in J is not explained.*

We included a loading control and explained the asterisk. These blots are now shown in Fig EV1J and K, respectively.

*6. P.8, "Hs AGO2 repressed the translation of this reporter without causing mRNA degradation at the concentration tested (Fig 2H-J)". Cannot find the concentration anywhere.*

This was described in the supplemental methods (now Appendix methods) under “Tethering assays and knockdowns”. The given values, however, do not represent concentrations but amounts of transfected plasmid. We therefore deleted the term concentration.

*7. P. 16, l. 3 - typo, "hypuristanol"*

This has been corrected.

*8. P.22, ll.6-7: "Therefore, it is possible that in addition to DDX6, other factors are involved in coupling deadenylation to decapping" - what is the evidence that DDX6 has any role in this in the first place? As the authors mention previously, the available evidence chiefly support a role of DDX6 in translational repression.*

We would like to stress that DDX6 represses translation of reporters that lack a poly(A) tail (MALAT reporters). In contrast, polyadenylated reporters are degraded by tethered DDX6 (as observed for AGO, GW182, NOT1 and miRNAs), therefore it is incorrect to say that “the available evidence chiefly supports a role of DDX6 in translational repression”. We agree with the reviewer that there is no direct evidence for a role of DDX6 in coupling deadenylation to decapping. However, there is strong evidence for a role of DDX6 in decapping (in particular in yeast and *Dm*

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cells). Furthermore, DDX6 interacts with decapping factors (EDC3, LSm14 and PatL1, in yeast, *Dm* and human cells) and with NOT1. Based on these interactions, it has been proposed that DDX6 could link deadenylation to decapping. We have clarified this point in the text.

*Additional suggestions*

*The authors have nice and comprehensive data, they might try to find a better way of presenting them to do them justice. For instance:*

1. *The results section starts out with a full figure on validation of the importance of the tryptophane-binding pockets on Ago for interaction with GW182/TNRC6. This is an important validation, but really background information and could easily go into the supplements.*

We have streamlined this part of the manuscript.

*2. It is not clear how the DDX6 experiments relate to the main body of the text. I would suggest incorporating the conclusions on DDX6 involvement in scanning-independent silencing in the main tethering experiment (AGOs, CCR4/NOT and GWs).*

Given that the other reviewers did not raise this comment, we decided to maintain the presentation as in the submitted version. In our view, this order makes more sense, in particular after the addition of Fig. 8.

*3. More generally, the use of cells from two different species and numerous reporters makes it sometimes very difficult for the reader to penetrate the figures. Why not try to find some color/pattern code to present the data, e.g., present results for human versus Drosophila cell lines in different colors so that it is obvious at a glance what is presented. Hatched vs. crosshatched vs shaded for different reporter types?*

We agree with the reviewer and have now indicated in all panels whether the experiments were performed in *Dm* or *Hs* cells. We believe that a color code superposed with different hatched patterns may be too confusing.

**Response to referee 2**

The referee states that: *"This paper does not provide any major new insight into how miRNAs repress translation (many groups have now reported that miRNA silencing requires AGO, GW182, CCR4-NOT and DDX6). However, it is an important body of work because it convincingly demonstrates that miRNA-mediated translational repression through DDX6 does not target eIF4A*

*or ribosomal scanning and is not impacted by mRNA 5' UTR secondary structure".*

We would like to clarify that our data do not rule out a role for eIF4A1 in silencing. Our data show that silencing can occur in the absence of scanning and that 5′-UTR structure does not impact the efficiency of silencing. However, we cannot exclude that eIF4A1 has additional roles in translation initiation or silencing. On the other hand, the role of eIF4A1 in silencing is difficult to analyze in cells due to pleotropic effects of the depletion, including cell death and mRNA destabilization. We have clarified this in the text.

*Ultimately, what would improve this paper is if the authors determined how DDX6 is repressing translation initiation. Multiple proteins can directly bind DDX6, including EDC3, LSM14, PATL1 and 4E-T. The authors could knock down these factors, either individually or in combinations and determine if any are required for DDX6-mediated translational inhibition.*

To address this comment, we performed additional experiments. We tested the activity of DDX6 mutants in complementation assays using a miRNA reporter. These new data are included in an additional Figure (Fig. 8). We show that a DDX6 mutant that interacts with NOT1 but does not interact with EDC3, LSm14, PatL1 and 4E-T cannot rescue silencing of a miRNA reporter in cells depleted of DDX6. Conversely, a DDX6 mutant that does not interact with NOT1 but binds to EDC3, LSm14, PatL1 and 4E-T is also inactive in silencing. The specific contribution of EDC3, LSm14, PatL1 and 4E-T to silencing cannot be determined in this assay because all these proteins bind to the same surface of DDX6, and it is not possible to design mutations that disrupt only one of these interactions specifically. Previous studies reported that depletion of individual decapping factors does not suppress silencing, and we therefore tested 4E-T depletion. However, this depletion did not suppress silencing of this reporter, which is in agreement with data published by Kamenska at al. These authors observed a slight suppression of silencing in cells depleted of 4E-T. Additionally, it is possible that DDX6 interacts with other, not yet identified, partners using the same binding surface. Thus, the precise molecular mechanism of DDX6-mediated translational repression remains to be elucidated. This question has remained open for the last 10 years and much more work by our group and other research teams is needed to dissect the precise molecular mechanism. Therefore, we think that the elucidation of how DDX6 represses translation is out of the scope of this manuscript.

**Response to referee 3**

The referee states that our *"....results clarify several areas of confusion about the mechanism of miRNA-mediated translational repression and advance our understanding of this process. The experiments are extensive, and several lines of evidence are used to support the major conclusions. Overall, this is an excellent study, with just a few issues that should be addressed before publication.*

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*Major points:*

*1. A primary focus of this manuscript is that miRNA-mediated repression is not dependent on ribosome scanning. To investigate this, the authors used reporter RNAs with a very short 5′ UTR or a TISU element such that translation initiation would occur without any ribosome scanning. If transcription were initiated upstream of the assumed transcription start site, the resulting transcript could have a 5′ UTR of sufficient length for scanning to occur, which would confound interpretation of these experiments. The authors mention 5′ RACE data and say it confirms the 5′ ends of the transcripts with a 8 nt 5′ UTR or a TISU element, but don't show any data from the RACE experiments. They should show these data in a way that the reader can see the fraction of transcripts that begin at the intended 5′ ends. The fact that a northern of a reporter transcript containing a TISU element seems to show two bands, one which runs at approximately the same molecular weight as the reporter with a 216 nt 5′ UTR (Figure S4C, reporter d), demonstrates the need for these data.*

As requested by the reviewer we now show the 5′ RACE data for the TISU reporter in Figure EV3A and Appendix Fig S2. In Fig EV3A, we cloned the 5′ RACE products and sequenced 13 independent clones, which all showed the expected 5′-UTR. In Appendix Fig S2, we directly sequenced the 5′ RACE products without cloning. The chromatograms show that the expected transcription start site is used in human cells. For the *Drosophila* reporter, we observed that 1 out of 13 independent clones had a longer 5′-UTR of 16 nt instead of 8 nt. This longer 5′-UTR is nevertheless too short to support scanning. Reporter d indeed shows extra bands in the northern blot. However, because the 5′-RACE shows that the transcription start site is correct, we performed RNase H experiments in the presence of oligo (dT), which showed that the bands collapse in a single band, indicating that heterogeneity is mainly at the level of the poly(A) tail (Fig EV3D, lower panel). Although we cannot explain this heterogeneity, this observation does not affect our conclusions. Indeed, this reporter is used in Fig 5C,D. This figure shows that tethered TNRC6, AGO2 and NOT1 cause degradation of the reporter, which is clearly shown in the northern blot in Fig 5D. We do not use this reporter to make conclusions about translational repression.

*Minor points:*

*2. Substantial translational repression was observed when the NOT1 mutant that does not interact with DDX6 was tethered to a reporter transcript with either a MALAT1 3′ end or a poly(A) tail (Figure 6 H, J). Might this be due to the residual binding of DDX6 (Figure 6G)? Otherwise, this result would seem to conflict with the conclusion that miRNA-mediated translational repression is mediated by DDX6 (including results from this manuscript and a previous manuscript from the lab). The authors should clarify in the text.*

We agree with the reviewer and have modified the text accordingly. The NOT1 mutant shows

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residual binding to DDX6, which could explain the results in Fig. 6H and 6J.

*3. The authors treat cells with silvestrol and make statements about its impact on translation at several points in the manuscript (i.e. Fig. 4A), but in all but one case they only measure changes in luciferase activity and do not seem to also look for changes in mRNA abundance. Additionally, in the case in which mRNA was measured, they found that the transcripts seemed to be stabilized by the treatment. To make conclusions about silvestrol inhibiting translation, the authors need to make mRNA abundance measurements in the corresponding samples.*

We agree with the reviewer and we have now included a representative northern blot corresponding to Fig 4A in Fig EV3I and the corresponding source data. The quantification of mRNA levels corresponding to Fig 4A in four independent experiments is shown in Fig EV3J. Silvestrol treatment stabilized the mRNA reporters 2–5-fold in HEK293T cells but more than 10-fold in HeLa cells. These results suggest that the inhibitory effect of silvestrol on translation is underestimated. Importantly, despite the stabilization observed in the absence of tethered proteins, tethered AGO2, GW182 and NOT1 degraded the reporter mRNAs in the presence of silvestrol.

*4. In the experiments where a decapping mutant is overexpressed, no accumulation of deadenylated species seem to occur in the control tethering experiments despite decapping being inhibited (Fig. 2 M, N). Do the authors have an explanation for this?*

This is indeed the case. This observation has been reported in several manuscripts from our lab and other labs. Only the reporters that are exclusively degraded via the 5′-to-3′ decay pathway accumulate in a deadenylated form when decapping is blocked. This implies that we see accumulation of deadenylated mRNAs when AGO, GW182, NOT1, Nanos and other proteins that recruit the CCR4-NOT complex are tethered. In the control, no accumulation is observed because bulk mRNA is normally degraded via the 3′-to-5′ decay pathway, and we do not observe a major contribution of the 5′-to-3′ decay pathway at steady-state for these mRNAs when decapping is blocked.

*5. Can the authors confirm that in cases where comparisons are made concerning absolute RNA abundance (i.e. page 15, ...the mRNA reporters were stabilized by the silvestrol treatment in the absence of the tethered proteins...) that the samples are on the same northern blot despite the panels being separated?*

We confirm that the samples were analyzed on the same northern blot. This is clear in Figure 5F. In Fig EV3M, the panels are separated because additional samples were loaded. However, the source data for Fig. EV3M clearly shows that the samples were analyzed on the same northern blot. In general, the inclusion of the source data for all northern and western blots clarifies this question.

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2nd Editorial Decision 29 January 2016

Thank you for submitting the revised version of your manuscript. It has now been seen by two of the original referees whose comments are shown below. As you will see they both find that all major criticisms have been sufficiently addressed and recommend the manuscript for publication, pending final minor revision. In addition to commenting on the remaining points from ref #2, I would ask you to address the following minor editorial points in a final revision of your manuscript.

Thank you again for giving us the chance to consider your manuscript for The EMBO Journal, I look forward to your revision.

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Referee #2:

The only lingering issue I have is Figure 8F. In the left panel, the let-7 targeted reporter is repressed roughly 10-fold (i.e. 90% silencing) as compared to the control reporter. However, in the right panel, when DDX6 is knocked down, the let-7 reporter is depressed roughly 22-fold according to the y-axis. Based on these levels of repression and derepression, something weird is going on. Either the y-axis in the right panel needs revision, or the left panel wasn't from the same experiment as the right panel. Can the authors briefly comment on this?

Referee #3:

The authors have fully addressed all of my concerns.

2nd Revision - authors' response 16 February 2016

Regarding the additional comment from referee 2 on Figure 8F: we thank the referee for noticing the inconsistency. The ‘y’ axis in the right panel has been revised. The left and right panels are from the same experiment. A revised version of Figure 8 has been submitted.

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| Manuscript Number: EMBOJ-2015-92901R |

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

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

## A- Figures

### 1. Data

**The data shown in figures should satisfy the following conditions:**

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- 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:
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  - are tests one-sided or two-sided?
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Any descriptions too long for the figure legend should be included in the methods section and/or with the source data.

**Please ensure that the answers to the following questions are reported in the manuscript itself. We encourage you to include a specific subsection in the methods section for statistics, reagents, animal models and human subjects.**

**In the pink boxes below, provide the page number(s) of the manuscript draft or figure legend(s) where the information can be located. Every question should be answered. If the question is not relevant to your research, please write NA (non applicable).**

## B- Statistics and general methods

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

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| 1.a. How was the sample size chosen to ensure adequate power to detect a pre-specified effect size? | NA. This manuscript does not include statistical analyses. |
| 1.b. For animal studies, include a statement about sample size estimate even if no statistical methods were used. | NA. This study does not include any animal experiments |
| 2. Describe inclusion/exclusion criteria if samples or animals were excluded from the analysis. Were the criteria pre-established? | NA. We did not apply any formal rejection criteria. |
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| For animal studies, include a statement about randomization even if no randomization was used. | NA. This study does not include any animal experiments |
| 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. We did not take any explicit measures, but data analysis was done in teams of at least two investigators. |
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| Is there an estimate of variation within each group of data? | NA. This manuscript does not include statistical analyses. |
| Is the variance similar between the groups that are being statistically compared? | NA. This manuscript does not include statistical analyses. |

## C- Reagents

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| 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). | Antibodies are listed with catalog numbers in Appendix Table S4. |
| 7. Identify the source of cell lines and report if they were recently authenticated (e.g., by STR profiling) and tested for mycoplasma contamination. | HeLa, HEK293T and S2 cells were purchased at ATCC and are regularly checked for mycoplasma contamination. |

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

## D- Animal Models

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| | |
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| 18. Provide accession codes for deposited data. See author guidelines, under 'Data Deposition'.<br><br>Data deposition in a public repository is mandatory for:<br>a. Protein, DNA and RNA sequences<br>b. Macromolecular structures<br>c. Crystallographic data for small molecules<br>d. Functional genomics data<br>e. Proteomics and molecular interactions | NA. This study does not describe new protein, DNA or RNA sequences. This study does not include novel structural and crystallographic data, functional genomics or proteomics data. |
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