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Efficient RNA virus control in Drosophila requires the RNA methyltransferase Dnmt2

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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.)

Editor: Alejandra Clark

1st Editorial Decision 30 October 2012

Thank you for the submission of your research manuscript to EMBO reports. We have now received the full set of reports on it, which I copy below.

As you will see, all referees agree that the study is potentially interesting and novel. Overall referees #1 and #2 are positive about the work pointing to specific issues that will need to be further clarified in the text. However, referee #3 who is an expert in the field of Drosophila immunity raises major concerns about the technical quality and conclusiveness of the work. In particular he/she is concerned that the current experimental approach for the infection experiments is inappropriate suggesting that infections must be carried out with a preparation of pure DCV on mutant and control lines cured from persistent DCV infection. In addition, this opinion is also shared by referee #2 who after we invited all referees to cross comment on each other's reports, a standard procedure for all our decisions, agreed that pure stocks of virus should be used in the infection assay. Furthermore, referee #3 indicates that the history of the fly stocks should be described in more detail.

Given the potential interest of the novel findings and considering that all referees provide

constructive suggestions on how to move the study forward, I would like to give you the opportunity to revise the manuscript, with the understanding that the referees' concerns, in particular those of referee #3, have to be fully addressed as this would be essential for the conclusiveness of the study. Acceptance of the manuscript would entail a second round of review. I would like to point out that it is EMBO reports policy to allow a single round of revision only, and that thus acceptance or rejection of the manuscript will depend on the outcome of the next final round of peer-review.

Revised manuscripts should be submitted within three months of a request for revision; they will otherwise be treated as new submissions. If you feel that this period is insufficient to address the referees' concerns I can potentially extend this period slightly. Also, the length of the revised manuscript should not exceed roughly 30,000 characters (including spaces). Should you find the length constraints to be a problem, you may consider including some peripheral data in the form of Supplementary information. However, materials and methods essential for the understanding of the key experiments should be described in the main body of the text and may not be displayed as supplemental information only.

I look forward to seeing a revised form of your manuscript when it is ready. Should you in the meantime have any questions, please do not hesitate to contact me.

REFEREE REPORTS:

Referee #1

The paper by Durdevic et al. reports a new role for the RNA methyltransferase Dnmt2 in the innate immune response in Drosophila. The authors show that flies lacking the protein are defective in controlling the Drosophila C virus (DCV) infection. To investigate the role of Dnmt2 in host immune response they established an elegant system based on natural route of DCV infection by feeding flies with carcasses of previously infected flies. They show the increased accumulation of the viral RNA in the mutant flies which correlates with the delayed activation of the immune response genes. They also demonstrate specific translocation of the protein to compact cellular structures upon infection and show that restoration of Dnmt2 expression alleviates defects of immune response, while expression of the catalytically inactive form of Dnmt2 fails to protect flies form DCV infection. Co-IP experiments revealed that Dnmt2 likely directly binds DCV RNA. While the paper does not demonstrate the direct mechanism how Dnmt2 contributes to the proper activation of the innate immune response, it is certainly a very interesting observation opening the way to multiple new future research directions and should be published in EMBO reports. The paper is written clearly, experimental data are excellent and conclusions are justified. I have a couple of minor comments that I wish the authors would address in the final version:

Introduction, page 2, last paragraph: "Small interfering RNA pathway activity has been linked to sub-cellular membranes (Lee et al, 2009) and membranes are important for viral entry and replication (Cherry & Silverman, 2006). This indicates that infected cells use compartmentalization to sequester invading pathogens and to mount siRNA-mediated defenses."
It is generally accepted in virology that association of replication complexes of positive strand RNA viruses with membranes is a strategy beneficial for the viruses, not the way for the cells to control infection. I the authors would like to challenge this view they have to present more compelling evidence.

Fig. S4 is an important piece of data showing restoration of control of DCV infection in mutant flies with ectopically expressed functional Dnmt2. Unfortunately the version available to the reviewer does not show the comparison of DCV replication with the wt flies, contrary to what is described in the text on p.6: "Expression of Dnmt2 from the ubiquitin promoter (pUbq>>Dnmt2-EGFP; D2-OE) during infection suppressed DCV RNA levels below wild type control levels (Fig S4C)."
I suggest that the authors add the wt control to that figure and incorporate it in the main figures in the text.

Referee #2

The authors commence by investigating the function of Dmnt2 in a relatively unbiased way, and finish with quite convincing data showing a role in antiviral defense. A weakness of the MS is that the actual mechanism involved is unclear. However, the conclusions will interest many researchers in the field, and I am sure that almost anyone who reads it will find the story very appealing (if slightly gruesome). I would recommend acceptance in EMBO Rep.

Minor points:

1) The term dnmt2 mutants is used throughout the MS, without a clear description of the mutant allele(s) .

2) Fig. S4: It appears that the catalytically inactive mutant may confer considerable suppression of DCV relative to dnmt2 (null?) mutant used in earlier experiments. If so, this should be made explicit, with a clear indication of the relative efficiencies, since it would indicate that RNA methylation is not the major pathway in resistance.

2) Fig. 1B: The legend is difficult to follow. What do X and O indicate? P values are missing from my PDF.

Referee #3

The authors address here the role of DNMT2, an RNA methyltransferase, in the control of viral infection in the model organism Drosophila melanogaster. They show that Dnmt2 mutant flies exhibit stress (melanotic tumors), express immune markers, and contain increased levels of bacteria and viruses. However, since the history of the fly stocks used is not known (note that this a particular concern for the transgenic rescue line, which must be described in more detail), this information is of little use and could simply reflect exposure to bacteria or viruses at different times and concentration in the past.
The authors then performed natural infections of their mutant and control stocks by feeding them with homogenates of wild-type, Dnmt2 mutant and rescue flies. Based on the monitoring of the DCV viral load by QRT-PCR, Northern blot or western blot, they claim that Dnmt2 controls DCV infection. The assay used by the authors, which involves challenging flies with an ill-characterized mixture of viruses and bacteria is not appropriate to draw firm conclusions. They must perform the infections with a preparation of pure DCV, on mutant and control lines cured from persistent DCV infection. Only in these conditions will it be possible to assess the role of DNMT2 in the control of viral infection.

1st Revision - authors' response 11 December 2012

Reviewer #1

1) Introduction, page 2, last paragraph: "Small interfering RNA pathway activity has been linked to sub-cellular membranes (Lee et al, 2009) and membranes are important for viral entry and replication (Cherry & Silverman, 2006). This indicates that infected cells use compartmentalization to sequester invading pathogens and to mount siRNA-mediated defenses." It is generally accepted in virology that association of replication complexes of positive strand RNA viruses with membranes is a strategy beneficial for the viruses, not the way for the cells to control infection. If the authors would like to challenge this view they have to present more compelling evidence.

>>> We agree with the reviewer and have no intention to challenge the view that some viruses use cellular membrane systems for their replication. Since the association of siRNA pathway activity with multivesicular membranes has not yet been discussed or studied in the context of anti-viral

cellular activity we would like to keep our statement about the possibility of anti-viral siRNA mechanisms at the site of virus replication in the manuscript. To make this introductory point more clearly, we have re-phrased the respective paragraph and have added two references that report on and review the importance of endosomes for the activation of innate immune responses.

2) Fig. S4 is an important piece of data showing restoration of control of DCV infection in mutant flies with ectopically expressed functional Dnmt2.

>>> We would like to clarify a mis-reading of the reviewer. The overexpression of catalytically active Dnmt2 was performed in the wild-type genetic background (not in the *Dnmt2* mutant background). The rational for this experiment was to show that ectopic wild-type Dnmt2 can suppress the DCV levels even further than a wild-type fly strain (D2+/+) already can. To restore DCV control a transgenic fly line that expressed Dnmt2-EGFP under the endogenous Dnmt2 promoter in the Dnmt2 mutant background was used and these experiments are described in Fig 3. The over-expression of catalytically mutant Dnmt2 was performed in the Dnmt2 mutant background in order to test whether the catalytic activity or just the presence of a lot of Dnmt2 contributes to virus control. Although the description of these genotypes were present and correct in the manuscript we apologize for the confusion.

Unfortunately the version available to the reviewer does not show the comparison of DCV replication with the wt flies, contrary to what is described in the text on p.6: "Expression of Dnmt2 from the ubiquitin promoter (pUbq>>Dnmt2-EGFP; D2-OE) during infection suppressed DCV RNA levels below wild type control levels (Fig S4C)."

>>>To aid the reading of these passages we have added a more detailed description of the used genotypes to the main text. To address the concern that DCV replication data represented in Fig S4 could not be compared to DCV replication data in wild-type flies, we have expanded Fig S4 by two graphs that compare DCV levels in all genotypes at day 1 and 10 after oral infection (Fig S4D, E). This addition should allow to clarify the statements contained in the main text.

I suggest that the authors add the wt control to that figure and incorporate it in the main figures in the text.
>>>We have not added these data to the main text, as suggested by the reviewer, but used this space instead to include new experimental data from intra-thoracic infections with pure DCV (see also response to reviewer #3).

Reviewer #2

Minor points:

1) The term dnmt2 mutants is used throughout the MS, without a clear description of the mutant allele(s).

>>> The *Dnmt2* mutant alleles that were used in this study have been described previously and have been all published. We have cited their origin and references in the material and methods section contained in the supplemental data. We also added „null mutation“ as description of the used Dnmt2 mutation to the first text passage, in which we introduce *Dnmt2* mutant flies (p. 3).

2) Fig. S4: It appears that the catalytically inactive mutant may confer considerable suppression of DCV relative to dnmt2 (null?) mutant used in earlier experiments. If so, this should be made explicit, with a clear indication of the relative efficiencies, since it would indicate that RNA methylation is not the major pathway in resistance.

>>> We agree with the reviewer that our data set obtained after oral infection does not support the notion that RNA methylation is the major activity of Dnmt2-mediated DCV suppression. To compare the relative efficiencies of the used genotypes in DCV suppression, we have expanded Fig S4 by two graphs that compare DCV levels in all genotypes at day 1 and 10 after oral infection (Fig S4D, E).

On the other hand, our experiments using intra-thoracic infection with pure DCV shows that catalytically inactive Dnmt2 flies are significantly more sensitive to virus in this experimental paradigm (Fig 3D and 3E). Here, catalytically inactive flies die almost at rates of *Dnmt2* null mutant flies. We have therefore maintained our statement that the catalytic activity of Dnmt2 (=RNA methylation) contributes to virus control (see also response to reviewer #3)

2) Fig. 1B: The legend is difficult to follow. What do X and O indicate? P values are missing from my PDF.

>>> To better understand Fig 1B, we have re-organized it. First, we have replaced all O marks with X marks. The column that previously contained O marks (and now X) and encompasses all listed genes has been separated from the rest of the table. This column lists all genes that can be linked to stress by GO annotation or by using „Flybase“. We have also changed the legend description accordingly.

Reviewer #3

1) The authors address here the role of DNMT2, an RNA methyltransferase, in the control of viral infection in the model organism Drosophila melanogaster. They show that Dnmt2 mutant flies exhibit stress (melanotic tumors), express immune markers, and contain increased levels of bacteria and viruses. However, since the history of the fly stocks used is not known (note that this a particular concern for the transgenic rescue line, which must be described in more detail), this information is of little use and could simply reflect exposure to bacteria or viruses at different times and concentration in the past.

>>> We agree with the reviewer that the origin of the used fly lines is of importance. All fly stocks involving Dnmt2 have been created in the w^{1118} background and have been kept in the same stock collection since their establishment. We have added the details about the w^{1118} background to the description of our materials and methods in the supplemental data. The origin and phenotypes of the transgenic rescue line (D2-TG) has been previously published. We have added the correct reference and cited it in the main text. In addition, we would like to point out that DCV levels in all recipients at the time of oral infection was comparable (Fig S3D) and the differences in the levels of endogenous bacteria were very low, which we take as support for the notion that none or no significant difference in persistent DCV infection or bacterial load was present in these recipient flies.

The authors then performed natural infections of their mutant and control stocks by feeding them with homogenates of wild-type, Dnmt2 mutant and rescue flies. Based on the monitoring of the DCV viral load by QRT-PCR, Northern blot or western blot, they claim that Dnmt2 controls DCV infection. The assay used by the authors, which involves challenging flies with an ill-characterized mixture of viruses and bacteria is not appropriate to draw firm conclusions. They must perform the infections with a preparation of pure DCV, on mutant and control lines cured from persistent DCV infection. Only in these conditions will it be possible to assess the role of DNMT2 in the control of viral infection.

>>> We thank the reviewer for these suggestions and agree that infections with pure DCV would allow firmer conclusions about a role of Dnmt2 in viral control. We have therefore invited expert help as co-autors and performed intra-thoracic infections on *Dnmt2* mutant and transgenic rescue flies (D2-TG). The experimental data are now included as Fig 3D in the manuscript. The results support our claim that Dnmt2 plays a significant role for virus control in *Drosophila*. In addition, we added data which indicate that the methyltransferase function of Dnmt2 contributes significantly to the survival of infected flies (Fig 3E). Importantly, since the contribution of the catalytic domain of Dnmt2 to the control of DCV was not convincing after oral infection (see response to reviewer #2) but could be documented after intra-thoracic infections, we are especially thankful for this constructive comment by the reviewer.

2nd Editorial Decision 03 January 2013

Thank you for submitting your revised version of the manuscript. As you will see from the comments copied below, the referees agree that their concerns have been fully addressed. I am therefore very pleased to accept your manuscript for publication in the next available issue of EMBO reports. Thank you for your contribution to our journal.

At the end of this email I include important information about how to proceed. Please ensure that you take the time to read the information and complete and return the necessary forms to allow us to publish your manuscript as quickly as possible.

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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.

REFEREE REPORTS:

Referee #1
The authors addressed all the reviewer's concerns; I believe this manuscript should now be published in EMBO reports.

Referee #3
The authors have addressed the key point I raised in my first review. I now recommend publication of the manuscript.

