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Multilayer checkpoints for microRNA authenticity during RISC assembly

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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

28 February 2011

Thank you for the submission of your manuscript to EMBO reports. Please accept my apologies for the delay in getting back to you but we have only now received the full set of referee reports that is copied below.

As you will see, while referee 1 is more critical, referees 2 and 3 feel that the study is potentially suitable for publication in EMBO reports. The referees do raise some important concerns though, which need to be addressed before the manuscript can be considered for publication in our journal.

Both referees 1 and 3 point out that the physiological significance of the results needs to be strengthened and they suggest different experiments to do so. Both referees also think that the phosphorylation efficiency of 5' dT-duplexes needs to be shown experimentally. Referee 1 further indicates missing controls and quantifications and feels that the generalization of the findings needs to be experimentally confirmed. Referees 2 and 3 mention that the proposed model should be discussed in light of published data and referee 2 further indicates that the conclusion that the thermodynamic asymmetry rule is secondary needs to be corroborated by additional data.

Given these evaluations and the constructive referee comments, I would like to give you the opportunity to revise your 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. 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 apologize again for my late reply and I look forward to seeing a revised version of your manuscript when it is ready.

Yours sincerely

Editor
EMBO Reports

REFEREE REPORTS:

Referee #1:

Kawamata et al.

Multilayer checkpoints for microRNA authenticity during RISC assembly

In this manuscript, Kawamata et al. first analyse the function of the 5' phosphates of a miRNA duplexes in RISC loading. Using a native gel shift assay, which they have published before, they show that the 5' phosphate is required for RISC loading. They further show that the 5' nucleotide is not only required for mature RISC formation but is also important for pre-RISC formation. In addition, the authors analyze the function of the 5' nucleotide in pre- and mature RISC assembly. They suggest that the 5' nucleotide is more important when mismatches are present either in the 5' or the 3' part of the duplex.

This short manuscript is mainly an extension of previous work published by the same authors. However, it has been shown before that the 5' phosphate is required for RISC formation and consequently for its activity. Furthermore, many of the experiments are not as clear as the authors state.

Specific points

1. The authors claim that a 5' dT is less efficiently phosphorylated as has been shown before. The authors should show phosphorylation efficiency in their own experiments or better use a 5' methylated dT.
2. This manuscript is solely based on an in vitro gel shift assay. The authors need to test their findings in a physiological relevant system. For example, they should generate reporters for the small RNAs that they use and measure activity in a tissue culture system.
3. The gel shifts are generally not fully convincing. For example, Figure 1D is of rather low quality. They claim that the complexes marked with asterisks are unrelated small RNA complexes. Therefore, it would be expected that these complexes are similar in all experiments. However, in some experiments these complexes are visible and in other experiments they are not. Lanes 2 and 4 of Figure 1D are absolutely empty. The authors should load and show the free labeled RNA in their assays, otherwise it is not possible to distinguish between degradation of the RNA and RISC loading.
4. All experiments are based on a few single duplexes. To make such general statements, it would be important to repeat the experiments with a number of different sequences.
5. In Figure 2, the authors claim that pre-RISC formation is rather independent of the 5' terminal nucleotide for duplex A (although there are clear differences shown on the gel). Using an excess of cold duplexes the authors monitor mature RISC formation and find that mature RISC formation

depends on the 5' nucleotide. Generally, without a solid quantification of the radioactive signals of pre and mature RISC it is not possible to conclude anything from these figures.

6. Why did the authors use duplex A, which forms pre-RISC only in Figure 1, for their assays on mature RISC formation shown in Figure B?

7. The authors propose a model, in which the identity of the 5' nucleotide is important when mismatches are present within the miRNA duplex. The authors should appreciate that Ago2 loads small RNAs that originate from rather perfectly matched precursors and furthermore, also Ago2-bound small RNAs show a clear U bias at their 5' ends.

Referee #2:

The authors describe multilevel checkpoints for microRNA authenticity during RISC assembly. Using a biochemical approach the authors show that 5' phosphate is required for RISC loading. Furthermore the authors provide evidence that the 5' nucleotide of the guide and base-pairing function as additional anchors.

Minor issues:

1. page 3. line 6 : reference Hafner et al. (2010) Cell should be added to the list of reference describing miRNAs are load in all four human Agos.

2. On page 6 the authors argue that the 5' phosphate of the guide is strictly required and precedes the "thermodynamic" asymmetry rule. If this is indeed the case, a pre-RISC loading experiment (as described in Figure 2C+D) using radiolabeled duplex 3 of Figure 1D should be repeated in the presence of increasing molar amounts of cold duplex 4 of Figure 1A or duplex 3 of Figure 1D in a competition experiment. If the asymmetry rule is secondary, either of the two cold duplexes should compete in the same manner at equal molar amounts.

3. page 8+9. the authors should discuss their finding that the 5' nucleotide function as an anchor in the light of Frank et al (2010) Nature, in which the structure of the human Ago2 MID domain shows a preference for U and A.

4. Figure legend 2.

Probably it should say in (E) and (F) the same experiments as in (C) rather (B).

Referee #3:

The authors of the manuscript "Multilayer checkpoints for microRNA authenticity during RISC assembly" give novel insights into the mechanism and requirements of miRNAs to become loaded into the Ago1-RISC complex in *Drosophila* embryo extracts. The experiments described herein use a native gel system, previously published by the same authors in NSMB, that makes possible to distinguish pre-RISC (containing miRNA-miRNA* duplexes) from mature RISC (harboring mature miRNA) assembly. Two open questions concerning main features of miRNAs, which is the presence of a 5' monophosphate and the frequent occurrence of a uridine at position 1 of the guide strand, were addressed in this manuscript. One of the findings is the requirement of the 5' Phosphate on the guide strand for its incorporation into mature Ago1-RISC, a fact that has been well studied in the past for siRNA incorporation into Ago2-RISC in *Drosophila* as well as for siRNA incorporation into mammalian RISC. However, the key finding is the neglect of the asymmetry rule by Ago1-RISC (in contrast to Ago2-RISC), meaning the incorporation of a 5' Phosphate-containing passenger strand into mature RISC originated from a duplex in which the guide strand harbors a non-phosphorylatable 5' OH. The authors describe also other checkpoints, such as the identity of the first nucleotide of the guide strand as well as the base-pairing status in the seed and the 3' mid-region, that are sensed by RISC in order to ensure accurate loading of miRNAs. Overall the manuscript describes novel aspects of miRNA assembly into RISC (at least) *in vitro*. However, there are certain issues that have to be addressed by the authors.

1) The authors do not show any data concerning the impairment in phosphorylation of 5' dT-

duplexes by the cellular RNA kinase, but rather refer to 'Nykanen et al., 2001' and "data not shown". For completeness of the manuscript it would be important to provide these data (as Supplemental information) since RISC assembly in dependency of the 5' Phosphate group is a crucial part of the paper.

2) The authors state as a possible explanation for the observation in Fig. 1C that "... the 5' phosphate of the guide strand is dispensable for RISC loading but becomes essential after unwinding; therefore duplex A and B were loaded into Ago1 normally..." This statement does not hold true and thus should not be given since duplex A2 (Fig. 1C) which (in addition to a 5' dT-OH on the passenger) does not have a 5' Phosphate on the guide fails to become incorporated into pre-RISC.

3) Figs. 2C and 2D: In order to claim that the identity of the 5' nucleotide does not critically impact on pre-RISC loading but is decisive for the efficiency of mature RISC formation, it is crucial to show a quantification of the bands representing pre-Ago1-RISC (Fig. 2C) and then determine the ratio between pre-Ago1-RISC and mature Ago1-RISC over time (Fig. 2D).

4) An important claim the authors made is the necessity of having a Uridine base at position 1 in the guide strand of a miRNA duplex with mismatches in the 3' mid- or seed region (in addition to a central mismatch) for the assembly into pre-Ago1-RISC. Duplexes with unfavorable bases are only incorporated into pre-Ago1-RISC when there is base-pairing in the seed and 3'-mid region (Fig. 2D), functioning as an additional anchor for Ago1. However, have the authors performed RISC assembly experiments using a duplex containing base mismatches in all three regions (central, seed and 3' mid)? This would be an important control experiment, the prediction being that less unfavorable nucleotides at position 1 (for example Adenine, see Figs. 2E and F) would now entirely become unfavorable in case the anchor functions in cumulative way, and for example exclusively duplexes containing Uridine at position 1 would become assembled. As most endogenous miRNAs contain mismatches in all three regions (central, seed and 3' mid), performing such an experiment could also reflect the significance of the results obtained by the author's in vitro system for the in vivo situation.

5) Apart from the miRNA assembly checkpoints described by the authors (5' Phosphate and identity of the nucleotide at position 1), the 2-nt 3' overhang of a miRNA could also represent a main checkpoint. Have the authors ever checked pre- and mature RISC assembly using blunt and overhang structures other than 2-nt 3' overhang? Including these results would complete their analysis on the main structural features of miRNAs for the current manuscript.

6) Some minor issues:

Which target mRNAs did the authors use for Figs. 2C-F? It is not mentioned in the Methods section.

Supplementary Table 1: All passenger strands that contain a 3' end-label carry a 3' H and not a 3' OH group. Please correct in the scheme.

1st Revision - authors' response

22 April 2011

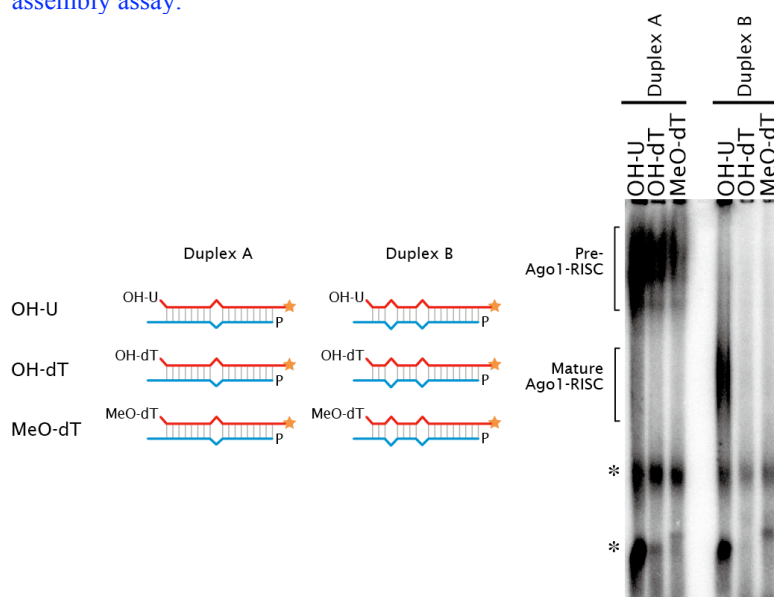
Point-by-point responses to the Referees' comments

Referee #1:

1. The authors claim that a 5' dT is less efficiently phosphorylated as has been shown before. The authors should show phosphorylation efficiency in their own experiments or better use a 5' methylated dT.

We have experimentally confirmed that 5' dT RNAs are barely phosphorylated in our standard assay conditions, which is now shown in the lower panels of Figure 1 (note that 5'-P-dT RNA migrates faster than 5'-OH-dT RNA) and Supplementary Figure 1B. In addition, as shown below,

we have confirmed that 5'-MeO-dT duplex and 5'-OH-dT duplex behave identically in our RISC assembly assay.



2. This manuscript is solely based on an in vitro gel shift assay. The authors need to test their findings in a physiological relevant system. For example, they should generate reporters for the small RNAs that they use and measure activity in a tissue culture system.

We appreciate the Referee's comment. Because flies have two Ago proteins with distinct RNA preference and functionality, a reporter assay in a fly cell culture system (typically S2 cells are used) to precisely measure the Ago1-RISC activity requires near-complete knockdown of Ago2 and/or Dcr-2, simultaneously with the reporter assay. Although this may be possible, given that a small fraction of Ago2 and/or Dcr-2 could still be present after knockdown and that Ago2 is so catalytically active, a causal interpretation of such a fastidious experiment would be complicated. Instead, for Figure 1, we have performed cleavage assays of complementary target RNAs (it is well established that target cleavage assays reflect in vivo relevance) in *dcr-2* null embryo lysate, which precisely measures the amount of mature Ago1-RISC. As expected, the results from the cleavage assays (now shown in Supplementary Figure 2A) agree well with the gel shift assays, reinforcing the validity of the gel shift assays. Unfortunately, cleavage assay for Figure 2 is technically challenging, because we will need to synthesize a 5'-capped, 5-nitroindole-containing target RNA (5'-cap stabilizes the target RNA, which is obligatory for target cleavage assay in lysate, and 5-nitroindole normalizes the contribution of the 5' nucleotide for target recognition). We would like to note that all of our current and previous results strongly support that the gel shift assays quantitatively reflect the amount of mature RISCs formed (Kawamata et al., 2009 and Yoda et al., 2010). Moreover, the gel shift assay is the only available experimental system that can differentiate and quantitate pre-RISCs and mature RISCs, which is vital for this study.

3. The gel shifts are generally not fully convincing. For example, Figure 1D is of rather low quality. They claim that the complexes marked with asterisks are unrelated small RNA complexes. Therefore, it would be expected that these complexes are similar in all experiments. However, in some experiments these complexes are visible and in other experiments they are not. Lanes 2 and 4 of Figure 1D are absolutely empty. The authors should load and show the free labeled RNA in their assays, otherwise it is not possible to distinguish between degradation of the RNA and RISC loading.

Our unpublished data suggest that these complexes with asterisks are involved in RNA degradation and, as expected from the nature of nucleases, do have preferences for RNA structures and/or sequences. However, we have previously confirmed that formation of these complexes does not affect the Ago1-RISC assembly pathway (Kawamata et al., 2009). Therefore, the presence or absence of these complexes does not preclude our analysis. In addition, we have experimentally confirmed the integrity of RNAs after incubation in lysate, excluding the possibility that radiolabeled RNAs were degraded preferentially in some experiments but not others. The data are

now included in Figure 1 (lower panels). Please also see our response to the comment 5 by the Referee #3.

4. All experiments are based on a few single duplexes. To make such general statements, it would be important to repeat the experiments with a number of different sequences.

We have performed target cleavage assays for a completely unrelated duplex (derived from the firefly luciferase sequence), which is now shown in Supplementary Figure 2B. The data well supported our conclusion.

5. In Figure 2, the authors claim that pre-RISC formation is rather independent of the 5' terminal nucleotide for duplex A (although there are clear differences shown on the gel). Using an excess of cold duplexes the authors monitor mature RISC formation and find that mature RISC formation depends on the 5' nucleotide. Generally, without a solid quantification of the radioactive signals of pre and mature RISC it is not possible to conclude anything from these figures.

Quantifications for Figure 2 with error bars from 3–5 independent trials are now shown in Supplementary Figure 4.

6. Why did the authors use duplex A, which forms pre-RISC only in Figure 1, for their assays on mature RISC formation shown in Figure B?

Because duplex A unwinds more slowly than duplex B, duplex A makes it easier to quantitatively monitor the transition from pre-Ago1-RISC to mature Ago1-RISC. We have added this statement in the text.

7. The authors propose a model, in which the identity of the 5' nucleotide is important when mismatches are present within the miRNA duplex. The authors should appreciate that Ago2 loads small RNAs that originate from rather perfectly matched precursors and furthermore, also Ago2-bound small RNAs show a clear U bias at their 5' ends.

To our knowledge, fly Ago2 prefers 5' C rather than 5' U (Ghildiyal et al., 2009; Okamura et al., 2009; Czech et al., 2009). Moreover, we do not intend to argue that the 5'-nucleotide identity is important only when internal mismatches are present. Rather, we argue that 5'-nucleotide identity and internal base-pairing can each act as an additive anchor.

Referee #2:

1. page 3. line 6 : reference Hafner et al. (2010) Cell should be added to the list of reference describing miRNAs are load in all four human Agos.

This paper is now cited in the text.

2. On page 6 the authors argue that the 5' phosphate of the guide is strictly required and precedes the "thermodynamic" asymmetry rule. If this is indeed the case, a pre-RISC loading experiment (as described in Figure 2C+D) using radiolabeled duplex 3 of Figure 1D should be repeated in the presence of increasing molar amounts of cold duplex 4 of Figure 1A or duplex 3 of Figure 1D in a competition experiment. If the asymmetry rule is secondary, either of the two cold duplexes should compete in the same manner at equal molar amounts.

We appreciate the Referee's comment. Although the proposed competition experiment is attractive, our standard assay system (using *dcr-2* embryo lysate) contains substantial amount of endogenous siRNAs and microRNAs, which already compete with the radiolabeled duplex and may thus potentially complicate the quantitative interpretation of the experiment. Moreover, we envision that the P/OH duplex and the (flipped) OH/P duplex are not necessarily incorporated into pre-Ago1-RISC with completely identical efficiency, because their sequences and structures in the steric context toward Ago1 are different, which can influence anchoring points other than those we identified in this study. Instead, we have performed simple target cleavage assays, which agree well with the native gel analyses and confirm that the 5' phosphate of the guide strand is strictly required and the roles of the two strands can be flipped (Supplementary Figure 2).

3. page 8+9. the authors should discuss their finding that the 5' nucleotide function as an anchor in the light of Frank et al (2010) Nature, in which the structure of the human Ago2 MID domain shows a preference for U and A.

We have added discussion in the text.

4. Figure legend 2.

Probably it should say in (E) and (F) the same experiments as in (C) rather (B).

We appreciate the Referee's comment. We have amended the Figure Legend.

Referee #3:

1) The authors do not show any data concerning the impairment in phosphorylation of 5' dT-duplexes by the cellular RNA kinase, but rather refer to 'Nykanen et al., 2001' and "data not shown". For completeness of the manuscript it would be important to provide these data (as Supplemental information) since RISC assembly in dependency of the 5' Phosphate group is a crucial part of the paper.

We have experimentally confirmed that 5' dT RNAs are barely phosphorylated in our standard assay conditions, which is now shown in the lower panels of Figure 1 (note that 5'-P-dT RNA migrates faster than 5'-OH-dT RNA) and Supplementary Figure 1B. Please also see our response to comment 1 by Referee #1.

2) The authors state as a possible explanation for the observation in Fig. 1C that "... the 5'; phosphate of the guide strand is dispensable for RISC loading but becomes essential after unwinding; therefore duplex A and B were loaded into Ago1 normally..." This statement does not hold true and thus should not be given since duplex A2 (Fig. 1C) which (in addition to a 5' dT-OH on the passenger) does not have a 5' Phosphate on the guide fails to become incorporated into pre-RISC.

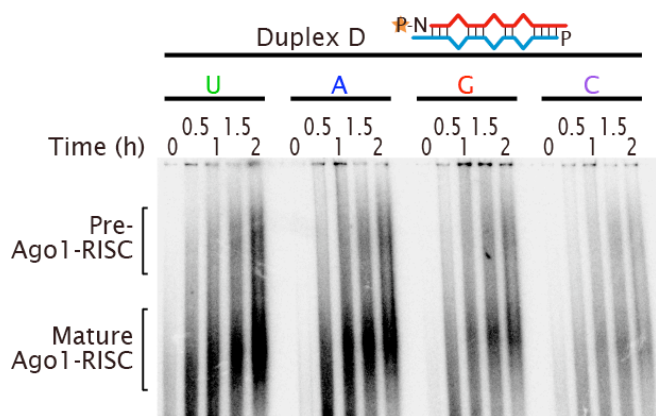
We appreciate the Referee's comment. We have deleted this statement.

3) Figs. 2C and 2D: In order to claim that the identity of the 5' nucleotide does not critically impact on pre-RISC loading but is decisive for the efficiency of mature RISC formation, it is crucial to show a quantification of the bands representing pre-Ago1-RISC (Fig. 2C) and then determine the ratio between pre-Ago1-RISC and mature Ago1-RISC over time (Fig. 2D).

Quantifications for Figure 2 with error bars from 3–5 independent trials are now shown in Supplementary Figure 4.

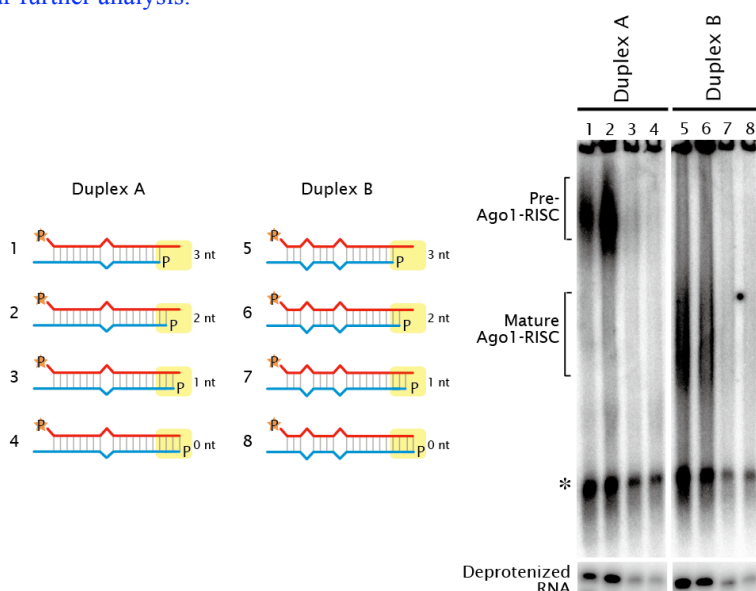
4) An important claim the authors made is the necessity of having a Uridine base at position 1 in the guide strand of a miRNA duplex with mismatches in the 3' mid- or seed region (in addition to a central mismatch) for the assembly into pre-Ago1-RISC. Duplexes with unfavorable bases are only incorporated into pre-Ago1-RISC when there is base-pairing in the seed and 3'-mid region (Fig. 2D), functioning as an additional anchor for Ago1. However, have the authors performed RISC assembly experiments using a duplex containing base mismatches in all three regions (central, seed and 3' mid)? This would be an important control experiment, the prediction being that less unfavorable nucleotides at position 1 (for example Adenine, see Figs. 2E and F) would now entirely become unfavorable in case the anchor functions in cumulative way, and for example exclusively duplexes containing Uridine at position 1 would become assembled. As most endogenous miRNAs contain mismatches in all three regions (central, seed and 3' mid), performing such an experiment could also reflect the significance of the results obtained by the author's in vitro system for the in vivo situation.

We appreciate the Referee's comment. As shown below, our data suggest that such a duplex containing mismatches in all three regions is unwound so quickly in vitro even at 10°C that we cannot quantitatively determine the amount of pre-Ago1-RISCs formed. For mature Ago1-RISCs, we observed a preference of U≥A>G≥C as expected from the in vivo situation, but it does not seem like 5'-U duplex is "exclusively" assembled into RISC (the trend is no stronger than duplex B or duplex C). Taken together, although it is clear that "5' favorable nucleotide" and "the base-pairing status in the seed region and the middle of the 3' region" serve as additive anchors, we cannot determine whether or not "a mismatch in the seed region" and "a mismatch in the 3' region" function cumulatively. Given this limitation, we have added a statement "at this point it is difficult to quantitate the weighing of each anchor" in the text.



5) Apart from the miRNA assembly checkpoints described by the authors (5' Phosphate and identity of the nucleotide at position 1), the 2-nt 3' overhang of a miRNA could also represent a main checkpoint. Have the authors ever checked pre- and mature RISC assembly using blunt and overhang structures other than 2-nt 3' overhang? Including these results would complete their analysis on the main structural features of miRNAs for the current manuscript.

We have tried a blunt duplex and duplexes with different overhang structures. However, the stability of these duplexes in lysate was very heterogeneous as shown below (lower panels), which precluded our further analysis.



This contrasted with the duplexes with 2-nt 3' overhangs used in this study, which have comparable stability in lysate (the data are now included in Figure 1, lower panels).

6) Some minor issues:

Which target mRNAs did the authors use for Figs. 2C-F? It is not mentioned in the Methods section.

We used a 31-nt 2'-O-methyl antisense oligonucleotide with a 5-nitroindole at position 26, right across from the 5' nucleotides of the guide strands. This information is now added in the Methods section.

Supplementary Table 1: All passenger strands that contain a 3' end-label carry a 3' H and not a 3' OH group. Please correct in the scheme.

We appreciate the Referee's comment. We have amended this.

2nd Editorial Decision

23 May 2011

Thank you for the submission of your revised manuscript to EMBO reports. I apologize for the delay in getting back to you but we have only recently received the enclosed reports from the referees that were asked to assess it. Given that referee 1 still has several concerns, I asked referee 3 to comment on these. Referee 3 agrees with some of the concerns about low quality images and requests now that the quality of supplementary figure 1B needs to be improved and that full gels for figure 2C-F need to be shown. The referee also indicates where further discussion of the data is necessary. I would like you to address and incorporate these suggestions in a revised manuscript before we can proceed with the official acceptance of your manuscript.

I also noticed that the figure legend for supplementary figure 4 does not define the error bars, please include this information. The supplementary tables 1-3 further need to be removed from the main manuscript file to the supplementary information.

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:

Kawamata et al.

Multilayer checkpoints for microRNA authenticity during RISC assembly

The authors have revised their manuscript and responded to all the points that I had raised on the previous version of the manuscript. In general, my points have been adequately addressed.

However, I still think that some of the images are of low quality. The authors show now in Figure 1D, that the RNA is not degraded. However, it is not clear why lane 4, for example, is an empty lane. All unrelated bands should be there as well. This holds true for a number of Figures.

Sometimes the authors show gels with unrelated bands. Other gels have been cut and only the expected complexes are shown.

The fact that a 5' phosphate is required for RISC loading is not new, although the authors add some novel details to it. The second part is novel, but the data is not as clear as the authors claim.

Referee #3:

The authors have addressed my concerns in full detail.
In my opinion, the manuscript is now ready for publication in EMBO Reports.

Additional comments from Referee 3 in relation to Referee's 1 criticisms.

Critical observations:

1) In response to Referee #1: lane 4 is empty because the asymmetry and the lack of 5'P play against loading onto the passenger strand, the labeled one. It is in fact the worst configuration regarding pre- and mature RISC formation. The same goes for lane 2, with double OH. None of the remaining duplexes in Fig. 1 show that combination.

2) Supp. Fig. 1B: poor quality, the lack of phosphorylation is not clear enough.

3) Please show full gels for Fig. 2 C-D-E-F to be able to visualize fast migrating complexes (see below).

Observations regarding the unspecific, asterisk-labeled complexes:

1) In Fig. 1B, the lower complexes seem related to the bulge in position 5, and the presence or absence of P/OH plays no role.

2) In Supp. Fig. 1, duplexes 2 and 4 show strong signals for lower complexes, depending on dT or U; in this case, the extra bulge does not matter. Still in Supp. Fig. 1, duplex 1 is similar to duplex 2 and duplex 3 is similar to duplex 4 for the formation of pre-RISC or mature-RISC, indicating that the base does not matter as long as the Phosphate group is present.

The authors may want to comment on these issues in their final response.

2nd Revision - authors' response

24 May 2011

Point-by-point response to the Referees' comments

Referee #1:

However, I still think that some of the images are of low quality. The authors show now in Figure 1D, that the RNA is not degraded. However, it is not clear why lane 4, for example, is an empty lane. All unrelated bands should be there as well. This holds true for a number of Figures.

Sometimes the authors show gels with unrelated bands. Other gels have been cut and only the expected complexes are shown.

As Referee #3 clearly points out, duplexes 2 and 4 in Figure 1D are very "bad" duplexes for pre-RISC or mature RISC detection using the radiolabeled strand. We have now shown full gels for Figs. 2C-F, and clearly stated that there is no correlation between formation of the nonspecific asterisk-labeled complexes and formation of pre-RISC and mature RISC in the Figure Legends.

Referee #3:

1) In response to Referee #1: lane 4 is empty because the asymmetry and the lack of 5'P play against loading onto the passenger strand, the labeled one. It is in fact the worst configuration regarding pre- and mature RISC formation. The same goes for lane 2, with double OH. None of the remaining duplexes in Fig. 1 show that combination.

We appreciate the Referee's clarification.

2) Supp. Fig. 1B: poor quality, the lack of phosphorylation is not clear enough.

We have adjusted the contrast of Supplementary Fig. 1B. We have also added a new data showing that 5'-OH-dT duplexes behave similarly to phosphorylation-resistant 5'-MeO-dT duplexes in our RISC assembly assays (Supplementary Fig. 1C; originally included in our previous response to the Referees).

3) Please show full gels for Fig. 2 C-D-E-F to be able to visualize fast migrating complexes (see below).

We have now shown full gels for Figs. 2C–F, and separated the model in Fig. 3.

Observations regarding the unspecific, asterisk-labeled complexes:

1) In Fig. 1B, the lower complexes seem related to the bulge in position 5, and the presence or absence of P/OH plays no role.

2) In Supp. Fig. 1, duplexes 2 and 4 show strong signals for lower complexes, depending on dT or U; in this case, the extra bulge does not matter. Still in Supp. Fig. 1, duplex 1 is similar to duplex 2 and duplex 3 is similar to duplex 4 for the formation of pre-RISC or mature-RISC, indicating that the base does not matter as long as the Phosphate group is present.

The authors may want to comment on these issues in their final response.

We appreciate the Referee's clarification. As the Referee points out, there is no correlation between formation of the nonspecific asterisk-labeled complexes and formation of pre-RISC and mature RISC. We have clearly stated this point in the Figure Legends.

Correspondence – Referee #3's comments

25 May 2011

1) In response to Referee #1: lane 4 is empty because the asymmetry and the lack of 5'P play against loading onto the passenger strand, the labeled one. It is in fact the worst configuration regarding pre- and mature RISC formation. The same goes for lane 2, with double OH. None of the remaining duplexes in Fig. 1 show that combination.

We appreciate the Referee's clarification.

Referee #3: Fine.

2) Supp. Fig. 1B: poor quality, the lack of phosphorylation is not clear enough.

We have adjusted the contrast of Supplementary Fig. 1B. We have also added a new data showing that 5'-OH-dT duplexes behave similarly to phosphorylation-resistant 5'-MeO-dT duplexes in our RISC assembly assays (Supplementary Fig. 1C; originally included in our previous response to the Referees).

Referee #3: The contrast is not the issue here. Phosphorylation is very poor (and also only becomes apparent after 60 min incubation time, meaning later than the usual assay duration for the native gel analysis) and combined with degradation. The authors should show 5' phosphorylation assays with perfect duplexes (as positive controls) and, most importantly, with duplex B, simply because it gets into mature RISC (OH-U, lane 4 Supp. Fig. 1C) and therefore it should be phosphorylatable in order to allow RISC assembly.

3) Please show full gels for Fig. 2 C-D-E-F to be able to visualize fast migrating complexes (see below).

We have now shown full gels for Figs. 2C-F, and separated the model in Fig. 3.

Observations regarding the unspecific, asterisk-labeled complexes:

1) In Fig. 1B, the lower complexes seem related to the bulge in position 5, and the presence or

absence of P/OH plays no role.

2) In Supp. Fig. 1, duplexes 2 and 4 show strong signals for lower complexes, depending on dT or U; in this case, the extra bulge does not matter. Still in Supp. Fig. 1, duplex 1 is similar to duplex 2 and duplex 3 is similar to duplex 4 for the formation of pre-RISC or mature-RISC, indicating that the base does not matter as long as the Phosphate group is present. The authors may want to comment on these issues in their final response.

We appreciate the Referee's clarification. As the Referee points out, there is no correlation between formation of the nonspecific asterisk-labeled complexes and formation of pre-RISC and mature RISC. We have clearly stated this point in the Figure Legends.

Referee #3: I disagree with the authors: I did not point out a lack correlation between *complexes and pre- or mature RISC. I would like the authors to explain/speculate on the behavior of those complexes in Figure 1B and Supp. Fig. 1 in relation to the presence of P vs. OH and deoxy vs. ribonucleotides.

Correspondence – Authors' response

27 May 2011

I think that simply omitting the 5'-OH-U duplex data in Supplementary Figure 1B will solve all the unnecessary confusion (please see attached).

What we need as a control to support the data in Figure 1 is that "in the lysate we routinely use to study Ago1-RISC assembly (overnight embryo lysate from *dcr-2* null flies), 5'-dT duplexes were barely phosphorylated" (page 5).

The 5'-OH-dT data in Supplementary Fig. 1B clearly indicate that the 5'-OH-dT duplex remain non-phosphorylated at least for 60 min, a sufficient length for the experiments in Figure 1 where incubation was always only for 30 min. Moreover, the newly added lower panels in Figure 1 demonstrate that not only the duplex A (whose phosphorylation time course was tested in Supplementary Fig. 1B) but also all the 5'-OH-dT duplexes used in Figure 1 remained non-phosphorylated during the course of our experiments.

"How quickly the 5'-OH-U duplexes are phosphorylated" (this is obviously what the Referee #3 concerns) is not the issue at all here; it is rather an issue for characterizing the nature of the endogenous small RNA kinase, which is clearly out of scope of this study.

I would like to emphasize that, in Figure 1, we simply compared "~100% pre-phosphorylated 5'-P-dT duplexes" and "5'-OH-dT duplexes", which remained non-phosphorylated as demonstrated by all of our control experiments. Of note, in Supplementary Fig. 1A, we compared "~100% pre-phosphorylated 5'-P-U duplexes" and "~100% pre-phosphorylated 5'-P-dT duplexes", which behaved identically and justified us to use the series of dT duplexes in Figure 1.

I am very pleased to accept your manuscript for publication in the next available issue of EMBO reports. Thank you for your contribution to our journal.

Note that the modifications to figure legends and supplementary figures that you proposed in response to the referee's comments will be included in the final version of the manuscript.

Thank you again 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