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GW182 proteins cause PABP dissociation from silenced miRNA targets in the absence of deadenylation

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1st Editorial Decision 15 October 2012

Thank you for submitting your manuscript for consideration by The EMBO Journal. It has now been seen by three referees whose comments are shown below.

As you will see from the reports, while referees #2 and #3 express positive interest in your findings and highlight their importance and general relevance, referee #1 is more critical and raises a number of concerns that you will have to address in full before submitting a revised version of the manuscript. In addition, referees #2 and #3 also request that a number of minor changes and additional clarifications be performed and included.

Given the referees' positive recommendations, we offer you the opportunity 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 to the full satisfaction of the referees in this revised version. Please do not hesitate to contact me if you have questions related to the review process and the requests made by the referees

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We generally allow three months as standard revision time. As a matter of policy, competing manuscripts published during this period will not negatively impact on our assessment of the conceptual advance presented by your study. However, we request that you contact the editor as soon as possible upon publication of any related work, to discuss how to proceed. Should you foresee a problem in meeting this three-month deadline, please let us know in advance and we may be able to grant an extension.

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

REFEREE REPORTS:

Referee #1:

miRNAs are small non-coding RNAs that post-transcriptionally regulate protein production either through direct translational repression and/or accelerated mRNA deadenylation, decapping, and decay. After associating with an Argonaute protein, miRNAs recruit several protein factors to the 3' UTRs of targeted mRNAs to mediate gene silencing. These factors include GW182, the translation factor poly(A)-binding protein (PABP), and the CCR4-NOT and PAN2-PAN3 deadenylase complexes. Whilst many recent publications have provided insight into the mechanisms of miRNA-mediated mRNA decay, the process by which miRNAs repress translation remains enigmatic. In this paper, Zekri and colleagues use a co-immunoprecipitation-based approach coupled with RT-PCR to determine how efficiently silenced versus unsilenced reporter mRNAs associate with the translation initiation factors eIF4E, eIF4G, and PABP. They find that miRNAs reduce the association between the three initiation factors with reporter mRNAs, although eIF4E and eIF4G dissociation appear to be a consequence of mRNA deadenylation, decapping, and decay. The authors propose that PABP dissociation requires CCR4-NOT recruitment through direct contact between GW182 and NOT1, the scaffolding subunit of the CCR4-NOT complex. This paper presents some interesting and novel findings with regards to the role of PABP in translational repression by miRNAs. However, several key experiments need to be performe to support their conclusions. In particular, it would be pertinent to show that the mRNA is indeed decapped, to explain why eIF4E cannot bind to it. There are also a number of inconsistencies in the paper as detailed below:
Major points:
1) The current model describing miRNA action proposes that translational repression occurs first (typically within the first hour), followed by the deadenylation, decapping, and decay of the miRNA target to consolidate the initial translational repression. The experiments presented in this work were done in vivo at steady state, where mRNA decay dominates. For example, eIF4E and eIF4G dissociate from miRNA targets after decapping and deadenylation, respectively. However, it is not clear whether the translational repression observed by other groups at early time points involves CCR4-NOT-dependent PABP dissociation, because the data presented here were produced at steady state. Providing evidence for CCR4-NOT-dependent PABP dissociation using a polyadenylated reporter construct in an in vitro cell extract at early time points would strongly support their conclusions. Also, because the temporal order of PABP dissociation is not directly addressed by the experiments in this paper, it is suggested that sentences which describe PABP dissociation as an "early" event in miRNA-mediated gene silencing be rephrased.
2) Figure 1C: Can the authors provide RT-qPCR data for the GW182 association with miRNA targets? Also, it needs to be explained how the data showing GW182 dissociation from the RISC (Zekri et al, Mol Cell Biol 2009) reconcile with the data in this manuscript.
3) Figure 2:
i) Figure 2E: It was previously reported that GW182 dissociates from mRNA targets at steady state. Here, it is shown that PABP overexpression causes GW182 to remain on targeted mRNAs. Can the authors explain why this should be the case?
ii) Figure 2B: It appears that PABP overexpression not only stabilizes mRNA levels, but also protects the poly (A) tails of targeted mRNAs. Therefore, is it possible that PABP overexpression merely protects the mRNA from degradation, thereby allowing for more eIF4E, eIF4G, and PABP to associate with miRNA targets?

4) Figure 3: It is pertinent to check the levels of eIF4G and PABP in cells where decapping factors are knocked down, to ensure that the reduction in initiation factor binding to miRNA targets is due to dissociation and not differences in protein levels.
5) Figure 4:
i) Compare Figures 4A, 4C, 4D, and 4E, with Figures 1B, 1G, 1H, and 1I: When directly tethering GW182 to the reporter mRNA, the same degree of eIF4E, eIF4G, and PABP dissociation is seen as for the reporter silenced by miR-9b. However, based on Figures 1B and 4A, the directly tethered GW182 represses protein synthesis ~20% less than the reporter silenced by miR-9b. If the same degree of translation factor dissociation is seen in both cases, why are they different in terms of degree of gene silencing?
ii) Figures 4A, 4B, and 4I: In these figures, it appears as though miRNA-mediated gene silencing can be entirely explained through mRNA degradation. Are the effects on translation factor dissociation physiologically relevant with respect to direct translational repression, if tethering GW182 seems to only silence the reporter through mRNA decay?
6) Figure 5G: When GW182 is tethered to the reporter mRNA containing an internalized poly (A) tail, PABP dissociates, whilst eIF4G remains bound. In Figures 3D-3F, a reporter mRNA with a standard poly (A) tail is shown to have less bound eIF4G and PABP in the presence of miR-9b. The authors propose in the results and discussion sections that eIF4G dissociates from mRNAs undergoing deadenylation because PABP is not present to stabilize the interaction between eIF4G and mRNA. Whilst this explanation is plausible in isolation, it does not explain why PABP dissociation in Figure 5G is not accompanied by eIF4G dissociation, as well.
7) Figure 6:
i) Figures 6B and 6E: Figure 6E shows that both the CIM2 mutant and the CIM1 + CIM2 double mutant similarly impair PABP dissociation. However, in Figure 6B, it is shown that miRNA-mediated gene silencing is more significantly impaired in the CIM1 + CIM2 double mutant. This suggests that PABP dissociation is not the only determinant for translational silencing, and calls into question the impact of these findings.
8) The manuscript contains several results where PABP dissociates from an mRNA reporter; however, there is always some PABP remaining on the mRNA. Is partial PABP dissociation sufficient for maximal miRNA silencing?
9) Figure 7: It is possible that tethering a component of the CCR4-NOT complex, which is likely to recruit the entire complex, merely hinders PABP association with the mRNA due to steric constraints. Does tethering another control protein (perhaps -galactosidase or PAN3) to a reporter mRNA have any effect on PABP association/dissociation?
10) Depleting NOT1 in S2 cells and monitoring PABP dissociation on an mRNA reporter would provide information, which could support the authors' proposed model.
Minor points:
1) Comparing Figures 2 and S2: Can the authors explain why PABP overexpression impairs miRNA-mediated gene silencing more strongly than GW182 depletion?
2) Why is eIF4E and eIF4G binding not sufficient for efficient luciferase activity in Figure 5?
3) Typo: In the second sentence of the introduction, the human GW182 paralogs are incorrectly named TNRCA, B, and C, as opposed to TNRC6A, B, and C.
For the reference of Kozlov et al. Gehring is misspelled

Referee #2:

This is an elegant study which extends previous published evidence from Hentze's lab indicating that GW182 protein is involved in active displacement of PABP from mRNA repressed by either natural miRNAs or by tethering of miRNA pathway components. The data provide strong indications that PABP displacement may be mediated by the CCR4-NOT complex components. The authors also provide interesting information about possible steps leading to displacement of elF4G from mRNA, with this step being, possibly, associated with mRNA remodeling occurring during active deadenylation. The strength of the manuscript is in a comprehensive character of the analysis, involving different reporters and a series of complementary approaches and systems.
Detailed comments
1. The authors should make it clear in the text of Results that the IP data are normalized when tethering of proteins results in increase of the mRNA input (e.g., Figures 6E and 7C-E).
2. Please discuss apparent discrepancy between the data shown in Fig. 6B (relatively small effect of

CIM-1 or CIM-2 mutation on degree of repression) and Fig. 6E (complete or nearly complete rescue of PABP association with mRNA target).
3. Fig. 4E. The effect on PABP is minimal, unlikely to be significant. This should be indicated in Results.
4. P. 15, l. 4 bottom. To my knowledge, Cooke et al. did not study NOT1 tethering.
5. It would be interesting to discuss in Discussion:
A. Is there any evidence in the literature of the CCR4-NOT (or its associating proteins) interaction with PABP?
B. Is there any prior evidence of elF4G dissociation from elF4-F during translation, translational regulation, or translational repression?
6. Reference to Pillai et al. 2005 should be added on p. 19, l. 2 top.
7. It would be helpful the authors clearly indicate in the figure when the HA-tagged or endogenous 4E, 4G, and PABP are monitored. Sometimes this is confusing. For example, in Fig. 2 legend it stands that HA-tagged proteins are used but in the figures itself (panels G-E) they are annotated as -elF4E, -PABP, etc.

Referee #3:

This paper describes a well conducted set of experiments designed to address the mechanism of translational repression during miRNA mediated silencing in Drosophila melanogaster cells. The work dissects the order of events that occur during silencing of a model target mRNA and represents an important contribution to resolving the currently conflicting data on the primary mechanisms involved in miRNA promoted silencing.

By using constructs, downregulation and tethering the authors examine the importance of deadenylation, decapping and specific interaction regions of GW182 proteins in bringing about translational repression in Drosophila cells. In particular they examine the requirements for the dissociation of PABP and the eIF4F proteins, eIF4E and eIF4G from the mRNA target.

The work shows that the miRNA-promoted silencing of a normally polyadenylated mRNA target is associated with deadenylation of the mRNA and release of PABP, eIF4E and eIF4G. However, prevention of the decapping process by downregulation blocks the release of eIF4E, suggesting that release of this protein during silencing is secondary to cap removal. However, under these conditions eIF4G and PABP still dissociate.

To examine the role of deadenylation of the target in these processes the authors used an mRNA target with an internal poly(A) sequence in the 3'UTR (which can't be deadenylated). This work produced the novel and unexpected result that in the absence of deadenylation PABP dissociation still occurred during silencing. However, under these conditions both eIF4G and eIF4E remained associated with the silenced target. This result, together with that from the experiments where the mRNA cap was artificially retained, suggests that the mechanisms responsible for the interaction and dissociation of eIF4G are more complicated than simple consequences of the association of this protein with its binding partners eIF4E and PABP. However, in the experiment with deadenylation-resistant mRNA, it is just possible that, if the ratio of eIF4G relative to PABP normally associated with mRNA is low, the residual low level of PABP remaining during silencing could still support its retention.

A major conclusion from this study is that the interaction of GW182 with the CCR4-NOT complex is sufficient for PABP dissociation but does not directly cause dissociation of eIF4G and eIF4E from mRNA during silencing. The data from the tethering experiments suggest that interaction of GW182 with PABP is dispensable for PABP dissociation, rather that the interaction with the CCR4-NOT complex is primarily responsible, probably by displacement. This was an unexpected and novel result.

The work is of very high technical standard and the data from co-immunoprecipitation studies are supported by a good set of controls for non-specific binding.

In general the paper is well written with clear explanations of the aims of each set of experiments and the conclusions drawn from them. The work has important general implications for the mechanism of silencing, well beyond the Drosophila system, and will be read by many workers using mammalian cells. I think many such readers would appreciate some brief background in the introduction on the similarity and differences between the DM eIF4F proteins and the more widely studied human homologues, and maybe a little more clarity on the Materials and Methods on which constructs, proteins and antibodies relate to Drosophila or human systems.

It would also be helpful if the authors could include some approximate indication of the extent to which the expression of HA tagged eIF4E, eIF4G and PABP increases the overall expression of each of these proteins - however, it is recognised that consistent results were obtained by co-immunoprecipitation of endogenous proteins in some experiments.

1st Revision - authors' response 30 December 2012

Response to comments of Reviewer 1

In the general comment, the referee mentions that it would be pertinent to show that the mRNA is indeed decapped to explain why eIF4E cannot bind to it. However, this experiment is in the manuscript. In fact, decapped mRNAs are immediately degraded *in vivo* and do not accumulate as decapped decay intermediates. In other words, the fraction of the mRNA target that is not degraded is capped. The only way to show that an mRNA undergoes decapping is by showing that degradation is inhibited in cells depleted of decapping factors. This experiment is shown in Figure 3. Because we found that eIF4E binds to the target when decapping and decay are inhibited (Figure 3), the reduced association of eIF4E observed in control cells can be, at least in part, explained through the degradation of the reporter. Additionally, it is well established in the literature that miRNA targets undergo decapping, and it is also known that eIF4E must dissociate from the cap structure for decapping to occur; however, it is not known how eIF4E is displaced from the cap structure.

Major points:

1) The current model describing miRNA action proposes that translational repression occurs first (typically within the first hour), followed by the deadenylation, decapping, and decay of the miRNA target to consolidate the initial translational repression. The experiments presented in this work were done in vivo at steady state, where mRNA decay dominates. For example, eIF4E and eIF4G dissociate from miRNA targets after decapping and deadenylation, respectively. However, it is not clear whether the translational repression observed by other groups at early time points involves CCR4-NOT-dependent PABP dissociation, because the data presented here were produced at steady state. Providing evidence for CCR4-NOT-dependent PABP dissociation using a polyadenylated reporter construct in an in vitro cell extract at early time points would strongly support their conclusions. Also, because the temporal order of PABP dissociation is not directly addressed by the experiments in this paper, it is suggested that sentences which describe PABP dissociation as an "early" event in miRNA-mediated gene silencing be rephrased.

We agree with the reviewer that our experiments were performed at steady state and we therefore do not have temporal resolution. Our conclusion that PABP dissociation is an early event was inferred based on previous reports indicating that translational repression precedes deadenylation and on our observation that PABP dissociation occurs in the absence of deadenylation. Nevertheless, we have rephrased our statements as requested by the reviewer. We also agree that it would be interesting to determine the kinetics of PABP dissociation *in vivo*. These experiments would require a different experimental set up and the use of inducible reporters as shown in the manuscripts from the Green and Filipowicz labs (Béthune et al, 2012; Djuranovic et al, 2012). However, we think these experiments are not feasible *in vivo* because at early time points (within less than 1 hr after induction), the expression levels of the inducible reporters are very low; thus, the efficiency of the immunoprecipitation is likely to be compromised, and the results will not be reliable with the

reporters expressed at very low levels. Finally, it is important to note that the experiment requested by the reviewer was previously performed by Moretti and co-workers using *Drosophila* cell-free extracts. Indeed, Moretti et al. (2012) showed that PABP displacement occurs at early time points prior to the onset of deadenylation *in vitro*.

2) Figure 1C: Can the authors provide RT-qPCR data for the GW182 association with miRNA targets? Also, it needs to be explained how the data showing GW182 dissociation from the RISC (Zekri et al, Mol Cell Biol 2009) reconcile with the data in this manuscript.

We thank the reviewer for this comment and included the requested experiment in Figures 2E and Supplementary Figure S3D. The manuscript by Zekri et al. (2009) showed that GW182 was detected in association with silenced targets in cells depleted of NOT1, wherein target degradation is inhibited. Similarly, in this manuscript, we show that GW182 coimmunoprecipitates the target in cells depleted of decapping factors, in which decapping and subsequent degradation are inhibited (Figure 3C). We also show that GW182 remains bound to the target in cells overexpressing PABP, in which silencing and consequently target degradation are inhibited (Figure 2F). Thus, GW182 association with mRNA targets is observed when target degradation is inhibited either by depletion of deadenylation factors (Zekri et al, 2009), decapping factors (Supplementary Figure S3D), or by the overexpression of PABP (Figure 2F). In contrast to AGO1, GW182 does not preferentially co-immunoprecipitate silenced targets in control cells. These observations suggest that GW182 dissociates from miRNA targets after degradation. Alternatively, GW182 may not be accessible to the antibodies when bound to a target mRNA in control cells. However, we consider this last possibility unlikely, because previously we could not coimmunoprecipitate silenced targets with GW182 proteins carrying N-terminal HA or GFP (green fluorescent protein) tags or a C-terminal V5 tag, although these proteins complemented silencing (Zekri et al, 2009). Moreover, as shown in Figures 2E and 2F, we obtained similar results when we analysed the association of endogenous GW182 using polyclonal antibodies. We discuss the observations reported by Zekri et al. (2009) more extensively to clarify that they are consistent with those described in this manuscript.

3) Figure 2:

i) Figure 2E: It was previously reported that GW182 dissociates from mRNA targets at steady state. Here, it is shown that PABP overexpression causes GW182 to remain on targeted mRNAs. Can the authors explain why this should be the case?

As mentioned in the response to point 2, PABP overexpression inhibits deadenylation and subsequent degradation of the mRNA target. Under these conditions, GW182 remains bound to the mRNA target as reported by Zekri et al. (2009).

ii) Figure 2B: It appears that PABP overexpression not only stabilizes mRNA levels, but also protects the poly (A) tails of targeted mRNAs. Therefore, is it possible that PABP overexpression merely protects the mRNA from degradation, thereby allowing for more eIF4E, eIF4G, and PABP to associate with miRNA targets?

PABP overexpression does indeed counteract deadenylation and subsequent degradation of the mRNA target. Consequently, the mRNA target is no longer degraded in the presence of the miRNA (Figure 2B, compare lanes with and without miRNA in the presence of PABP). The referee is concerned that we see more eIF4E, eIF4G and PABP association because there is more RNA; however, this is not the case because the association of the mRNA target with eIF4E, eIF4G, and PABP in the presence of the miRNA is normalized to the values obtained in the absence of the miRNA. In other words, if PABP overexpression enhanced eIF4E, eIF4G and PABP binding to the target, this should occur both in the presence and absence of the miRNA. Accordingly, Figure 2D shows that in cells overexpressing PABP, binding of eIF4E, eIF4G and PABP to the mRNA target is not changed regardless of the presence of the miRNA despite the fact that both AGO1 and GW182 bind to the target when the miRNA is expressed (Figures 2D and 2F). These figures are particularly

important because they show that a target is not silenced by miRNAs when PABP displacement and target degradation are prevented, although AGO1 and GW182 are bound to the mRNA.

4) Figure 3: It is pertinent to check the levels of eIF4G and PABP in cells where decapping factors are knocked down, to ensure that the reduction in initiation factor binding to miRNA targets is due to dissociation and not differences in protein levels.

We agree with the reviewer and performed the requested experiment, the results are now shown in Figure S3B. Endogenous levels of eIF4E, eIF4G and PABP are not affected in the depleted cells.

5) Figure 4:

i) Compare Figures 4A, 4C, 4D, and 4E, with Figures 1B, 1G, 1H, and 1I: When directly tethering GW182 to the reporter mRNA, the same degree of eIF4E, eIF4G, and PABP dissociation is seen as for the reporter silenced by miR-9b. However, based on Figures 1B and 4A, the directly tethered GW182 represses protein synthesis ~20% less than the reporter silenced by miR-9b. If the same degree of translation factor dissociation is seen in both cases, why are they different in terms of degree of gene silencing?

We think that the two reporters cannot be directly compared. They have different 3' UTRs and the contribution of translational repression and degradation to silencing is different for the two reporters. For example, the Nerfin reporter is degraded to 60% of control levels but protein expression is reduced to 20%. In contrast, tethered GW182 causes a similar reduction in protein and mRNA levels. For this reporter, the reduction in the association of eIF4E, eIF4G and PABP observed in control cells can be explained through degradation of the reporter; thus, only the results obtained in cells in which mRNA degradation is prevented can be interpreted for this reporter (Figures 4F–H). We clarified this in the text.

ii) Figures 4A, 4B, and 4I: In these figures, it appears as though miRNA-mediated gene silencing can be entirely explained through mRNA degradation. Are the effects on translation factor dissociation physiologically relevant with respect to direct translational repression, if tethering GW182 seems to only silence the reporter through mRNA decay?

As mentioned above, we agree with the reviewer that the dissociation of eIF4E, eIF4G and PABP observed in the tethering assays in control cells are difficult to interpret and it is not possible to draw any conclusion regarding translational repression. Only the experiments performed in cells in which degradation of the reporter is prevented are conclusive (Figure 4F–H). These experiments show that eIF4G dissociates as a consequence of deadenylation, whereas eIF4E remains bound to a target which is also bound by AGO1 and GW182. These findings rule out models of silencing suggesting that AGOs compete with eIF4E for the cap structure. We clarified this in the text.

6) Figure 5G: When GW182 is tethered to the reporter mRNA containing an internalized poly (A) tail, PABP dissociates, whilst eIF4G remains bound. In Figures 3D-3F, a reporter mRNA with a standard poly (A) tail is shown to have less bound eIF4G and PABP in the presence of miR-9b. The authors propose in the results and discussion sections that eIF4G dissociates from mRNAs undergoing deadenylation because PABP is not present to stabilize the interaction between eIF4G and mRNA. Whilst this explanation is plausible in isolation, it does not explain why PABP dissociation in Figure 5G is not accompanied by eIF4G dissociation, as well.

We agree with the reviewer that destabilization of eIF4G after PABP dissociation does not explain why eIF4G remains bound to the target that does not undergo deadenylation and decapping. One possible explanation for this observation is that the residual levels of PABP remaining are sufficient

to retain eIF4G in one reporter but not the other. An alternative explanation is that eIF4G dissociation occurs only on mRNAs undergoing deadenylation and may reflect changes in the mRNP composition when deadenylation is coupled to decapping. We have added a discussion of alternative explanations; however, we do not have a definitive answer for why eIF4G behaves differently when the reporter undergoes deadenylation relative to the reporter that is not deadenylated.

7) Figure 6:

i) Figures 6B and 6E: Figure 6E shows that both the CIM2 mutant and the CIM1 + CIM2 double mutant similarly impair PABP dissociation. However, in Figure 6B, it is shown that miRNA-mediated gene silencing is more significantly impaired in the CIM1 + CIM2 double mutant. This suggests that PABP dissociation is not the only determinant for translational silencing, and calls into question the impact of these findings.

We agree with the reviewer that the CIM-2 and the CIM-1+2 mutants both fail to dissociate PABP, but the silencing activity of the double mutant is more significantly impaired. This is consistent with the observation that NOT1 binding is also more significantly impaired for the double mutant. Furthermore, we have not claimed that PABP dissociation is the only determinant for translational repression. To address the question of the relevance of PABP displacement to silencing, we have performed additional experiments that are shown in Figures 8 and 9 and Supplementary Figure S6 of the revised manuscript.

First, we extended the observations described in the first version of the manuscript showing that TNRC6C-SD mutants that cannot release PABP are impaired in silencing. In the revised version of this manuscript, we demonstrate that these mutants are impaired in silencing and do not discriminate between unadenylated and polyadenylated targets (Figures 8E and 8F), whereas the wild-type SD displaces PABP and more efficiently silences polyadenylated targets (Figure 8C).

Second, we substituted the internal poly(A) in our reporter with six MS2 binding sites to tether PABP to the reporter and thus prevent its dissociation. We show that tethered PABP enhances translation (6-fold) and stabilizes the mRNA reporter (1.4-fold; Supplementary Figures S6E and S6F). However, in contrast to the reporter to which PABP binds to an internal poly(A), tethered PABP cannot be displaced by GW182, and silencing is less efficient (Figure 8H–J), suggesting that PABP displacement contributes to silencing. In these experiments, we estimate the contribution of PABP displacement to silencing to be 1.7-fold. This is consistent with the observation that the CIM-2 mutant, which does not displace PABP, is 2-fold less active in tethering assays relative to the wild type TNRC6C-SD (Figure 6C).

Third, we observed that PABP is not displaced if it binds to an internal poly(A) inserted upstream of the GW182 binding site. This observation provides an opportunity to estimate the contribution of PABP dissociation to silencing. We observed that the reporter to which PABP binds upstream of GW182 is 1.7-fold less efficiently repressed relative to the corresponding reporter to which PABP binds downstream of GW182 and is released (Figure 9).

Although the numbers we obtained for the contribution of PABP dissociation to silencing are a rough estimate, they are surprisingly consistent using different reporters and indicate that the contribution of PABP dissociation to silencing is approximately 2-fold. This effect is not negligible given that miRNA repression is in the range of 2- to 5-fold.

8) The manuscript contains several results where PABP dissociates from an mRNA reporter; however, there is always some PABP remaining on the mRNA. Is partial PABP dissociation sufficient for maximal miRNA silencing?

We agree with the reviewer that we always observe some PABP bound to the reporter. As mentioned above, we estimate that the contribution of this partial PABP dissociation to silencing is approximately 2-fold. It is unclear whether full dissociation would provide a more important contribution and whether it can occur *in vivo*, where PABP can most likely rebind the target.

9) Figure 7: It is possible that tethering a component of the CCR4-NOT complex, which is likely to recruit the entire complex, merely hinders PABP association with the mRNA due to steric constraints. Does tethering another control protein (perhaps β-galactosidase or PAN3) to a reporter mRNA have any effect on PABP association/dissociation?

We have performed the experiment suggested by the reviewer. We show that tethered PAN3 or an AGO1 mutant that does not interact with GW182 (the F2V2 mutant) do not cause PABP dissociation (Figure S5). Furthermore, as mentioned above, when PABP binds upstream of GW182 (or of NOT1) it is not released, suggesting that the reduction in PABP binding indeed reflects dissociation rather than reduced accessibility to the antibodies due to steric effects. Furthermore, it is important to note that the poly(A) stretch was inserted 288 residues downstream of the BoxB hairpins in the reporter from which PABP dissociates (Figure 5A), whereas when PABP does not dissociate, the poly(A) stretch is only 37 residues upstream of the BoxB hairpins (Figure 9A). These results indicate that the antibodies can access PABP molecules bound 37 residues apart from the GW182 (or NOT1) binding site.

10) Depleting NOT1 in S2 cells and monitoring PABP dissociation on an mRNA reporter would provide information, which could support the authors' proposed model.

We have attempted the experiment suggested by the reviewer. However, NOT1 depletion inhibits cell proliferation and causes cell death, thus the levels of the reporter are strongly reduced in these cells and the results of the PABP immunoprecipitations are not reliable (due to the dynamic range of the assay). We think that the results obtained with GW182 mutants that do not bind NOT1 are more conclusive.

Minor points:

1) *Comparing Figures 2 and S2: Can the authors explain why PABP overexpression impairs miRNA-mediated gene silencing more strongly than GW182 depletion?*

The reviewer is comparing apples and oranges. It is not possible to compare PABP overexpression and GW182 knockdown. These are two different proteins, and in one case one protein is overexpressed, while in the other the protein is depleted. In GW182-depleted cells, the levels of endogenous GW182 are reduced to 10% of control levels (Supplementary Figure S2C), thus there is still some residual GW182 in these cells, and this residual amount may be sufficient to sustain silencing to some extent.

2) Why is eIF4E and eIF4G binding not sufficient for efficient luciferase activity in Figure 5?

The reviewer asks why the reporter with the internal poly(A) stretch is silenced despite the fact that eIF4E and eIF4G remain bound. We think that this observation indicates that miRNAs do not interfere with eIF4F complex binding to the cap structure and therefore that miRNAs inhibit translation at a step downstream of cap recognition.

3) Typo: In the second sentence of the introduction, the human GW182 paralogs are incorrectly named TNRCA, B, and C, as opposed to TNRC6A, B, and C.

For the reference of Kozlov et al. Gehring is misspelled

The typos have been corrected.

Response to comments of Reviewer 2

Reviewer 2 is very positive and only requested text changes.

1. The authors should make it clear in the text of Results that the IP data are normalized when tethering of proteins results in increase of the mRNA input (e.g., Figures 6E and 7C-E).

We have clarified in the text that the IP data are not normalized to the inputs. The IP data are normalized to the control IP (e.g., IP with tethered lN-HA or in the absence of miRNA). We have decided not to normalize to the inputs because the IP efficiencies are not directly proportional to the input. For example, POP2 stabilizes the reporter 2.2-fold but association with eIF4E and eIF4G increases 1.5-fold (Figure 7). Because when POP2 is tethered to the reporter we observe PABP dissociation despite the stabilization of the reporter, we assume that we should be able to detect PABP dissociation with the CIM mutants if they were able to do so, despite the increase in reporter levels.

2. Please discuss apparent discrepancy between the data shown in Fig. 6B (relatively small effect of CIM-1 or CIM-2 mutation on degree of repression) and Fig. 6E (complete or nearly complete rescue of PABP association with mRNA target).

As mentioned above, we have introduced a sentence clarifying that PABP dissociation is not the only determinant for translational repression. We have performed additional experiments that indicate that PABP dissociation contributes to the repression approximately 2-fold (see response to referee 1, point 7).

3. Fig. 4E. The effect on PABP is minimal, unlikely to be significant. This should be indicated in Results.

We agree with the reviewer that the effects observed in Figures 4C–E can be explained by the degradation of the reporter and are inconclusive. We have clarified this in the text.

4. P. 15, l. 4 bottom. To my knowledge, Cooke et al. did not study NOT1 tethering.

We agree with the reviewer and have deleted reference to Cooke et al. in this sentence.

5. It would be interesting to discuss in Discussion:

A. Is there any evidence in the literature of the CCR4-NOT (or its associating proteins) interaction with PABP?

B. Is there any prior evidence of elF4G dissociation from elF4-F during translation, translational regulation, or translational repression?

We have extended the discussion to include references showing that the CCR4-NOT complex interacts with PABP (Zekri et al, 2009) and cited studies showing that translational repressors other than miRNAs target PABP and eIF4G.

6. Reference to Pillai et al. 2005 should be added on p. 19, l. 2 top.

This reference is now included.

7. It would be helpful the authors clearly indicate in the figure when the HA-tagged or endogenous 4E, 4G, and PABP are monitored. Sometimes this is confusing. For example, in Fig. 2 legend it stands that HA-tagged proteins are used but in the figures itself (panels G-E) they are annotated as α-elF4E, α-PABP, etc.

We thank the reviewer for detecting this mistake. We have corrected the legend to figure 2 and modified the figures to indicate when the HA-tagged or endogenous proteins were used.

Response to comments of Reviewer 3

Reviewer 3 is very positive and requests only text changes and a western blot control showing the expression levels of the HA-tagged proteins which is now included in Figure S1A.

I think many such readers would appreciate some brief background in the introduction on the similarity and differences between the DM eIF4F proteins and the more widely studied human homologues, and maybe a little more clarity on the Materials and Methods on which constructs, proteins and antibodies relate to Drosophila or human systems.

We have revised the text accordingly.

It would also be helpful if the authors could include some approximate indication of the extent to which the expression of HA tagged eIF4E, eIF4G and PABP increases the overall expression of each of these proteins - however, it is recognized that consistent results were obtained by co-immunoprecipitation of endogenous proteins in some experiments.

We have performed the control experiment requested by the reviewer and included the corresponding western blots in Figure S1A. We estimate that the HA-tagged proteins increase the overall expression of each protein approximately 6-fold. Indeed, the western blot signals for the HA and endogenous proteins are comparable but the transfection efficiency is approximately 30%.

2nd Editorial Decision 28 January 2013

Thank you again for submitting a revised version of your manuscript and my apologies that it has taken a little longer than usual for the referees to return their recommendations.

We have now received reports from two of the original referees as shown below, and you will see that they are both broadly in favour of publication, pending satisfactory minor revision. While ref #2 finds that all original criticisms have been adequately addressed, ref #1 still has concerns regarding

the conclusion in fig 2B that PABP does not alter mRNA stability and therefore asks that you provide data normalizing the mRNA levels for PABP over-expression to values for GST over-expression. I would therefore ask you to provide this additional control as a supplemental figure and comment on it in the main manuscript.

In addition, there are a few editorial issues as outlined below that I would ask you to address before submitting a final version of the manuscript.

First of all we need a short segment stating the author contributions.
Secondly, we noticed that a substantial amount of the Materials and Methods section is included as supplementary information. It is EMBO Journal policy that M&M for all central experiments should be provided in the main manuscript text, and while it is fine to leave the description of plasmid constructions and amounts transfected in the supplementary info, I would ask you to move the description of IP and RT-PCR conditions to the main text.
In addition, I have to ask that all figure legends provide information on the nature of the error bars indicated in each figure and on the number of replicas used for statistical calculations.

Finally, we now encourage the publication of source data, particularly for electrophoretic gels and blots, with the aim of making primary data more accessible and transparent to the reader. Would you be willing to provide files comprising the original, uncropped and unprocessed scans of all gels used in the figures? We would need 1 file per figure (which can be a composite of source data from several panels) in jpg, gif or PDF format, uploaded as "Source data files". The gels should be labelled with the appropriate figure/panel number, and should have molecular weight markers; further annotation would clearly be useful but is not essential. These files will be published online with the article as a supplementary "Source Data". Please let me know if you have any questions about this policy.

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

REFEREE REPORTS:

Referee #1:

The revised manuscript submitted by Zekri et al. is significantly improved. The authors have provided satisfactory responses to many of my comments, and have included new data to strengthen their conclusions regarding the dissociation of PABP from miRNA-targets in vivo. However, there is one point that remains to be clarified:

ii) Figure 2B: It appears that PABP overexpression not only stabilizes mRNA levels, but also protects the poly (A) tails of targeted mRNAs. Therefore, is it possible that PABP overexpression merely protects the mRNA from degradation, thereby allowing for more eIF4E, eIF4G, and PABP to associate with miRNA targets?

Response: PABP overexpression does indeed counteract deadenylation and subsequent degradation of the
mRNA target. Consequently, the mRNA target is no longer degraded in the presence of the iRNA (Figure 2B, compare lanes with and without miRNA in the presence of PABP). The referee is concerned that we see more eIF4E, eIF4G and PABP association because there is more RNA; however, this is not the case because the association of the mRNA target with IF4E, eIF4G, and PABP in the presence of the miRNA is normalized to the values obtained in the absence of the miRNA. In other words, if PABP overexpression enhanced eIF4E, eIF4G and PABP binding to the target, this should occur both in the presence and absence of the miRNA. Accordingly, Figure 2D shows that in cells overexpressing PABP, binding of eIF4E, eIF4G and PABP to the mRNA target is not changed regardless of the presence of the miRNA despite the fact that both AGO1 and GW182 bind to the target when the miRNA is expressed (Figures 2D and 2F). These figures are particularly important because they show that a target is not silenced by miRNAs when PABP displacement and target degradation are prevented, although AGO1 and GW182 are bound to the mRNA.

New Critique: Normalizing the data to a non-targeted mRNA may not be sufficient, since the stability of the non-targeted mRNA would likely be less sensitive to PABP overexpression when compared to the miRNA-targeted reporter. Furthermore, the amount of mRNA input appears greater for eIF4E, eIF4G, and PABP pulldowns in the PABP overexpression experiment when compared to GST-overexpressing cells. In particular, the data for both GST and PABP overexpressing cells seem to show that roughly the same amount of mRNA is associated with eIF4E as the input. These data would suggest that changes in mRNA levels are, perhaps to a significant extent, responsible for the increased association of mRNA with eIF4E, eIF4G, and PABP.

To clarify whether the observed association of eIF4E, eIF4G, and PABP is not primarily due to the stabilization of the miRNA-targeted reporter in PABP overexpressing cells, we ask that the authors provide a figure that normalizes mRNA levels in the PABP overexpression experiment to the GST overexpression experiment. Doing so would indicate whether there is a true enhancement of eIF4E, eIF4G and PABP association, or whether this is just due to more miRNA-targeted mRNA being present as a result of enhanced mRNA stability in PABP-overexpressing cells.

Referee #2

I am satisfied with the revised revision

2nd Revision - authors' response 05 February 2013

We are pleased that the referees are in favour of publication and we have clarified the remaining comment of reviewer 1.

In addition, we have revised the text as suggested in your letter.

In particular:

1. We included a paragraph stating author contributions in the Acknowledgements.

2. We moved the description of IP and RT-PCR conditions to the main text.

3. We provide information on the nature of the error bars in the legend to Figure 1. Because all figures refer to Figure 1 we believe is not necessary to repeat the same sentence in all figures.

4. We uploaded Source data files for the main figures as requested.

Here is our answer to the point raised by reviewer 1.

Reviewer 1 is concerned that the restoration of eIF4E, eIF4G and PABP binding to the miRNA target in cells overexpressing PABP reflects a preferential stabilization of the miRNA reporter in the presence of miR-9b and requested that we normalize data obtained in PABP expressing cells to the control cells expressing GST. The referee assumes that the stability of the non-targeted reporter would be less sensitive to PABP overexpression when compared to the miRNA-targeted reporter. However, the northern blot in Figure 2B clearly shows that this is not the case (the targeted and the non-targeted reporter accumulate to similar levels). Furthermore, the normalization requested by the reviewer will be misleading because in control cells the targeted reporter is degraded but not in cells expressing PABP. Consequently, we should compare the targeted reported to the non-targeted reporter under the same conditions (in control cells or in cells expressing PABP). Furthermore, it is important to note that any potential nonspecific effect of PABP overexpression on mRNA levels are normalized out in our experiments because F-Luc levels are first normalized to R-Luc levels for

each sample. Then we compare the normalized F-Luc levels in the absence and presence of the miRNA under the same conditions. Finally, PABP dissociation can be observed even when the reporter is stabilized. For example POP2 stabilizes the reporter 2.2-fold but when POP2 is tethered to the reporter PABP dissociates, thus we assume that we should be able to detect PABP dissociation if this was happening.

We clarified this in the text.