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Inorganic phosphate blocks binding of pre-miRNA to Dicer-2 via its PAZ domains

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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 20 November 2013

Thank you for submitting your manuscript for consideration by The EMBO Journal and my apologies for the delay in communicating our decision to you. Your study has now been seen by three referees, whose comments are shown below.

As you will see from the reports, all referees express interest in the findings reported in your manuscript and raise only minor concerns that should be addressed before they can support publication of a revised manuscript.

I would ask you to especially focus your efforts on the following points:
-> discuss and clarify the technical concerns raised by ref #1
-> elaborate the discussion of how phosphate binds Dicer-2, how protein co-factors may affect the outcome of this binding, and how the presence of phosphate could alter RNA binding through the dsRBDs (as pointed out by refs 1 and 2)
-> elaborate the discussion of possible functional requirements that could underlie the need to retain dual substrate specificity of Dicer (ref #3)
-> provide additional experimental evidence for the possible sequential application of the Dicer-2 dual activities during processing of long dsRNA subtrates (as requested by ref #3).

In addition, I would ask you to revise the manuscript text to accommodate all minor changes and clarifications requested by the three referees.

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.

When preparing your letter of response to the referees' comments, please bear in mind that this will form part of the Peer-Review Process File, and will therefore be available online to the community.

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.

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

REFEREE REPORTS

Referee #1:

This is a very careful and thorough analysis of the effects of phosphate on dsRNA processing by Drosophila Dicer enzymes. The findings help to explain how flies use two Dicer enzymes to faithfully generate distinct small RNA populations and suggest new avenues for controlling Dicer activity in vivo. The manuscript contains the following major points:

• The authors demonstrate that although Dicer-2 misprocesses pre-miR-8 and -79 in vitro, it does not appear to process/misprocess pre-miRs in vivo, underscoring the idea that mechanisms regulating Dicer-2 activity are at work in the living animal.

• When processing short dsRNAs, Dicer-2 relies on recognition of the 5' phosphate (and two-nucleotide, 3' overhang) and recognition likely occurs in the PAZ domain. This is an important and surprising result because it was previously proposed that Dicer-2 does not possess a 5' phosphate recognition site, which conveniently explained why Dicer-2 does not process pre-miRs in vivo.

• Phosphate inhibits Dicer-2 processing of short dsRNAs (but not long dsRNAs) by inhibiting binding, increasing Km and decreasing kcat.

• Inhibition of Dicer-2 by phosphate is independent of stimulatory effects of ATP when cleaving long dsRNAs and even in the absence of ATP phosphate does not inhibit processing of long dsRNA substrates, suggesting that phosphate does not act at the ATP-binding site.

The study is very well executed and my comments/suggestions are mostly cosmetic or aimed at clarifying points I found confusing:

1) When describing processing of long dsRNA, it is not clear if only the first cleavage (from the end being examined) is being observed or if the authors are reporting on the first and all subsequent cleavages. This is an important point because the first cleavage leaves a 5' phosphate and 3' overhang, which presumably will be used in secondary and tertiary cleavage events. The methods describe the preparation of both end and body labeled dsRNAs, but it is not clear to me which of these was used in each experiment.

2) It is not clear how much dicer protein is used in the dicing reactions. Plotting rates in terms of nmol product per nmol or μg of protein would provide more information and clarify some issues for the reader. For example, pre-let-7 processing efficiency varies 3-5 fold between Figures 2, 4 and 5. Is this because different amounts of enzyme were used in each experiment or does this reflect variations in the specific enzymatic activity between Dicer-2 preparations? Perhaps different substrate concentrations were used in each experiment?

3) Likewise, comparison of panels B and C of Figure S4 leave the reader under the impression that cleavage of the 30 bp dsRNA is 150 times faster than the 104 bp dsRNA under saturating

conditions, which is not a true refection of the difference in kcat (according to page 13).

4) The abstract states, "Dicer-2 cleavage of short dsRNA requires a 5' terminal phosphate..." but in some figures (Figures 4 and 7) a 5' phosphate does not appear to be a strict requirement for cleavage...?

5) Examination of Figure 2A suggests that ATP may inhibit Dicer-2 cleavage of pre-miRs, pre-let-7 in particular. Is this effect strong enough to be worth mentioning?

6) The authors suggest that phosphate inhibits Dicer-2 by occupying the 5' phosphate-binding site and interfering with short dsRNA binding/positioning. Did they ever examine the effects of phosphate on UV crosslinking using a dsRNA with a 5' hydroxyl where this should not then be an issue?

7) The title indicates that inorganic phosphate interferes with binding of pre-miRNA via PAZ and dsRBDs. The model for inorganic phosphate occupying the 5' phosphate binding site in the PAZ domain feels plausible, but I see no direct evidence indicating that phosphate interferes with the dsRBDs.

8) Page 3: "The Dicer-2 helicase domain comprises DExDx and Helicase C domains"
The Dicer helicase belongs to the RIG-I family of helicases, which have more recently been shown to be composed of three domains (often termed Helicase 1, Helicase 2i and Helicase 2 or sometimes Domain 1, Domain 3 and Domain 2). See:
Nishino et al. (Structure 2005); http://www.ncbi.nlm.nih.gov/pubmed/15642269
Lau et al. (NSMB 2012); http://www.ncbi.nlm.nih.gov/pubmed/22426548
Jiang et al. (Nature 2011); http://www.ncbi.nlm.nih.gov/pubmed/21947008
Luo et al. (Cell 2011); http://www.ncbi.nlm.nih.gov/pubmed/22000018

9) A few references seem to be misplaced:

Page 4, "...contain a carboxy-terminal, canonical, dsRBD, which is thought to enhance affinity for substrate." Should cite:
Zhang et al. (Cell 2004); http://www.ncbi.nlm.nih.gov/pubmed/15242644;
and maybe Provost et al. (EMBO Journal 2002); http://www.ncbi.nlm.nih.gov/pubmed/12411504.

Page 16, "Since long dsRNA can be recognized by the helicase domain
and/or the central dsRNA binding domain..." this declaration is unreferenced. I believe there are indications of this in the literature, but I am uncertain as to how the authors came to this idea.

Page 18, "Recombinant Dicer-1 and Dicer-2 were expressed and purified as described {Lee et al., 2004, #56996; Zamore et al., 2000, #87396}. These references appear to be incorrect (the history discovery in the RNAi field would be very different if Zamore had a method for expressing and purifying recombinant forms of Dicer in 2000).

Figure 8 contains a cartoon schematic of Dicer-2 that is well aligned with the previously reported structural model of Dicer-2, and therefore should cite Lau et al. (NSMB 2012);
http://www.ncbi.nlm.nih.gov/pubmed/22426548.

Referee #2:

This is a thorough and meticulous analysis of an aspect of Dicer enzymology. The experiments are well designed and controlled, and the data is of uniform high quality. The manuscript is extremely well written. The subject of the work is how and perhaps why inorganic phosphate inhibits Dicer-2 activity on a form of RNA substrate that resembles pre-miR structures. The authors solve this puzzle, providing compelling evidence that phosphate acts through Dicer-2's PAZ domain to inhibit substrate recognition via its overhanging 5'-monophosphate end. The connection is made via correlation of specificity of phosphate inhibition and specificity of a point mutant in Dicer-2's PAZ domain. Circumstantial evidence is discussed pointing to a phosphate located in the crystal structure of the analogous domain in mammalian Dicer. It would have been great if the authors had shown

phosphate binding to the PAZ domain of Dicer-2 and its dependence on the two arginines. However, this experiment is not simple, and the burden of proof they provide is sufficient to support their model.

The findings are interesting because in vitro, physiological concentrations of inorganic phosphate are sufficient to inhibit the ability of Dicer-2 to process pre-microRNAs. Perhaps this is a natural mechanism cells use to increase substrate specificity of Dicer-2 away from microRNAs. Of course, the experiments have not included the Dicer binding protein isoforms of Loqs, and so such interpretations are qualified. I would recommend a brief mention of these issues in the discussion.

I have otherwise only minor comments:

1. Cleavage of long dsRNA by Dicer-2 is if anything, a little stronger when phosphate is present (Fig 2A). Although the effect is not ATP dependent, it does depend on the G31 residue inside the helicase domain, which normally binds ATP. Perhaps phosphate acts as a weak stimulator of Dicer-2 by mimicking ATP in some way. The authors should point this curious result out to the reader, and also perform a statistical test to see if the effect is significant.

2. Speaking of statistics, the authors describe the results of lots of tests but do not describe what those tests are. For every test result, define the test used.

3. Figure 6A - please expand the Y axis scale for the top right and bottom left plots. They are unnecessarily compressed.

4. Figure 6B - A great way to present the data from A. Please make these plots much bigger because they convey rich information.

5. The results from Figure 6 experiments seem to show a graded change in ATP and Pi dependence with different RNA substrates. The authors interpret the data as a more sharp transition in both results and discussion. The trends are very convincing, but how they trend, sharp versus graded, is more ambiguous. They should downplay that aspect of the interpretation.

6. In the methods section, they cite Lee et al 2004 as a reference for making small RNA libraries for sequencing and production of purified recombinant Dicers. This paper did neither (nor for that matter did Zamore et al 2000, which was referenced also for recombinant proteins. Please correct citation errors in the Methods section.

Referee #3:

The authors present convincing evidence using in vitro assays that fly Dicer-2 employs two distinct mechanisms to recognize short (<38 bp) and long dsRNA. In the first mode, Dicer-2 uses PAZ and C-terminal dsRBD to bind short dsRNA by recognizing the 5' phosphate and 2nt 3' overhang. This mode of action is inhibited by inorganic phosphate and does not require ATP. In the second mode of action, Dicer-2 recognizes long dsRNA through its helicase domain and requires ATP. The RNA terminal structure and inorganic phosphate does not affect processing.

In the authors' previous paper (Mol Cell, 2011), they showed that inorganic phosphate selectively inhibits Dicer-2's processing of pre-miRNA but not that of long dsRNA. The main novelty in the current work is that Dicer-2 has a preference for the end structure (5' phosphate and 2nt overhang) in the case of short duplex and that the PAZ domain (two basic residues in the domain) and c-terminal dsRBD are involved specifically in the recognition of short duplex. The authors propose that inorganic phosphate inhibits Dicer-2 processing by binding to the 5' phosphate pocket.

I suggest that the authors provide further explanation/speculation as to why Dicer-2 retains the first mechanism for short duplex recognition. One would expect that the 5' phosphate pocket would be mutated and lost during evolution if the first mode is not necessary. Could it be that there is a physiologically relevant short substrate in vivo?

The assay in this study was designed to measure the amounts of processed substrates. But I am

curious if Dicer-2 uses both action mechanisms for long duplex, which may not have been detected in this assay. Can the authors test a possibility that Dicer-2 initially uses the second mode but later switches to the first mode (or use both modes)? It would be informative to use internally labelled long dsRNA to monitor intermediate products. It would also be interesting to test the PAZ domain mutant (R943A/R956A) and dsRBD mutant (del1653-1722) and ask how the intermediate products are affected by the mutations.

Figure 7B. It is not clear from this result that the R943/R956 are directly responsible for the recognition of terminal phosphate because the mutant cannot process any of the short dsRNA.

Figure 4. pre-miR-87 was not processed sufficient. It would be better to use miR-8 or miR-79.

1st Revision - authors' response 04 December 2013

Responses to Reviewers' Critiques

Reviewer 1

1) When describing processing of long dsRNA, it is not clear if only the first cleavage (from the end being examined) is being observed or if the authors are reporting on the first and all subsequent cleavages. This is an important point because the first cleavage leaves a 5' phosphate and 3' overhang, which presumably will be used in secondary and tertiary cleavage events. The methods describe the preparation of both end and body labeled dsRNAs, but it is not clear to me which of these was used in each experiment.

Figure S4 (originally S3) used 5' terminally labeled dsRNA to monitor the first cleavage event, whereas Figures 2, 4, 7 and S5 (originally S4) used uniformly labeled substrates to monitor all cleavage events. In the original manuscript, we noted this in the legends for Figures 2 and 4 ['a 104 bp dsRNA with a twonucleotide, 3' overhanging end (100 nM, uniformly 32P-radiolabeled)' and 'uniformly 32P-radiolabeled 104 or 106 bp dsRNA (100 nM)'], but we failed to indicate the labeling strategy used in Figures 7, S3, and S4. We have now corrected this error by adding the additional information to those figure legends: '104 bp dsRNA (100 nM uniformly 32P-radiolabeled)' for Figure 7 legend; '5' 32Pradiolabeled 30, 38, 52, or 73 bp long dsRNA' for Figure S4; and 'Uniformly 32Pradiolabeled 104 bp dsRNA' for Figure S5.

2) It is not clear how much dicer protein is used in the dicing reactions. Plotting rates in terms of nmol product per nmol or µg of protein would provide more information and clarify some issues for the reader. For example, pre-let-7 processing efficiency varies 3- 5 fold between Figures 2, 4 and 5. Is this because different amounts of enzyme were used in each experiment or does this reflect variations in the specific enzymatic activity between Dicer-2 preparations? Perhaps different substrate concentrations were used in each experiment?

We had noted the Dicer concentrations used in each Figure legend. As indicated in the legends, the Dicer-2 concentration used for pre-let-7 processing in Figures 2, 4, and 5 were 8, 8, and 7, nM, respectively; 100 nM substrate RNA was used in all the experiments.

We prefer to present the rate data (nM substrate cleaved) rather than normalizing to enzyme concentration (nmol product per nmol enzyme) because a different Dicer-2 preparation was used in Figure 5 versus the other figures. There is currently no method for determining the specific activity of purified Dicer-2 (because the enzyme does not display burst kinetics), so comparisons between preparations are not formally appropriate. The different processing efficiencies most likely reflect small variations in the specific enzymatic activities between preparations. We now note this in the Materials and Methods (page 19).

3) Likewise, comparison of panels B and C of Figure S4 leave the reader under the impression that

cleavage of the 30 bp dsRNA is 150 times faster than the 104 bp dsRNA under saturating conditions, which is not a true refection of the difference in kcat (according to page 13).

We first determined *Vmax* from the graphs in Figure S5 (originally S4), then determined *kcat* by dividing *Vmax* by the enzyme concentration (Table 1). Keeping the graphs as they are more faithfully reflects the procedure that we used to obtain these values.

4) The abstract states, "Dicer-2 cleavage of short dsRNA requires a 5' terminal phosphate..." but in some figures (Figures 4 and 7) a 5' phosphate does not appear to be a strict requirement for cleavage...?

We agree that a 5' phosphate is not strictly requirement for cleavage of short dsRNA, but is required for efficient processing of short dsRNA. We have changed the abstract to read, "Efficient processing by Dicer-2 of short dsRNA requires a 5' terminal phosphate and a two-nucleotide, 3' overhang, but does not require ATP."

5) Examination of Figure 2A suggests that ATP may inhibit Dicer-2 cleavage of premiRs, pre-let-7 in particular. Is this effect strong enough to be worth mentioning?

There was no statistically significant difference between the pre-let-7 processing rates in the presence and absence of ATP (two-tailed Student's t-test p-value = 0.14).

6) The authors suggest that phosphate inhibits Dicer-2 by occupying the 5' phosphatebinding site and interfering with short dsRNA binding/positioning. Did they ever examine the effects of phosphate on UV crosslinking using a dsRNA with a 5' hydroxyl where this should not then be an issue?

Although we have not used UV crosslinking to examine binding of a dsRNA bearing a 5' hydroxyl, we did test a dsRNA with a 5' hydroxyl in the processing assay (Figures 4, 6, and 7). Inorganic phosphate inhibits processing of a short dsRNA bearing either a 5' hydroxyl or a 5' monophosphate. We describe these findings on page 16: "Our data suggest that the bound inorganic phosphate also blocks binding of a short dsRNA with a 5' hydroxyl end, perhaps because the phosphate oxygen and the terminal hydroxyl group occupy the same portion of the phosphate binding pocket (Figures 4 and 7B)."

7) The title indicates that inorganic phosphate interferes with binding of pre-miRNA via PAZ and dsRBDs. The model for inorganic phosphate occupying the 5' phosphate binding site in the PAZ domain feels plausible, but I see no direct evidence indicating that phosphate interferes with the dsRBDs.

We have changed the title to "Inorganic phosphate blocks binding of pre-miRNA to Dicer-2 via its PAZ domain."

8) Page 3: "The Dicer-2 helicase domain comprises DExDx and Helicase C domains" The Dicer helicase belongs to the RIG-I family of helicases, which have more recently been shown to be composed of three domains (often termed Helicase 1, Helicase 2i and Helicase 2 or sometimes Domain 1, Domain 3 and Domain 2). See: Nishino et al. (Structure 2005); http://www.ncbi.nlm.nih.gov/pubmed/15642269 Lau et al. (NSMB 2012); http://www.ncbi.nlm.nih.gov/pubmed/22426548 Jiang et al. (Nature 2011); http://www.ncbi.nlm.nih.gov/pubmed/21947008 Luo et al. (Cell 2011); http://www.ncbi.nlm.nih.gov/pubmed/22000018

To avoid confusing readers with an interest in helicases generally, we prefer to use the standard nomenclature, which is also employed by the SMART database of protein motifs (http://smart.embl-heidelberg.de/).

9) A few references seem to be misplaced:
Page 4, "...contain a carboxy-terminal, canonical, dsRBD, which is thought to enhance affinity for

substrate." Should cite: Zhang et al. (Cell 2004); http://www.ncbi.nlm.nih.gov/pubmed/15242644; and maybe Provost et al. (EMBO Journal 2002); http://www.ncbi.nlm.nih.gov/pubmed/12411504.

We have added these references.

Page 16, "Since long dsRNA can be recognized by the helicase domain and/or the central dsRNA binding domain..." this declaration is unreferenced. I believe there are indications of this in the literature, but I am uncertain as to how the authors came to this idea.

We now reference Lau et al., 2012.

Page 18, "Recombinant Dicer-1 and Dicer-2 were expressed and purified as described {Lee et al., 2004, #56996; Zamore et al., 2000, #87396}. These references appear to be incorrect (the history discovery in the RNAi field would be very different if Zamore had a method for expressing and purifying recombinant forms of Dicer in 2000).

We have corrected our mistakes and now cite Cenik et al. (2011) and Fukunaga et al. (2012) for the methods.

Figure 8 contains a cartoon schematic of Dicer-2 that is well aligned with the previously reported structural model of Dicer-2, and therefore should cite Lau et al. (NSMB 2012); http://www.ncbi.nlm.nih.gov/pubmed/22426548.

In the Figure 8 legend, we added the sentence "The model well aligns well with the previous structural model of Dicer-2 (Lau et al., 2012)."

Reviewer 2

The findings are interesting because in vitro, physiological concentrations of inorganic phosphate are sufficient to inhibit the ability of Dicer-2 to process pre-microRNAs. Perhaps this is a natural mechanism cells use to increase substrate specificity of Dicer-2 away from microRNAs. Of course, the experiments have not included the Dicer binding protein isoforms of Loqs, and so such interpretations are qualified. I would recommend a brief mention of these issues in the discussion.

Dicer-2 binds to its partner proteins, R2D2 and Loquacious-PD, via its helicase domain (Hartig and Forstemann, 2011 and Nishida et al, 2013). We previously found that R2D2, but not Loquacious-PD, inhibits Dicer-2 from processing premiRNA (Cenik et at, 2011). The inhibitory effects of R2D2 and inorganic phosphate were additive, suggesting that they act independently, consistent with the idea that inorganic phosphate binds to the PAZ domain of Dicer-2. Dicer-2 achieves the highest substrate specificity in the presence of both R2D2 and inorganic phosphate, which we propose is the in vivo situation. We now discuss these ideas in the text (page 17).

1. Cleavage of long dsRNA by Dicer-2 is if anything, a little stronger when phosphate is present (Fig 2A). Although the effect is not ATP dependent, it does depend on the G31 residue inside the helicase domain, which normally binds ATP. Perhaps phosphate acts as a weak stimulator of Dicer-2 by mimicking ATP in some way. The authors should point this curious result out to the reader, and also perform a statistical test to see if the effect is significant.

There was no statistically significant difference in the rates of processing long dsRNA in the presence or absence of inorganic phosphate for wild-type Dicer-2 with ATP (p-value = 0.33), wild-type Dicer-2 without ATP (p-value = 0.37), or G31R mutant Dicer-2 with ATP (p-value = 0.45; all two-tailed Student's t-test).

2. Speaking of statistics, the authors describe the results of lots of tests but do not describe what those tests are. For every test result, define the test used.

We used two-tailed Student's t-test for all statistical analyses. We added this to the Materials and Methods (page 20).

3. Figure 6A - please expand the Y axis scale for the top right and bottom left plots. They are unnecessarily compressed.

We modified the figure as suggested by the Reviewer.

4. Figure 6B - A great way to present the data from A. Please make these plots much bigger because they convey rich information.

We modified the figure as suggested by the Reviewer.

5. The results from Figure 6 experiments seem to show a graded change in ATP and Pi dependence with different RNA substrates. The authors interpret the data as a more sharp transition in both results and discussion. The trends are very convincing, but how they trend, sharp versus graded, is more ambiguous. They should downplay that aspect of the interpretation.

We tempered our discussion (page 15) as suggested by the Reviewer: "Our data suggest that the boundary between short and long dsRNAs is >30 bp but <38 bp, although more extensive work will be required to know if the transition between "short" and "long" occurs sharply or gradually over dsRNA length."

6. In the methods section, they cite Lee et al 2004 as a reference for making small RNA libraries for sequencing and production of purified recombinant Dicers. This paper did neither (nor for that matter did Zamore et al 2000, which was referenced also for recombinant proteins. Please correct citation errors in the Methods section.

We have corrected our mistakes and now cite Cenik et al. (2011) and Fukunaga et al. (2012) for the Methods.

Reviewer 3

The authors present convincing evidence using in vitro assays that fly Dicer-2 employs two distinct mechanisms to recognize short (<38 bp) and long dsRNA. In the first mode, Dicer-2 uses PAZ and C-terminal dsRBD to bind short dsRNA by recognizing the 5' phosphate and 2nt 3' overhang. This mode of action is inhibited by inorganic phosphate and does not require ATP. In the second mode of action, Dicer-2 recognizes long dsRNA through its helicase domain and requires ATP. The RNA terminal structure and inorganic phosphate does not affect processing.

In the authors' previous paper (Mol Cell, 2011), they showed that inorganic phosphate selectively inhibits Dicer-2's processing of pre-miRNA but not that of long dsRNA. The main novelty in the current work is that Dicer-2 has a preference for the end structure (5' phosphate and 2nt overhang) in the case of short duplex and that the PAZ domain (two basic residues in the domain) and c-terminal dsRBD are involved specifically in the recognition of short duplex. The authors propose that inorganic phosphate inhibits Dicer-2 processing by binding to the 5' phosphate pocket.

I suggest that the authors provide further explanation/speculation as to why Dicer-2 retains the first mechanism for short duplex recognition. One would expect that the 5' phosphate pocket would be mutated and lost during evolution if the first mode is not necessary. Could it be that there is a physiologically relevant short substrate in vivo?

Dicer-2 functions not only in cleaving long dsRNA into siRNAs, but also in loading those siRNA duplexes into Argonaute2. Thus, Dicer-2 does need to recognize and bind short (19 bp) dsRNA for loading. We now speculate in the Discussion section (page 17) that the 5' phosphate pocket is required for binding to an siRNA duplex during Argonaute loading.

The assay in this study was designed to measure the amounts of processed substrates. But I am

curious if Dicer-2 uses both action mechanisms for long duplex, which may not have been detected in this assay. Can the authors test a possibility that Dicer-2 initially uses the second mode but later switches to the first mode (or use both modes)? It would be informative to use internally labeled long dsRNA to monitor intermediate products. It would also be interesting to test the PAZ domain mutant (R943A/R956A) and dsRBD mutant (del1653-1722) and ask how the intermediate products are affected by the mutations.

We used uniformly (internally) labeled long dsRNA in Figures 2, 4, 7 and S5. Neither inorganic phosphate nor the mutations caused accumulation of intermediate products. We conclude that Dicer-2 uses only the second mode in long dsRNA cleavage. We added a sentence "We were unable to detect intermediates for the long dsRNAs in the presence or absence of inorganic phosphate" (page 15).

The reviewer might have meant for us to measure two separate rates for the long (104 bp) dsRNA cleavage; the rate to produce the first siRNA (*k1*) by using endlabeled long dsRNA substrate and the rate to produce a subsequent siRNA along the long dsRNA, for example, the fourth siRNA (*k4*), perhaps by using sitespecifically internally labeled long dsRNA substrates as in Cenik et al. (2011). However, Dicer-2 is processive enzyme (i.e., intermediate products are not detectable for a 104 bp dsRNA in the presence or absence of inorganic phosphate or using mutant Dicer-2), so *k1* and *k4* cannot be distinguished: *k1* is rate determining and $k1 << k2, k3, k4 \ldots kn$. We now discuss this in the manuscript (page 10):

"Even in the presence of ATP, production of the first siRNA from the end of a long dsRNA is rate determining for Dicer-2 (Cenik et al., 2011). Thus, subsequent production of siRNAs from the interior of a long dsRNA appears to proceed at the same rate as production of the first, terminal siRNA. All the long dsRNA substrates used here were diced at the similar rates (Figure 4), supporting the idea that production of the first siRNA limits the rate of producing internal siRNAs from substrates with all possible termini (Cenik et al., 2011). Consistent with this observation, we were unable to detect intermediates for any long dsRNA used here, irrespective of its terminal structure."

Figure 7B. It is not clear from this result that the R943/R956 are directly responsible for the recognition of terminal phosphate because the mutant cannot process any of the short dsRNA.

We agree that the result does not directly prove that the two arginine residues are directly responsible for the recognition of terminal phosphate. The only direct test we can image would be to solve the three-dimensional structure of Dicer-2 bound to a short dsRNA. However, we believe our model is the most plausible, given both our data and the human Dicer PAZ domain crystal structure, which suggests that arginine residues are directly involved in the recognition of the terminal phosphate (Park et al., 2011).

Figure 4. pre-miR-87 was not processed sufficient. It would be better to use miR-8 or miR-79.

We tested pre-miR-307a, which was processed sufficient, obtained similar conclusions, and added as a new figure (Supplementary Figure 3). The revised text now reads, "The rate of cleavage by Dicer-2 of 5″ monophosphorylated pre-miR-307a was 43-fold faster than that for 5″ hydroxy pre-miR-307a and the cleavages were inhibited by inorganic phosphate (Supplementary Figure 3)" (page 10).

