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# Unc-13 homolog D mediates an antiviral effect of the chromosome 19 microRNA cluster miR-517a

Kamil Krawczynski, Yingshi Ouyang, Jean-Francois Mouillet, Tianjiao Chu, Carolyn B. Coyne and Yoel Sadovsky
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**Original submission**

First decision letter

MS ID#: JOCES/2020/246769

MS TITLE: Unc-13 Homologue D Mediates an Antiviral Effect of the Chromosome 19 MicroRNA Cluster miR-517a

AUTHORS: Kamil Krawczyński, Yingshi Ouyang, Jean-Francois Mouillet, Tianjiao Chu, Carolyn Coyne, and Yoel Sadovsky
ARTICLE TYPE: Short Report

We have now reached a decision on the above manuscript.

To see the reviewers' reports and a copy of this decision letter, please go to: https://submit-jcs.biologists.org and click on the 'Manuscripts with Decisions' queue in the Author Area. (Corresponding author only has access to reviews.)

As you will see, the reviewers raise a number of substantial criticisms that prevent me from accepting the paper at this stage. They suggest, however, that a revised version might prove acceptable, if you can address their concerns. If you think that you can deal satisfactorily with the criticisms on revision, I would be pleased to see a revised manuscript. We would then return it to the reviewers.

*We are aware that you may currently be unable to access the lab to undertake experimental revisions. If it would be helpful, we encourage you to contact us to discuss your revision in greater detail. Please send us a point-by-point response indicating where you are able to address concerns raised (either experimentally or by changes to the text) and where you will not be able to do so within the normal timeframe of a revision. We will then provide further guidance. Please also note that we are happy to extend revision timeframes as necessary.*

Please ensure that you clearly highlight all changes made in the revised manuscript. Please avoid using 'Tracked changes' in Word files as these are lost in PDF conversion.

I should be grateful if you would also provide a point-by-point response detailing how you have dealt with the points raised by the reviewers in the 'Response to Reviewers' box. Please attend to

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all of the reviewers' comments. If you do not agree with any of their criticisms or suggestions please explain clearly why this is so.

Reviewer 1

*Advance summary and potential significance to field*

In this report, Krawczynski and co-workers explore the mechanism by which the C19MC miRNA miR-517a induces autophagy and viral resistance and provide evidence that Unc-13 Homologue D plays a critical role. In general, this is an interesting and well-written paper adding some new information on the mechanistic underpinnings of the anti-viral effect of C19MC, contributing significantly to the field.

*Comments for the author*

There are a few aspect that needs clarification:

Comments:
1. The objective of the current study is to define pathways that link C19MC miRNA and autophagy and the data identifying UNC13D (to which miR-517a binds) as one of these mechanistic links is compelling. However, a major part of the report concerns exploring the effect of ss-miRNA on TLR8 and NFκB/p65 signaling. The problem is that no rationale is provided for this focus in the context C19MC and autophagy and this data is not discussed in relation to virus resistance and autophagy. As a result, the paper falls into two distinct parts that is poorly integrated.
2. Page 6, lines 30-31: It is stated that "These ss miRNA mimics may better represent mature miRNAs delivered to cells via exosomes.." Please provide rationale for this statement – are exosomal miRNAs predominantly single stranded? Moreover, in experiments exploring the effect of miR-517a on UNC13D (Figure 3), it is unclear if ss or ds miRNA was used. If ds, what is the rationale to now not use ss and what effects does ss-517a have?
3. Although ss-517a robustly activated NFκB/p65 signaling (as determined using p65-Luc reporter plasmids) the resulting increase in IL-8 and CXL10 expression was modest and ss-517a did not increase TNF-alpha expression or CXCL 10 secretion into media. The authors may want to discuss this apparent relative 'dissociation' between the effect on NFκB/p65 and downstream targets.
4. Albeit a minor weakness, the study falls short of unequivocally demonstrate that the activation of autophagy is the critical mechanistic link between miR-517a and decreased VSV expression. In fact, one could argue that lack of effect of ITGB4 silencing on VSV expression, despite robust activation of autophagy may suggest that the link between miR-517a and virus resistance does not require activation of autophagy.

Minor Comments:

1. Summary Statement: The wording '…identified a direct placental microRNA target' is ambiguous in that I could be read as the target (rather than the miRNA) is placenta-specific. Suggest rephrasing.
2. Figure 1: The way statistical differences in TLR expression among 293XL cell clone data is displayed is confusing and it is not immediately clear which groups are different from each other. Authors may consider using letter superscripts (where different letters represent statistical differences).
3. Page 9, Line 5: mammalian target of rapamycin (mTOR) should probably be mechanistic target of rapamycin.

Reviewer 2

*Advance summary and potential significance to field*

In this manuscript the authors present data supporting the concept that host miRNA miR-517a can target host UNC13D (a regulator of autophagy), and that this targeting reduces replication of the

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RNA virus VSV. Although the manuscript provides some new insights into miR-517a targeting, as a whole the data and descriptions in the manuscript are scattered about and inconsistent, which makes the manuscript feel thrown together and difficult to follow. Some of the new data on miR-517a targeting of UNC13D and other mRNAs is interesting and the initial demonstration of miR-517a reduction of VSV RNA is encouraging; however the authors have not shown that miR-517 targeting of UNC13D is a requirement for the antiviral effect of miR-517 on VSV.

*Comments for the author*

1. The figures and text use “ss” vs. “miR” designations to indicate single stranded miRNAs vs. ds miRNA mimics. While this is easy enough to understand from the text, the designations are problematic as the native mature miRNA (which is single stranded) is specifically named “miR-517a”. Thus it would be more appropriate to refer to the single stranded version as “miR-517a” (or ss miR-517a) and the double stranded perhaps as “pre-miR-517a” or “ds miR-517a mimic” or even “ds miR-517a”.

2. It is unclear throughout what cells the authors are using and whether their TLR expression data and/or conclusions are correct. The specific descriptions of the cell lines are limited in the text here, but presuming that the 293XL/0 cells from InvivoGen are the control 293XL/null cells, this line should not express any of the TLRs. The authors contradict this. For example, line 16 states that the authors have validated the expression of TLR3 in all three 293XL lines; however the 293XL/null line is a control cell line that should not express TLR3. This indicates that the 293XL/0 cells are not true controls cells, or calls into question their conclusions from RNA expression data. Consistent with this, the addition of poly I:C, which should normally induce a very strong TLR3 response, causes a weak induction in 293XL/0 cells (Fig. 2A right panel inset). These findings all serve to suggest that more rigorous testing needs to be performed. Although Fig. 1 shows data demonstrating that PHTs express TLR3, TLR7 and TLR8 RNAs; a more rigorous demonstration would be to show protein levels for these TLRs. Moreover, the use of specific cell lines is varied and is many cases is inconsistently noted in the text. For example, Fig. S2A does not indicate anywhere what cell line is used. Due to the reliance upon comparison between different cell lines through the manuscript, individual cell lines for every panel should be clearly indicated on each figure.

3. The authors repeatedly state that they have performed CLASH analysis of Ago-associated miRNA-mRNA hybrids in PHT cells, and include methods for the assay.
However, no quality control data or experimental data from the CLASH analyses have been included in this manuscript. If this authors wish to include CLASH analyses in this manuscript then they need to include relevant control and experimental data and full data sets. These findings should elaborate and provide more specific information about the entire data sets, and should additionally include specific information about miR-517a: How many miR-517a targets were identified? What was the representation of the targets tested here?

4. Although the data in the manuscript (eg, Fig 4F) clearly suggest that miR-517a may have an antiviral effect on VSV (via repression of gene expression or genome replication) in U2OS cells, this conclusion would be significantly strengthened by a more robust measure of virus propagation such as a plaque assay.

5. Some basic information about the virus that is the basis of these studies (aside from the name), should be included in the manuscript. In addition, when referring to “VSV RNA” it would be useful to explain which portion of the 11 kb VSV genome is being tested.

6. The authors have not shown that miR-517 targeting of UNC13D is a requirement for the antiviral effect of miR-517 on VSV. This should be shown in some capacity in order to support the mechanistic conclusion that the authors wish to make.

MINOR

Line 11 states that Fig.1 shows that the PHT cells respond to the TLR ligands – however Fig.1 only shows RNA expression data, not ligand responsiveness.

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Reviewer 3

*Advance summary and potential significance to field*

Krawczynski et al. show that chromosome 19 miRNA cluster specifically mirRNA-517a affects antiviral immunity through post-transcriptional regulation of Unc13. Interestingly, the first part of the paper describes how single-stranded mature miRNAs activate the TLR8 pathway and downstream genes. The first two figures and the last two figures focus on separate findings with a common theme of miRNAs affecting innate and antiviral immunity. Fig-1 & 2 show single-stranded mature miRNAs activate the TLR8 pathway. These data are very preliminary and not sufficiently controlled to support their claims. I strongly suggest that the authors should remove these data from this manuscript. Figs 3 & 4 show that miR-517a regulates Unc13D which is important in antiviral immunity, has promising data that should be further strengthened. I would advise the authors to focus on the UNC13 part of the story and strengthen these data.

*Comments for the author*

Krawczynski et al. show that chromosome 19 miRNA cluster specifically mirRNA-517a affects antiviral immunity through post-transcriptional regulation of Unc13. Interestingly, the first part of the paper describes how single-stranded mature miRNAs activate the TLR8 pathway and downstream genes. The first two figures and the last two figures focus on separate findings with a common theme of miRNAs affecting innate and antiviral immunity. Fig-1 & 2 show single-stranded mature miRNAs activate the TLR8 pathway. These data are very preliminary and not sufficiently controlled to support their claims. I strongly suggest that the authors should remove these data from this manuscript. Figs 3 & 4 show that miR-517a regulates Unc13D which is important in antiviral immunity, has promising data that could be further strengthened.

Specific comments for Figs :

The authors extensively describe the CLASH technique, which led to the identification of miR-517 targets. However, no data obtained from the sequencing are presented in this manuscript. An additional figure with the sequencing data needs to be presented. I also could not find the accession number for the sequencing data that should be deposited (probably I missed it).

Figs 3 & 4: Deletion of endogenous miR-517 is critical to show that the effect on unc13D and VSV is physiological. These experiments need to be added to the manuscript.

Adding a schematic of a model would help the readers appreciate the study.

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**First revision**

Author response to reviewers' comments

*Please note that all text locations cited in this document refer to the highlighted version of the manuscript.*

General editorial notes:
1. The word count of our revised manuscript is now 3,000, which meets the requirements for a Short Report.
2. All submission checklist questions are now complete.
3. Table S1 is now in the Supplement.
4. The information on all funding sources is now complete, within the revised text and on the web site.
5. The number of supplementary figures is three, which matches the number of figures in the main text.

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General comments about the manuscript's structure:
1. We have bolstered our results by doing additional experiments and overexpressing UNC13D. We also enhanced the links between the two parts of the paper. This led to the general re-ordering of our figures:
a. Fig. 1: Inactivation of UNC13D attenuates VSV and enhances LC3B levels.
b. Fig. 2: Overexpression of UNC13D results in increased viral replication and decreased autophagy.
c. Fig. 3: The effect of mature miRNA on TLR8-mediated NFκB signaling.
d. The three supplemental figures and Table 1 logically follow this order.

**Reviewer 1:** In this report, Krawczynski and co-workers explore the mechanism by which the C19MC miRNA miR-517a induces autophagy and viral resistance and provide evidence that Unc-13 Homologue D plays a critical role. In general, this is an interesting and well-written paper adding some new information on the mechanistic underpinnings of the anti-viral effect of C19MC, contributing significantly to the field.

*Comments:*
*1. The objective of the current study is to define pathways that link C19MC miRNA and autophagy and the data identifying UNC13D (to which miR-517a binds) as one of these mechanistic links is compelling. However, a major part of the report concerns exploring the effect of ss-miRNA on TLR8 and NFκB/p65 signaling. The problem is that no rationale is provided for this focus in the context C19MC and autophagy and this data is not discussed in relation to virus resistance and autophagy. As a result, the paper falls into two distinct parts that is poorly integrated.*

Response: We thank the reviewer for this important comment, which was also noted by Reviewer 3. We have restructured and refocused the revised text, as also noted in our general comment. We strengthened our analysis of the miR-517a-UNC13D axis and the role of UNC13D in autophagy. We placed the results of the miR-517a-UNC13D experiments as panels in the re- organized Figures 1-2. Importantly, we recently uncovered that trophoblastic small extracellular vesicles (sEVs, also known as exosomes), which we found to exhibit antiviral activity (Delorme- Axford *et al*, *PNAS* 2013), enter cells through the endocytic pathways (maropinocytosis and clathrin-mediated uptake) and ultimately deliver exosomal miRNA cargo (including miR-517a) to the P-body proteins Ago2 and GW182 (Li et al, *J Extracellular Vesicles*, in press). These data, which are now added to our text on page 5, lines 5-9 and page 7, line 31, through page 8, line 6, provide additional justification to our pursuit, because sEV miR-517a, which enters endosomes, would encounter endosomal TLR8 that recognizes single stranded RNA, including mature miRNA. In addition, given that certain miRNAs are able to activate endosomal TLR8-mediated signaling pathways such as NFκB/p65, we believed that we should have further examined whether miR-517a uniquely potentiates TLR8-initiated pathways.

*2. Page 6, lines 30-31: It is stated that "These ss miRNA mimics may better represent mature miRNAs delivered to cells via exosomes." Please provide rationale for this statement – are exosomal miRNAs predominantly single stranded? Moreover, in experiments exploring the effect of miR-517a on UNC13D (Figure 3), it is unclear if ss or ds miRNA was used. If ds, what is the rationale to now not use ss and what effects does ss-517a have?*

Response: We agree that miRNA mimics are commonly designed as double-stranded oligonucleotides, and are chemically enhanced to preferentially program the RISC complex, where the active single-stranded miRNA strand is loaded for target suppression. Mature (single stranded miRNA) have been used to study activation of TLR8 and TLR7 (Refs by Fabbri, *PNAS* 2012 and Heil, *Science*, 2004 within our manuscript, and references therein). Thus we used similar constructs to assess the effect of miR-517a in our TLR8 study. This modified text is now located on page 5, lines 5-9, and page 8, lines 2-6. Throughout the text and figures, we now use the designation mature miR-517a, for consistency.

*3. Although ss-517a robustly activated NFκB/p65 signaling (as determined using p65-Luc reporter plasmids) the resulting increase in IL-8 and CXL10 expression was modest and ss-517a did not increase TNFa expression or CXCL 10 secretion into media. The authors may want to discuss this apparent relative 'dissociation' between the effect on NFκB/p65 and downstream targets.*

Response: The artificial, amplification-based luciferase system is very sensitive, much more than

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cytokine ELISA system. We measured the cytokines (Fig. 3 and Fig. S3) to assess the effect of miR-517a based on qPCR and also the more physiological context, measured by ELISA (page 9, lines 3-4). Our data confirms our conclusion that miR-517a activated NFκB through TLR8 in target cells, but the activation was not restricted to C19MC miRNA, and other mature RNAs also induced TLR8 downstream pathways.

*4. Albeit a minor weakness, the study falls short of unequivocally demonstrate that the activation of autophagy is the critical mechanistic link between miR-517a and decreased*

*VSV expression. In fact, one could argue that lack of effect of ITGB4 silencing on VSV expression, despite robust activation of autophagy may suggest that the link between miR-517a and virus resistance does not require activation of autophagy.*

Response: Thank you for this important comment. We have showed that activation of autophagy was necessary for the antiviral activity of miR-517a (Delorme-Axford. et al, *PNAS* 2013). Our primary goal in the current study was to identify proteins that might be a part of this mechanism, and mediate the antiviral activity of miR-517a in non-placental cells such as U2OS. In our original submission we based our conclusions on the observations that miR-517a silenced the expression of *UNC13D* and that VSV vRNA levels were lower in *UNC13D* KO. We now added additional experiments indicating that VSV vRNA levels were increased upon overexpression of UNC13D in cells expressing miR-517a, and that UNC13D enhanced the TFEB phosphorylation and hence inactivation of the autophagy-promoting factor TFEB. At this point we cannot rule out the possibility that ITG4B4 and RPTOR play a role in other aspects of autophagy. The relevance of these possibilities to C19MC miRNA remains to be explored.

*Minor Comments:*
*1. Summary Statement: The wording '…identified a direct placental microRNA target' is ambiguous in that I could be read as the target (rather than the miRNA) is placenta specific. Suggest rephrasing.*

Response: We agree and changed to: "We previously showed that placenta-specific microRNA miR-517a attenuates viral infection. Here we identified that miR-517a directly targets UNC13D as a part of its antiviral function." (page 2, line 2).

*2. Figure 1: The way statistical differences in TLR expression among 293XL cell clone data is displayed is confusing and it is not immediately clear which groups are different from each other. Authors may consider using letter superscripts (where different letters represent statistical differences).*

Response: We agree and have changed the way we denote these changes (now Fig. S2.) In the legend of Fig. S2 we added: "The differences in TLR expression among the 293XL cell clones (with other cell types used as controls) were analyzed using…"

*3. Page 9, Line 5: mammalian target of rapamycin (mTOR) should probably be mechanistic target of rapamycin*

Response: We agree, and made the change to "Mechanistic" (page 6, line 27).

***Reviewer 2****: In this manuscript the authors present data supporting the concept that host miRNA miR-517a can target host UNC13D (a regulator of autophagy), and that this targeting reduces replication of the RNA virus VSV. Although the manuscript provides some new insights into miR-517a targeting, as a whole the data and descriptions in the manuscript are scattered about and inconsistent, which makes the manuscript feel thrown together and difficult to follow. Some of the new data on miR-517a targeting of UNC13D and other mRNAs is interesting and the initial demonstration of miR-517a reduction of VSV RNA is encouraging; however the authors have not shown that miR-517 targeting of UNC13D is a requirement for the antiviral effect of miR-517 on VSV.*

Response: Thank you. Please see below, and also comment #4 to reviewer 1. We believe that we provided convincing evidence for the role of UNC13D in the antiviral effect of miR-517a, now

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including overexpression of UNC13D. However, we clearly acknowledge that other proteins may be important for this effect. This is now added on page 7, lines 7-14, with additional discussion on page 9, lines 18-31.

Comments

1. *The figures and text use "ss" vs. "miR" designations to indicate single stranded miRNAs vs. ds miRNA mimics. While this is easy enough to understand from the text, the designations are problematic as the native mature miRNA (which is single stranded) is specifically named "miR-517a". Thus it would be more appropriate to refer to the single stranded version as "miR-517a" (or ss miR-517a) and the double stranded perhaps as "pre-miR-517a" or "ds miR-517a mimic" or even "ds miR-517a".*

Response: We agree with the reviewer and indeed changed the terms used throughout the text to "mature miRNA" or simply "miRNA". Please see also comment #2 to Reviewer 1.

2. *It is unclear throughout what cells the authors are using and whether their TLR expression data and/or conclusions are correct. The specific descriptions of the cell lines are limited in the text here, but presuming that the 293XL/0 cells from InvivoGen are the control 293XL/null cells, this line should not express any of the TLRs. The authors contradict this. For example, line 16 states that the authors have validated the expression of TLR3 in all three 293XL lines; however the 293XL/null line is a control cell line that should not express TLR3. This indicates that the 293XL/0 cells are not true controls cells, or calls into question their conclusions from RNA expression data. Consistent with this, the addition of poly I:C, which should normally induce a very strong TLR3 response, causes a weak induction in 293XL/0 cells (Fig. 2A right panel inset). These findings all serve to suggest that more rigorous testing needs to be performed. Although Fig. 1 shows data demonstrating that PHTs express TLR3, TLR7 and TLR8 RNAs; a more rigorous demonstration would be to show protein levels for these TLRs. Moreover, the use of specific cell lines is varied and is many cases is inconsistently noted in the text. For example, Fig. S2A does not indicate anywhere what cell line is used. Due to the reliance upon comparison between different cell lines through the manuscript, individual cell lines for every panel should be clearly indicated on each figure.*

Response: We apologize for causing this confusion. The data are now presented in Fig. S2, and the control cells we used for the InvivoGen 293XL/8 or 293XL/7 are the parental 293XL/0 cells that do express endogenous levels of TLR3 (and also TLR5 and NOD1, https://www.invivogen.com/293-control-cell). We clarified this in the Methods on page 11, line 11. Our PCR data in Fig. S2 supports the uniform level of TLR3 among the three 293XL cell lines. With regard to the response to poly I:C, while the effect was 6-fold on the p65-Luc reporter (now Fig. 3A, right panel), the effect was 10-30-fold on other reporter constructs (Fig. S3B). The expression of TLR3 in HEK293 cells has been extensively characterized (one example by Alexopoulou *et al*, *Nature* 2001). We did not repeat experiments designed to further characterize these cells, because we relied on our validation using the more sensitive and highly quantitative qPCR data and because TLR antibodyies are notoriously unreliable. As we re- structured our paper and placed much greater emphasis on UNC13D, we hope that the reviewer agrees that such an expansion of our experiments on a relatively more minor component of our revised paper might not be warranted.

3. *The authors repeatedly state that they have performed CLASH analysis of Ago associated miRNA-mRNA hybrids in PHT cells, and include methods for the assay. However, no quality control data or experimental data from the CLASH analyses have been included in this manuscript. If this authors wish to include CLASH analyses in this manuscript then they need to include relevant control and experimental data and full data sets. These findings should elaborate and provide more specific information about the entire data sets, and should additionally include specific information about miR-517a: How many miR-517a targets were identified? What was the representation of the targets tested here?*

Response: We appreciate this comment and the reviewer's interest. In this Short Report we focused on the function of UNC13D and other two targets of miR-517a, which served as negative controls. We have provided the necessary technical information in the Methods section (page 12, lines 14-29). Moreover, as requested, we have deposited the CLASH-seq data in the NIH Sequence Read Archive with BioProject accession #PRJNA659526 (page 12, line 27-29). We are currently pursuing

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other targets that did not meet the defined criteria established for this paper, and we will publish them along with an expanded methodological analysis.

*4. Although the data in the manuscript (eg, Fig 4F) clearly suggest that miR-517a may have an antiviral effect on VSV (via repression of gene expression or genome replication) in U2OS cells, this conclusion would be significantly strengthened by a more robust measure of virus propagation such as a plaque assay.*

Response: Thank you. We have previously demonstrated the antiviral activity of miR-517a using a plaque assay (Delorme-Axford et al, *PNAS* 2013). In this revised text we have performed additional experiments, and showed the effect of UNC13D using a range of MOIs (Fig. 1E). We have expanded our experiments to include overexpression of UNC13D and its effect on VSV vRNA levels (Fig. 2)

*5. Some basic information about the virus that is the basis of these studies (aside from the name), should be included in the manuscript. In addition, when referring to "VSV RNA" it would be useful to explain which portion of the 11 kb VSV genome is being tested.*

Response: Thank you. We briefly introduce the basic information of VSV (page 6, line 31 through page 7 line 1.) We also added the requested data on PCR amplification of the vRNA, which we performed using amplicons derived from Glycoprotein (G) region in the VSV genome as we described in our recent publication (Delorme-Axford E. et al, *PNAS* 2013) and VSV Nucleoprotein (N), which is an indispensable component to support VSV replication in host cells. VSV vRNA fold changes of either viral G or N RNA were comparable. We added this information in the Method (page 14, lines 12-15)

*6. The authors have not shown that miR-517 targeting of UNC13D is a requirement for the antiviral effect of miR-517 on VSV. This should be shown in some capacity in order to support the mechanistic conclusion that the authors wish to make.*

Response: Thank you for the important point. Based on this comment, we have performed additional experiments where we overexpressed UNC13D. As shown in the new Fig. 2, overexpression of UNC13D in U2OS cells significantly increased VSV vRNA levels when compared to control, GFP expressing cells, suggesting that UNC13D promoted viral replication. Moreover, we showed that UNC13D overexpression reversed, at least in part, the antiviral action of miR-517a, and enhanced VSV infection (Fig. 2A-C). Thus, when combined with our silencing data, and the additional new data in Fig. 2, we believe that we have offered multiple lines of evidence showing that UNC13D, one of miR-517a targets, mediates, at least in part, the antiviral activity of miR-517a in U2OS cells. The evidence is summarized in a new section of our Discussion on page 9, lines 18-24.

Minor Comments:
*1. Line 11 states that Fig.1 shows that the PHT cells respond to the TLR ligands, however Fig.1 only shows RNA expression data, not ligand responsiveness.*

Response: Thank you. We apologize for our error, and deleted this statement. We have restructured the text and figures by focusing on the miR-517a-UNC13D axis. Accordingly, we moved the Fig.1 results to the new Fig.S2.

**Reviewer 3**

*Krawczynski et al. show that chromosome 19 miRNA cluster specifically mirRNA-517a affects antiviral immunity through post-transcriptional regulation of Unc13. Interestingly, the first part of the paper describes how single-stranded mature miRNAs activate the TLR8 pathway and downstream genes. The first two figures and the last two figures focus on separate findings with a common theme of miRNAs affecting innate and antiviral immunity. Fig-1 & 2 show single- stranded mature miRNAs activate the TLR8 pathway. These data are very preliminary and not sufficiently controlled to support their claims. I strongly suggest that the authors should remove these data from this manuscript. Figs 3 & 4 show that miR-517a regulates Unc13D, which is important in antiviral immunity, has promising data that should be further strengthened. I would advise the authors to focus on the UNC13 part of the story and strengthen these data.*

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Response: We agree. Similar comments, in some variations, were made by the other reviewers. We took them to heart, and revised the entire manuscript. See also our general comment response. We performed new experiments and generated new data that bolstered our conclusions on the miR-517a-UNC13D axis. These data are now presented in Figs. 1-2. Importantly, we added data showing that overexpression of UNC13D in U2OS cells significantly increased VSV vRNA levels, consistent with the effect of UNC13D KO in the new Fig.1. Furthermore, we showed that overexpression of UNC13D in cells exposed to the antiviral effect of miR-517a, enhanced viral infection, suggesting that UNC13D, at least in part, mediates the effect of miR- 517a on VSV replication. These, and the additional new data in Fig. 2, can be summarized as following: (a) miR-517a silences the expression of *UNC13D*; (b) VSV vRNA levels are lower in *UNC13D* KO; (c) VSV vRNA levels are increased upon overexpression of UNC13D in cells expressing miR-517a; (d) UNC13D enhances the phosphorylation and hence inactivation of the autophagy-promoting factor TFEB (page 9, line 18-24). These data also buttress the link between the action of miR-517a and autophagy. While we agree that the section in our paper about TLRs (now in Fig. 3 and Fig. S1-2) can be viewed as a distinct part of our paper, we believe that this section is relevant to our findings even if our data indicated that C19MC miRNA-mediated TLR8 activation is not unique to this family of miRNAs. Our assessment is based on our recent discovery that trophoblastic small extracellular vesicles (sEVs, or exosomes) enter cells through discrete endocytic pathways (macropinocytosis and clathrin-mediated uptake) and ultimately deliver exosomal miRNA cargo (including C19MC miR-517a) to cytoplasmic P-body proteins Ago2 and GW182 (Li et al, *J Extracellular Vesicles*, in press). These findings, which are now added to our text on page 7, line 31, through page 8, line 2, provide additional justification to our pursuit, because sEV miR-517a, which enters endosomes, would encounter endosomal TLR8 that typically recognizes single stranded RNA, including mature miRNA. In addition, given that certain miRNAs are able to activate endosomal TLR8-mediated signaling pathways such as NFκB/p65, we believed that these data justified the pursuit of miRNA activation of TLR8 pathways.

Comments

1. Authors extensively describe the CLASH technique, which led to the identification of miR-517 targets. However, no data obtained from the sequencing are presented in this manuscript. An additional figure with the sequencing data needs to be presented. I also could not find the accession number for the sequencing data that should be deposited (probably I missed it).

Response: We appreciate the reviewer's comment. In this Short Report we focused on the function of UNC13D and two other targets of miR-517a (ITGB4 and RPTOR, both served as negative controls). As requested, we have deposited the CLASH-seq data in the NIH Sequence Read Archive with BioProject accession #PRJNA659526 (page 12, lines 27-29). We are currently pursuing other targets that did not meet our criteria for inclusion in this text, and we will publish them along with an expanded methodological analysis.

2. Figs 3 & 4: Deletion of endogenous miR-517 is critical to show that the effect on unc13D and VSV is physiological. These experiments need to be added to the manuscript.

Response: The C19MC miRNAs, including miR-517a, are expressed almost exclusively in the human trophoblasts. Our focus here was on the effect of these miRNAs on non-trophoblastic cells that do not endogenously express C19MC miRNAs (including miR-517a). We reported that these miRNAs can bestow an antiviral activity upon these non-placental cells, and we emphasize that these target cells, including the U2OS cells we pursued here, do not express miR-517a or any other C19MC miRNA (Delorme-Axford E. et al, *PNAS* 2013). Thus, we would not be able to delete endogenous miR-517a in U2OS cells and study the physiological consequence of this effect. In our future pursuits, we will produce a miR-517a knockdown or knockout in human trophoblasts and interrogate its effect on UNC13D and VSV infection.

3. *Adding a schematic of a model would help the readers appreciate the study.*

Response: We are grateful for this comment. We humbly feel that the data provided in this Short Report warrant further investigation before a model is proposed. Nonetheless, we hope that our revised text and re-organization of the figures help to provide a clearer explanation of our results and inferences.

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Second decision letter

MS ID#: JOCES/2020/246769</font>

MS TITLE: Unc-13 Homologue D Mediates an Antiviral Effect of the Chromosome 19 MicroRNA Cluster miR-517a

AUTHORS: Kamil Krawczynski, Yingshi Ouyang, Jean-Francois Mouillet, Tianjiao Chu, Carolyn Coyne, and Yoel Sadovsky
ARTICLE TYPE: Short Report

I am happy to tell you that your manuscript has been accepted for publication in Journal of Cell Science, pending some minor text changes suggested by the reviewers to improve clarity in some areas, correction of the BioProject issue, and standard ethics checks.

Reviewer 1

*Advance summary and potential significance to field*

After restructuring the narrative, focusing more on the link between C19MC miRNA and autophagy, the paper is more accessible and conveys an interesting story with new information on the mechanistic underpinning of the anti-viral effect of C19MC.

*Comments for the author*

The authors have responded appropriately and sufficiently, including adding some new experiments, to the previous critique. However, albeit a minor problem, by adding new experiments linking UNC13D to autophagy via TFEB it appears that an apparent inconsistency was introduced: TFED activation by Torin2 (mTOR inhibitor) reduced the virus-promoting activity of UNC13D, yet RPTOR silencing (mTORC1 inhibition) did not?
May need some brief discussion/explanation.

Reviewer 2

*Advance summary and potential significance to field*

This manuscript is substantially improved over the previous version due to the addition of new experiments and rearrangement of the data flow. The authors have addressed all of my major concerns appropriately.

*Comments for the author*

The one lingering issue of note is that the authors have indicated in the manuscript that they have made available their CLASH-seq data sets via the NIH BioProject resource; however, this BioProject number is not found. Please clarify that this is the correct number and that all data sets will be made available at least by the time of publication.

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**Second revision**

Author response to reviewers' comments

Reviewer 1 *Comments for the Author:*
*The authors have responded appropriately and sufficiently, including adding some new experiments, to the previous critique. However, albeit a minor problem, by adding new experiments linking UNC13D to autophagy via TFEB it appears that an apparent inconsistency was introduced: TFED*

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*activation by Torin2 (mTOR inhibitor) reduced the virus-promoting activity of UNC13D, yet RPTOR silencing (mTORC1 inhibition) did not? May need some brief discussion/explanation.*

Response: Thank you for the comments. Indeed, we found that although siRNA-mediated knockdown of RPTOR did not reduce VSV vRNA, UNC13D knockout reduced VSV vRNA and activation of TFEB by Torin 2 reduced the virus-promoting activity of UNC13D. We didn't examine whether silencing of RPTOR reduces the virus-promoting activity of UNC13D.

While mTORC1-mediated phosphorylation of S6K and 4E-BP1 requires RPTOR as a scaffolding protein to recruit these two substrates, mTORC1-mediated phosphorylation, and thereby inactivation of TFEB, is independent of RPTOR (Napolitano et al, *Nature* 2020, cited in our text). Thus, RPTOR and TFEB appear to mediate distinct axes of the mTOR complex with regard to the control of autophagy. Unlike RPTOR, which serves as an adaptor protein for recruitment of S6K and 4E-BP1, TFEB is a potent, direct activator of autophagy, acting by nuclear translocation and enhanced transcription of autophagy-related genes. Based on our data, we concluded that UNC13D increases VSV vRNA levels by promoting TFEB phosphorylation, leading to inactivation of TFEB and thereby inhibition of autophagy. The interaction of RPTOR with TFEB is likely less direct. We have modified the text on page 9, lines 24-30.

Reviewer 2 *Comments for the Author:*
*The one lingering issue of note is that the authors have indicated in the manuscript that they have made available their CLASH-seq data sets via the NIH BioProject resource; however, this BioProject number is not found. Please clarify that this is the correct number and that all data sets will be made available at least by the time of publication.*

Response: Thank you. BioProject accession #PRJNA659526, as indicated in the Methods (section on CLASH, page 12 lines 28-29) is correct and will be available upon publication of our work, per NIH BioProject setting guidelines. (www.ncbi.nlm.nih.gov/bioproject/docs/faq).

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