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Kinetic analysis reveals successive steps leading to miRNA-mediated silencing in mammalian cells

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(Note: With the exception of the correction of typographical or spelling errors that could be a source of ambiguity, letters and reports are not edited. The original formatting of letters and referee reports may not be reflected in this compilation.)

1st Editorial Decision 07 February 2012

Thank you for the submission of your manuscript to our journal. I am truly sorry for the delay in getting back to you, which is due to the fact that the referees are not in agreement about your manuscript. I have copied their reports below, and you will see that while referee 1 is positive, both referees 2 and 3 are concerned about the conclusiveness of the data. They raise critical issues that would have to be addressed in a revised version of the manuscript before it can be considered for publication in EMBO reports.

Referee 2 points out that the study does not exclude that deadenylation of mRNAs occurs at early time points and/or independent of translational repression. S/he also indicates that no evidence is provided to support the suggestion that deadenylation occurs as a consequence of translational repression. Upon further consultation with referee 3, this referee agrees that a precise order of events cannot be concluded from the current data. Both referees remark that experimental assays that accurately measure deadenylation and mRNA decay are essential to conclusively address the contribution and timing of these events.

From the referee comments it is clear that the current data are insufficient to support the main conclusion of the manuscript. Publication of the study in our journal can therefore not be considered at this stage. On the other hand, given the potential interest of your findings, I would like to give you

the opportunity to address the concerns and would be willing to consider a revised manuscript with the understanding that the referee concerns must be fully addressed and their suggestions (as detailed above and in their reports) taken on board. I realize that the referee concerns are major and require extensive revision, and I understand (but would regret) if you rather decided to submit the manuscript elsewhere. In this case, I would welcome a message to that effect.

Should you decide to embark on such a revision, acceptance of the manuscript will depend on a positive outcome of a second round of review and I should also remind you that it is EMBO reports policy to allow a single round of revision only and that, therefore, acceptance or rejection of the manuscript will depend on the completeness of your responses included in the next, final version of the manuscript.

I look forward to seeing a revised version of your manuscript when it is ready.

Yours sincerely,

Editor
EMBO Reports

REFEREE REPORTS:

Referee #1:

1. Do the contents of this manuscript report a single key finding? YES

The single key finding of this manuscript is a systematic analysis of the order of events during miRNA-guided gene silencing. They show that translational repression is the predominant miRNA function on newly synthesized targets. At steady state mRNA levels, deadenylation-dependent mRNA decay dominates over translational repression, as has been shown by others before.

2. Is the main message supported by compelling experimental evidence? YES

The message of the manuscript is clearly supported by the experimental data provided. There are some minor points that should be clarified (see point 6).

3. Have similar findings been reported elsewhere (e.g. on a closely related protein; in another organism or context)? NO

It has been shown that miRNAs can regulate gene expression by inducing translational repression as well as mRNA decay. It has been shown recently that under steady state conditions, translational repression contributes about 85% of the gene silencing effects while translational inhibition only about 15%. Here, the authors have analyzed mRNA decay and translational repression on newly synthesized mRNAs and find that under these conditions translational inhibition dominates. This has not been analyzed and reported before.

4. Is the main finding of general interest to molecular biologists? YES

It is still a major question in the field how much mRNA decay or translation effects contribute to overall miRNA-guided gene silencing. This manuscript clearly demonstrates the order of events and places both translational repression as well as mRNA decay at specific time points of the mRNA life cycle. It is therefore interesting for molecular biologists.

5. After appropriate revision, would a resubmitted manuscript be most suited for publication:

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Minor points that should be clarified:

1. In Figures 2B. C, D and 3B, protein and RNA levels are combined to one graph, which allows for the direct visualization of the linked effects. However, it is unclear from the Figure, what the 100% dashed line means. Is it mRNA or protein? There should be a reference line for both mRNA and protein.

2. Maybe the authors could extend the measurements between 0-2 h. This seems to be the most interesting time period in the experiments (maybe 45min, 1 h, 1:15h etc.). Differences between mRNAs (which even increase) and protein levels (which rapidly decrease could be visualized much better.

3. It is probably difficult to compare different 3' UTRs with one or more binding sites and conclude that one site guides mRNA decay and multiple sites translational repression. These are different 3' UTRs and many other factors could bind and influence expression. Ideally, the authors should generate a reporter containing one, two three artificial miRNA binding sites and measure mRNA decay vs. protein production. This issue should be discussed at the least.

Referee #2:

Animal miRNAs induce translational repression, mRNA deadenylation and degradation of mRNA targets. However how translational repression is achieved remains unclear. It is also not known whether translational repression precedes or follows deadenylation. In fact, it is not even known whether translational repression and deadenylation are coupled or represent two independent mechanisms.

In this manuscript Bethune et al. attempt to address this question using inducible miRNA reporters. Translational repression, deadenylation and decay were measured over time after induction. The authors conclude that translational repression precedes deadenylation and present a model in which deadenylation occurs as a consequence of translational repression. This model is surprising given that this and other groups have recently shown that the TNRC6s proteins, which are components of miRISC complexes, interact with deadenylases directly, and thus that deadenylation is a direct effect of miRNAs and not a consequence of a block in translation. Also there is ample evidence in the literature that deadenylation occurs in the absence of translation. Clearly, the authors observed different rates of translation inhibition and mRNA decay, and their data also suggest that at least for some reporters the rate of translational repression is faster. However, these observations do not provide evidence that deadenylation is a consequence of translation inhibition. For example, data presented in this manuscript do not rule out that deadenylation occurs independently of translational repression even if it happens later.

A major problem with this manuscript is that the authors set out to establish a precise order of events but the experimental approach allows them to measure precisely translational repression but not mRNA levels or deadenylation. Therefore the conclusion that translational repression occurs first is not supported by strong experimental evidence. In particular at early time points mRNA levels and deadenylation cannot be measured accurately with the methods used, e.g. qPCR and fractionation. Indeed it is unlikely that these methods can detect <2 fold reduction in mRNA levels or partial deadenylation. Thus the authors should use methods that allows them to measure mRNA levels and poly(A) tail length accurately.
Given these limitations, the paper does not provide significant novel insight and does not solve the controversy in the field.

Finally, there are some inaccuracies in the references to the literature:

1. In the discussion the authors mention that deadenylases interact directly with miRISCs and cite references 3-5 and also 13. However, reference 13 is misplaced in this context as reference 13 failed to detect any convincing interaction between deadenylases and TNRC6s proteins.
2. The authors do not give proper credit to the work described in references 7, 8, 9 and 10. These papers show that at least at the times they performed their measurements, mRNA destabilization

accounted for a large fraction of miRNA repression, which is what the authors see at later time points. None of these manuscripts ruled out the possibility of an earlier triggering event that repressed translation. In other words, the authors seem to exaggerate the differences and the controversy when in fact their data fits quite well with available data in the literature.
3. Reference 11 is also misrepresented. These authors did not exclude a contribution of translational repression. In fact, the model in reference 11 is very similar to the model proposed in this manuscript.
4. The authors claim that their model is similar to the model proposed by Djuranovic et al. As mentioned above, this is surprising as in this model deadenylation and decay happen mainly by default as a consequence of the repression of translation, what is inconsistent with a direct interaction with deadenylases.

Referee #3:

The study by Bethune et al. examines the relationship between translational inhibition and mRNA decay using inducible reporters. Despite intensive efforts to understand the mechanistic basis of these two processes, there is still controversy regarding the order of these events and the contribution of these processes to overall inhibition of genes by microRNAs. A high profile paper that has received much attention suggests that the vast majority of the microRNA effect is attributable to mRNA destabilization (Nature 466:835 2010). However, recent reports have raised questions bout the interpretation of the data in this report. Bethune et al. therefore addresses a critical issue in the microRNA field.

Bethune et al. use inducible reporters targeted by endogenous microRNAs to claim that translational inhibition is the dominant effect on newly synthesized mRNAs and that mRNA destabilization is the dominant effect at steady state (a time point when all the measurements were taken in Nature 466:835 2010). There is sufficient data in this paper to make the important contribution that translational inhibition is the dominant effect on newly synthesized mRNAs. However, the claim "that mRNA destabilization is the dominant effect at steady state" (abstract) is a misinterpretation of the data. A few changes are required prior to publication to allow accurate presentation of these studies. Specific recommendations are underlined below.

Fig. 1 described the system to assess these effects. The reporters using bi-directional promoters driving expression of two different reporters (one target of microRNAs, one not a target of microRNAs) is clever and representative for the process the authors wish to study. This data is acceptable with two minor caveats. First, this paper is about translational repression and mRNA stability. Therefore, characterization of this system should show the de-repression at protein and mRNA levels in Fig. 1D and 1E. Second, the responsiveness of this system to microRNAs was shown using siRNAs against the human GW182 isoforms, which have been implicated in microRNA activities. A better demonstration for the microRNA responsiveness of these reporters is to knock down Ago 1 and 2 as these are the established microRNA interacting proteins and thus better represent microRNA responses. This strategy was used previously by this group to demonstrate increased mRNA levels of genes targeted by microRNAs (NAR 34:4801, 2006). This is all the more important when considering that GW182 isoforms act downstream of Agos to recruit deadenylases. There are also roles for microRNA activity which does not depend upon GW182 and GW182 is implicated in other processes other than microRNA activity.

The main observation in support of authors' conclusion is Fig. 2B & C. Here induction leads to pure translational repression after 2 hours (there is no change in target mRNA but significant change in target protein levels, after which there is significant reduction of mRNA with marginal further reduction in protein levels). In Fig. 2B when comparing mRNA/protein levels at 2 hours and 4 hours, although target mRNA level drops 40% due to degradation, protein levels decrease only by 10%. This means that at least 30% of the mRNA that decreased was not making protein in the first place, indicating that it was translationally repressed at 2 hours and subsequently degraded at 4 hours. It is unclear whether the 10% decrease in protein levels was due to pure translational repression or mRNA degradation by 4 hours. From 4 hours to 6 hours, mRNA is unchanged while protein decreases 10%, indicating pure translational repression. From 8 hours to 24 hours, both protein and mRNA decrease by 10%, indicating that this additional 10% decrease in protein levels is

likely a result of the 10% decrease in mRNA levels. However, this 10% decrease in mRNA levels might have been a consequence of translational repression, as observed from 2 hours to 4 hours. In Fig. 2C comparing mRNA/protein levels at 2 hours and 3 hours, mRNA levels drop 20% yet the protein levels remain the same suggesting that that 20% mRNA was already translationally repressed at 2 hours and subsequently degraded without further influencing protein levels. Also in Fig. 2C comparing mRNA/protein levels at 8 hours and 24 hours, mRNA levels remain steady whereas the protein levels drop more than 20%. All these data show translation inhibition is the predominant effect even at later times when reduced mRNA levels are detected. The data show that there is a pool of mRNAs whose dramatic reduction confers little to overall inhibition. It is an unusual observation that protein levels are only slightly affected by large changes in mRNA levels. This suggests that many mRNAs are not involved in either translation or repression. The authors should comment on the likely causes and implications of these observations.

The central finding of the paper is that translational repression is the predominant effect on newly synthesized targets and also likely at steady state. This message is not clear and at times the description of the results is not accurate:

On page 5, second paragraph the authors state: "At each time-point, protein levels were lower than mRNA levels, indicating that silencing of the reporters is due to a combination of translational inhibition and mRNA decay." This conclusion is not supported by the data. It is true that at each time-point, protein levels were lower than mRNA levels. It is not true that silencing of the reporters is due to a combination of translational inhibition and mRNA decay. Fig. 2B, C, and D at time point 2 hours, the target mRNA is intact (i.e. 100% relative to the mutated mRNA target) whereas protein levels are already 20-40% lower than one hour earlier. On page 5 the next statement is "This indicates that miRNA-mediated repression starts shortly after mRNA export and first acts at the translational level, without inducing mRNA decay." The authors should correct these contradictory statements and remove the statement referring to mRNA export. The authors did not test mRNA export and do not have any way of knowing the kinetics of transport compared to translation inhibition. The authors should also accurately describe and interpret their data and state something along the lines of: effects on mRNA stability has little contribution over time to microRNA-mediated repression and the best interpretation of these data is that mRNA destabilization is a consequence of translational repression.

If the authors wish to test the claim that at steady state mRNA decay is the dominant effect, the authors could use a transcriptional inhibitor or use a repressible promoter to reduce effects from newly synthesized mRNAs and then introduce an anti-let-7 or anti-miR-30 or anti-miR-21 and re-test repression in the absence of new mRNA production.

On the surface these recommendations may seem minor but they are essential for an accurate interpretation of the data and need to be corrected prior to publication. It is also recommended that the authors comment on the timing of these studies in comparison to the timing of the studies in Nature 466:835 2010 which observed the opposite effects.

After cross-sending the referee reports Referee #3 added:

Reviewer #2 is technically right. When you get right down to it, Bethune et al cannot unambiguously claim that translational repression precedes deadenylation, even at 2 hours when the authors observe microRNA target protein reduction but no change in microRNA target mRNA levels. The authors used RT-PCR to assess mRNA levels which does not assess deadenylation and certainly partial deadenylation could have occurred which could have reduced translation of microRNA target mRNAs more than untargeted mRNAs. Personally, I think the most likely (parsimonious) explanation is that translational repression occurred first followed by deadenylation and then destabilization but an alternate order of event is possible.

To substantiate their claims (and make an important contribution to the literature), the authors should recover mRNAs using B-box element RNAs and Western blot for Agos, GW182 isoforms and deadenylases at the critical

timepoints (especially 2 hours). Filipowicz has published using these reporters. A complimentary experiment is over-expression of tagged Agos, GW182s and deadenylases followed by IP and RT-PCR/Northern and deadenylation assays. These experiments are feasible and can be accomplished within a reasonable period of time.

1st Revision - authors' response 04 May 2012

Response to referees comments.

Our responses are in blue colour, always below specific points raised by the referee.

Referee #1:

1. Do the contents of this manuscript report a single key finding? YES

The single key finding of this manuscript is a systematic analysis of the order of events during miRNA-guided gene silencing. They show that translational repression is the predominant miRNA function on newly synthesized targets. At steady state mRNA levels, deadenylation-dependent mRNA decay dominates over translational repression, as has been shown by others before.

2. Is the main message supported by compelling experimental evidence? YES

The message of the manuscript is clearly supported by the experimental data provided. There are some minor points that should be clarified (see point 6).

3. Have similar findings been reported elsewhere (e.g. on a closely related protein; in another organism or context)? NO

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1. In Figures 2B. C, D and 3B, protein and RNA levels are combined to one graph, which allows for the direct visualization of the linked effects. However, it is unclear from the Figure, what the 100% dashed line means. Is it mRNA or protein? There should be a reference line for both mRNA and protein.

This has been clarified in the figure legend. The dashed line applies for both protein and RNA levels (MUT protein and RNA levels are set to 100 at each time point)

2. Maybe the authors could extend the measurements between 0-2 h. This seems to be the most interesting time period in the experiments (maybe 45min, 1 h, 1:15h etc.). Differences between mRNAs (which even increase) and protein levels (which rapidly decrease could be visualized much better.

This is addressed on figure 3, where reporter expression levels are assessed every 20 min after a pulse of transcription.

3. It is probably difficult to compare different 3' UTRs with one or more binding sites and conclude that one site guides mRNA decay and multiple sites translational repression. These are different 3' UTRs and many other factors could bind and influence expression. Ideally, the authors should generate a reporter containing one, two three artificial miRNA binding sites and measure mRNA decay vs. protein production. This issue should be discussed at the least.

This speculation has been removed from the main text as it is not essential for the main message of this report.

Referee #2:

Animal miRNAs induce translational repression, mRNA deadenylation and degradation of mRNA targets. However how translational repression is achieved remains unclear. It is also not known whether translational repression precedes or follows deadenylation. In fact, it is not even known whether translational repression and deadenylation are coupled or represent two independent mechanisms.

In this manuscript Bethune et al. attempt to address this question using inducible miRNA reporters. Translational repression, deadenylation and decay were measured over time after induction. The authors conclude that translational repression precedes deadenylation and present a model in which deadenylation occurs as a consequence of translational repression.

1)
This model is surprising given that this and other groups have recently shown that the TNRC6s proteins, which are components of

miRISC complexes, interact with deadenylases directly, and thus that deadenylation is a direct effect of miRNAs and not a consequence of a block in translation.

1) The fact that TNRC6 proteins interact with deadenylases does not necessarily mean that deadenylation is a direct effect of miRNAs and not a consequence of a block in translation. Indeed, even if deadenylases are part of the miRISC, they may not be able to exert their exonuclease activity on poly(A) tails as long as translation is active. This would go along the idea that the mRNA closed loop structure protects both ends of mRNAs from degradation. The initial effect of miRISC on translation may then make the poly(A) tail more accessible to the recruited deadenylases. Deadenylation by the miRISC-associated deadenylases would then be a consequence of the miRNA-mediated inhibition of translation. In other words, though deadenylases are directly recruited by the miRISC and do stimulate deadenylation of target mRNAs, the initial effect of miRNAs on translation might facilitate their action by making the poly(A) tail more accessible. Alternatively, they could of course also work independently of the initial translational block. Our data do not exclude any of the two possibilities that we now keep clearly open in the description of our model (see comments below).

2)
Also there is ample evidence in the literature that deadenylation occurs in the absence of translation.

2) The referee makes allusion to a popular experiment which consists of expressing and analyzing a non-translatable RNA with miRNA sites. Indeed, such reporters are deadenylated and eventually degraded in a miRNA-dependent manner, which shows that deadenylation occurs in the absence of translation. This type of experiments has often been interpreted as evidence that deadenylation does not need translation to be inhibited since it is independent of translation. This interpretation is however misleading as a non-translatable RNA is actually a mimic of a translationally repressed RNA.

3)
Clearly, the authors observed different rates of translation inhibition and mRNA decay, and their data also suggest that at least for some reporters the rate of translational repression is faster. However, these observations do not provide evidence that deadenylation is a consequence of translation inhibition. For example, data presented in this manuscript do not rule out that deadenylation occurs independently of translational repression even if it happens later.

3) We agree with the criticism and indeed, kinetic analyses can not address whether deadenylation is a consequence of translational inhibition or if both reactions are completely independent but one is slower than the other.

We would like to point that in the original manuscript we did not claim that deadenylation was a consequence of the translational inhibition but only raised this possibility in our final model (page 8). The comment of the referee indicates that we were not clear enough and, in the revised version, we added a sentence describing the alternative mechanism (deadenylation independent of the initial translational block).

Nevertheless, we tried to address this issue as follows:

1. Reporters were analyzed under conditions where deadenylation is blocked

(either through knock-downs or expression of dominant negative deadenylase mutants). We observed that, upon interfering with deadenylation, reporters were still repressed at the protein level, but mRNA levels and poly(A) tail length were not affected (new Fig. 4, described on pages 6-7). Moreover, in kinetic analysis, we observed that, upon impaired deadenylation, the initial repression levels are maintained over time (whereas repression becomes stronger over time in control conditions) with stable mRNA levels and no detectable deadenylation. This strongly suggests that the initial translational block precedes and is completely independent from mRNA deadenylation and decay.

2. We have performed experiments where cell lines were treated with cyclohexamide or puromycin before inducting expression of the reporters. The aim was to analyze if the kinetic of deadenylation and decay would have been faster in the absence of translation (which would have implied that active translation tends to protect mRNAs from being deadenylated). However, the reporters were actually stabilized upon treatment with translation inhibitors. Such effects have been already described in the past in various systems and are difficult to explain. Therefore, these experiments remained inconclusive and were not included in this manuscript. To sum up, we were not able to technically address if deadenylation is a consequence or at least is stimulated by the initial translation inhibition, or if it happens independently of the translational block. We therefore explicitly keep both possibilities open in our final model (page 8).

4)

A major problem with this manuscript is that the authors set out to establish a precise order of events but the experimental approach allows them to measure precisely translational repression but not mRNA levels or deadenylation. Therefore the conclusion that translational repression occurs first is not supported by strong experimental evidence. In particular at early time points mRNA levels and deadenylation cannot be measured accurately with the methods used, e.g. qPCR and fractionation. Indeed it is unlikely that these methods can detect <2 fold reduction in mRNA levels or partial deadenylation. Thus the authors should use methods that allows them to measure mRNA levels and poly(A) tail length accurately. Given these limitations, the paper does not provide significant novel insight and does not solve the controversy in the field.

4) Using standard curves, we provide evidence that in the conditions we use, RT-qPCR is accurate and linear enough to detect and quantify subtle changes in mRNA levels in the time window where we detect no change (Shown in new Fig. S2). Hence, we are able to measure mRNA levels accurately enough to support our claims.

Moreover, we have measured poly(A) tail length with an orthogonal technique based on polyG/I extension and PCR amplification. Estimation of poly(A) tail length was performed using a high resolution microfluidic chip. With this technique, when comparing WT and MUT reporters, we also do not detect additional partial deadenylation at early time points when translational inhibition is already observed (shown in new Fig. 3B, described on pages 6-7).

The claim that initial translational inhibition is not coupled to partial deadenylation is further supported by the new experiments described in 3.1 above (and shown on new Fig. 4, described on pages 6-7) indicating that blocking deadenylation does

not affect the initial translational inhibition.

Finally, there are some inaccuracies in the references to the literature:

1. In the discussion the authors mention that deadenylases interact directly with miRISCs and cite references 3-5 and also 13. However, reference 13 is misplaced in this context as reference 13 failed to detect any convincing interaction between deadenylases and TNRC6s proteins.

Reference 13 has been moved to the right place in the sentence.

2. The authors do not give proper credit to the work described in references 7, 8, 9 and 10. These papers show that at least at the times they performed their measurements, mRNA destabilization accounted for a large fraction of miRNA repression, which is what the authors see at later time points. None of these manuscripts ruled out the possibility of an earlier triggering event that repressed translation. In other words, the authors seem to exaggerate the differences and the controversy when in fact their data fits quite well with available data in the literature.

This has been corrected in the main text to avoid misinterpretation of the data

3. Reference 11 is also misrepresented. These authors did not exclude a contribution of translational repression. In fact, the model in reference 11 is very similar to the model proposed in this manuscript.

The misrepresented reference 11 has been removed from the manuscript

4. The authors claim that their model is similar to the model proposed by Djuranovic et al. As mentioned above, this is surprising as in this model deadenylation and decay happen mainly by default as a consequence of the repression of translation, what is inconsistent with a direct interaction with deadenylases.

As described in Point 1, the direct interaction of miRISC with deadenylases does not prove that deadenylation happens by default. As discussed before, the poly(A) tail may need to be rendered more accessible through an initial translational block in order to make deadenylation more efficient. We (and others) could not address this point; therefore we keep both possibilities open in our final model.

Referee #3:

The study by Bethune et al. examines the relationship between translational inhibition and mRNA decay using inducible reporters. Despite intensive efforts to understand the mechanistic basis of these two processes, there is still controversy regarding the order of these events and the contribution of these processes to overall inhibition of genes by microRNAs. A high profile paper that has received much attention suggests that the vast majority of the microRNA effect is attributable to mRNA destabilization (Nature 466:835 2010). However, recent reports have raised questions bout the interpretation of the data in this report. Bethune et al. therefore addresses a critical issue in the microRNA field.

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5)
Fig. 1 described the system to assess these effects. The reporters using bi-directional promoters driving expression of two different reporters (one target of microRNAs, one not a target of microRNAs) is clever and representative for the process the authors wish to study. This data is acceptable with two minor caveats. First, this paper is about translational repression and mRNA stability. Therefore, characterization of this system should show the de-repression at protein and mRNA levels in Fig. 1D and 1E. Second, the responsiveness of this system to microRNAs was shown using siRNAs against the human GW182 isoforms, which have been implicated in microRNA activities. A better demonstration for the microRNA responsiveness of these reporters is to knock down Ago 1 and 2 as these are the established microRNA interacting proteins and thus better represent microRNA responses. This strategy was used previously by this group to demonstrate increased mRNA levels of genes targeted by microRNAs (NAR 34:4801, 2006). This is all the more important when considering that GW182 isoforms act downstream of Agos to recruit deadenylases. There are also roles for microRNA activity which does not depend upon GW182 and GW182 is implicated in other processes other than microRNA activity.

5) We have included data showing derepression at both protein and RNA levels (Fig. 1 D,E). We keep showing derepression upon knock down of GW182 isoforms as it is much more efficient than derepression upon Ago1/2 knock-down. Discussing it with colleagues in the field (e.g., G. Meister), we confirmed that the mild derepression we observe upon knocking down Ago1/2 is typical (about 30-40% recovery), and is most likely due to the fact that Ago2 is directly involved in RNAi as it is the slicer protein. Hence, expression of Ago2 can probably be knocked-down until a certain point at which RNAi is not efficient anymore. Thereafter, Ago2 levels most probably rise again until they reach a point where RNAi becomes efficient again. In other words, a sustainable knock-down of Ago2 seems to be inherently not possible to achieve through RNAi. By contrast, GW182 isoforms are necessary for miRNA-mediated repression but play no role in RNAi, thus by targeting them with siRNAs it is possible to get a durable knock-down, which we achieved. To our knowledge, GW182 isoforms are necessary for miRNA-mediated repression in mammalian cells (also confirmed here), and we are not aware of any known additional role of these proteins in other processes.
Of note, the derepression effects we show in the revised version of the manuscript are much more efficient than those we describe in the original submitted manuscript. This is because we switched to another transfection reagent (from nanofectin-siRNA to Hiperfect) and now reach much higher transfection efficiencies (the methods section was updated accordingly). Moreover, in the antimiRs experiment (Fig. 1E), we now use a combination of anti-miR21 and anti-miR590 to derepress the reck reporter as miR-21 and miR-590 share the same seed sequence and therefore can both target the reporter. This allowed us to completely

suppress repression of this reporter and is described in the figure legend.

6)
The main observation in support of authors' conclusion is Fig. 2B & C. Here induction leads to pure translational repression after 2 hours (there is no change in target mRNA but significant change in target protein levels, after which there is significant reduction of mRNA with marginal further reduction in protein levels). In Fig. 2B when comparing mRNA/protein levels at 2 hours and 4 hours, although target mRNA level drops 40% due to degradation, protein levels decrease only by 10%. This means that at least 30% of the mRNA that decreased was not making protein in the first place, indicating that it was translationally repressed at 2 hours and subsequently degraded at 4 hours. It is unclear whether the 10% decrease in protein levels was due to pure translational repression or mRNA degradation by 4 hours. From 4 hours to 6 hours, mRNA is unchanged while protein decreases 10%, indicating pure translational repression. From 8 hours to 24 hours, both protein and mRNA decrease by 10%,indicating that this additional 10% decrease in protein levels is likely a result of the 10% decrease in mRNA levels. However, this 10% decrease in mRNA levels might have been a consequence of translational repression, as observed from 2 hours to 4 hours. In Fig. 2C comparing mRNA/protein levels at 2 hours and 3 hours, mRNA levels drop 20% yet the protein levels remain the same suggesting that that 20% mRNA was already translationally repressed at 2 hours and subsequently degraded without further influencing protein levels. Also in Fig. 2C comparing mRNA/protein levels at 8 hours and 24 hours, mRNA levels remain steady whereas the protein levels drop more than 20%. All these data show translation inhibition is the predominant effect even at later times when reduced mRNA levels are detected. The data show that there is a pool of mRNAs whose dramatic reduction confers little to overall inhibition. It is an unusual observation that protein levels are only slightly affected by large changes in mRNA levels. This suggests that many mRNAs are not involved in either translation or repression. The authors should comment on the likely causes and implications of these observations.

6) We apologize for obviously not describing our data clearly enough as there is a misunderstanding here. The levels of the reporters are not directly comparable from one time point to another. Indeed, at each time point they are represented as % of the MUT reporter levels at this particular time point (in other words in % of the levels they would have had at this particular time point if they were not repressed). The levels of the MUT reporters (be it at RNA or protein levels) were arbitrary set to 100 at each time point, but in absolute values they get higher with time (see figure 1C). We chose to represent the levels of the reporters this way as it allows direct assessment of the contributions of translational repression and mRNA decay over time at each time point. Hence, for Fig. 2B, mRNA levels of the WT reporter do not drop 40% between 2h and 4h but at 2 h they represent 100% of the levels of the corresponding MUT (non-repressed) reporter at that time point, while at 4h they represent only 40% of the levels of the MUT reporter at that particular time point. Since levels of the MUT reporter are higher at 4 h than 2 h, it is not the same 100% at both time points. With this representation of the data, it is possible to say that at 2 h the 20% drop in protein levels (when compared to the MUT reporter at the same time point) is completely due to translation inhibition since relative RNA levels are 100% (of the non-repressed MUT reporter) at the same time point. On the other hand, at 4h, the 70% drop of protein levels (when compared to the levels of the MUT reporter at that particular time point) is to compare to the 40% drop in RNA level at the same time point. Hence at 4h, if translation efficiency was the same for the WT (repressed) and MUT (non-repressed) reporters, one would

expect to see a 40% drop in protein levels. Since the drop is higher, translation efficiency is lower for the WT reporter and thus repression is due to a combination of mRNA decay and translational inhibition. At this time point, mRNA decay represents 4/7 (57%) of the repressive effect seen at the protein level, and hence is already the dominant mechanism.

We now explain our normalization of the data more clearly in the main text (page 5).

8)
The central finding of the paper is that translational repression is the predominant effect on newly synthesized targets and also likely at steady state. This message is not clear and at times the description of the results is not accurate:

On page 5, second paragraph the authors state: "At each time-point, protein levels were lower than mRNA levels, indicating that silencing of the reporters is due to a combination of translational inhibition and mRNA decay." This conclusion is not supported by the data. It is true that at each time-point, protein levels were lower than mRNA levels. It is not true that silencing of the reporters is due to a combination of translational inhibition and mRNA decay. Fig. 2B, C, and D at time point 2 hours, the target mRNA is intact (i.e. 100% relative to the mutated mRNA target) whereas protein levels are already 20-40% lower than one hour earlier. On page 5 the next statement is "This indicates that miRNA-mediated repression starts shortly after mRNA export and first acts at the translational level, without inducing mRNA decay." The authors should correct these contradictory statements and remove the statement referring to mRNA export. The authors did not test mRNA export and do not have any way of knowing the kinetics of transport compared to translation inhibition.

7) Similar misunderstanding to that discussed (6). The data are correctly interpreted and it is true that for the hmga2 reporters, repression is due to a combination of translation repression and mRNA decay at later time-points. Indeed, this does not apply to early time-points when mRNA levels are not decreased and the sentence on page 5 has been corrected.
As to the mRNA export issue, since we observe an effect on translation as soon as we detect mRNA in the cytoplasmic fraction, it seemed reasonable to us to speculate that miRNA-mediated repression starts shortly after mRNA export. However, we have now removed it from the text, except one speculation on page 5.

8)
The authors should also accurately describe and interpret their data and state something along the lines of: effects on mRNA stability has little contribution over time to microRNA-mediated repression and the best interpretation of these data is that mRNA destabilization is a consequence of translational repression.

8) This comment is not valid considering responses to point 6 and 7. Our interpretation of the data is correct: as soon as mRNA decay is detectable is rapidly becomes the dominant mechanism explaining repression.

9)
If the authors wish to test the claim that at steady state mRNA decay is the dominant effect, the authors could use a transcriptional inhibitor or use a repressible promoter to reduce effects from newly synthesized mRNAs and then introduce an anti-

let-7 or anti-miR-30 or anti-miR-21 and re-test repression in the absence of new mRNA production.

9) With the correct interpretation of the data, this is not necessary. It is clear that mRNA decay is dominant at steady-state (see response to points 6 and 7).

10)
On the surface these recommendations may seem minor but they are essential for an accurate interpretation of the data and need to be corrected prior to publication. It is also recommended that the authors comment on the timing of these studies in comparison to the timing of the studies in Nature 466:835 2010 which observed the opposite effects.

10) This is now briefly discussed on page 6 of the main text. Due to space limitation we can unfortunately not include a more ample discussion.

After cross-sending the referee reports Referee #3 added:

11)
Reviewer #2 is technically right. When you get right down to it, Bethune et al cannot unambiguously claim that translational repression precedes deadenylation, even at 2 hours when the authors observe microRNA target protein reduction but no change in microRNA target mRNA levels. The authors used RT-PCR to assess mRNA levels which does not assess deadenylation and certainly partial deadenylation could have occurred which could have reduced translation of microRNA target mRNAs more than untargeted mRNAs. Personally, I think the most likely (parsimonious) explanation is that translational repression occurred first followed by deadenylation and then destabilization but an alternate order of event is possible.

11) See answer to point (3) of referee 2. We have shown the accuracy of our qPCR quantification and used a second technique plus additional controls to address the timing of poly(A) tail shortening and its influence on the initial translational inhibition.
As we could not show whether deadenylation is dependent or not on the initial translational block, we keep both possibilities open in our final model. Of note, in the original manuscript, we did not claim that deadenylation was a consequence of the initial translational block but only raised this possibility. Our claim was that translation inhibition precedes deadenylation which we believe is supported by our data.

12)
To substantiate their claims (and make an important contribution to the literature), the authors should recover mRNAs using B-box element RNAs and Western blot for Agos, GW182 isoforms and deadenylases at the critical timepoints (especially 2 hours). Filipowicz has published using these reporters. A complimentary experiment is over-expression of tagged Agos, GW182s and deadenylases followed by IP and RT-PCR/Northern and deadenylation assays. These experiments are feasible and can be accomplished within a reasonable period of time.

12) We have not performed experiments consisting of RNA pulldowns and mass spec analysis of associated proteins as a function of time. Such experiments would not address the main concerns of the referees as recent data indicate that

deadenylase complexes may not only be involved in deadenylation but also in the translational repression itself (data from the lab of E. Izaurralde and of ours (references 3 and 4, see also the report by Cooke et al, JBC 2010 from M. Wicken's lab). Hence, time of recruitment of GW182 or the CCR4-NOT complex can not be regarded as evidence supporting either deadenylation or translational repression. Moreover, this type of experiments are not trivial to perform due to low amounts of the starting material, poor recovery yields of RNA pull-downs, and low amounts of final recovered material to be used for Western or mass spec analyses. As a matter of fact, we have attempted some time ago to perform this type of experiments but failed to recover enough material to proceed with analyses.

2nd Editorial Decision 20 May 2012

Thank you for the submission of your revised manuscript to our journal. We have now received the enclosed reports from the referees. As you will see, both referees 1 and 2 support now acceptance of the manuscript for publication in our journal, while referee 3 still has a few suggestions that I would like you to address before we can proceed with the official acceptance of your manuscript.

I look forward to seeing a new revised version of your manuscript as soon as possible.

Yours sincerely,

Editor
EMBO Reports

REFEREE REPORTS:

Referee #1:

In their revised version of the manuscript entitled "Kinetic analysis reveals successive steps leading to miRNA-mediated silencing in mammalian cells", Bethune et al. have addressed the few minor points that I had raised on their previous version. I am satisfied with the revised version.

Referee #2:

The authors have included additional data and addressed most of the criticisms, therefore the paper is now acceptable for publication.

Referee #3:

Bethume et al. addressed several concerns raised in the initial review. It is notable that in this kinetic analysis timepoints cannot be compared to each other, even based on percentage reductions. In their reply they state that "The levels of the reporters are not directly comparable from one time point to another." Kinetic analysis is meant to compare rates of change at different timepoints. Apparently this is not possible in this kinetic analysis.

The representation of the data in Figs. 2B, C, D; Fig. 3C; and Figs. 4A, B are misleading. The data show accumulation rates of protein and RNA over time slower for repressed than for unrepressed

mRNAs. The bar graphs imply reduced protein and RNA levels over time. This is not the case. There is a more accurate way to present these data as they do in Fig. 1B, C and 2A. By graphing the accumulation of protein and RNA over time, the reader will be able to more readily compare effects on protein and mRNAs at different times. Points on graphs for mRNA and protein accumulation should be marked for percentage reduction at a given time point.

There are few inconsistencies between figures. For example, the authors quantify polyA tail length in Fig. 3. At 60 mins, the degree of full length WT polyA tail is ~ 2/3 full length MUT polyA tail (Fig. 3B right panel) but there is no change in full length WT polyA tail relative to full length MUT polyA tail in Fig. 3C. Similarly, at 180 mins., the degree of full length WT polyA tail is ~ 1/8 full length MUT polyA tail (Fig. 3B right panel). However, at 180 mins., full length WT polyA tail is ~ 1/2 full length MUT polyA tail according to Fig. 3C. The authors should check their quantitation here.

Overall, data presented by Bethume et al supports the authors' conclusion that translation repression represents the main mechanism for microRNA operation at early time points (1 hour) and that deadenylation and degradation occur at later times (3 hours). While a couple of points are still not clear, these data support the major conclusion and are consistent with recent previous reports coming to similar conclusions.

2nd Revision - authors' response 21 May 2012

Thank you for getting back to us with the referees' feedback. We have addressed the last comments of referee #3 as follow: an additional supplementary figure (Fig. S3) shows the accumulation of the reporters at the mRNA level over time for all three cell lines. Similar data showing accumulation of the reporters at the protein level were already shown in the submitted manuscript on Figures 1B, C and 2A. Now the reader can see the data both the way we presented them (showing the effects of miRNAs at the protein and RNA levels at each individual time point) and the way referee #3 favors (showing the raw protein and mRNA levels over time).

For the inconsistency pointed by the referee, we would like to point out that, as stated in the text, Fig. 3B represents qualitative data revealing the nature (but not the amount) of the population of polyadenylated reporters. As this method (polyG/I extension, followed by end-point PCR) is not quantitative it can not and was not used for such purposes (even including an internal quantification control would be at best semi-quantitative). As indicated in the text, the quantification shown on Fig. 3C is based on the fractionation procedure shown on Fig. S4 where the amounts of fully polyadenylated reporters were quantified by qPCR. This has now been further explained in the material and methods section.

We hope you will agree that we now addressed all the referees concerns and that the manuscript is suitable for publication in EMBO Reports.

3rd Editorial Decision 22 May 2012

I am very pleased to accept your manuscript for publication in the next available issue of EMBO reports.

Thank you for your contribution to EMBO reports and congratulations on a successful publication. Please consider us again in the future for your most exciting work.

Yours sincerely,
Editor
EMBO Reports

