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The tRNA pseudouridine synthase TruB1 regulates the maturation of let-7 miRNA

Ryota Kurimoto, Tomoki Chiba, Yoshiaki Ito, Takahide Matsushima, Yuki Yano, Kohei Miyata, Yuka Yashiro, Tsutomu Suzuki, Kozo Tomita, and Hiroshi Asahara
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Corresponding author(s): Hiroshi Asahara (asahara@scripps.edu)

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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. Depending on transfer agreements, referee reports obtained elsewhere may or may not be included in this compilation. Referee reports are anonymous unless the Referee chooses to sign their reports.)

1st Editorial Decision 25th Mar 2020

Thank you for submitting your manuscript proposing a role for TruB1 in let-7 maturation and function for consideration by The EMBO Journal. We have now received three reports on your study, which are included below for your information.

As you will see, the referees express interest in the proposed regulation of let-7 by TruB1. However, they are not yet fully convinced that this function is sufficiently supported by the experimental data currently included in the manuscript, and find that substantial additional analyses of how TruB1 acts on let-7 would be needed. For a revision, experiments that address which steps of let-7 maturation TruB1 affects (referee #2 points 1-4, referee#3 point 1) would therefore be important, as well as validating the specificity of DGCR8 recruitment and competitive binding with Lin28B (ref#2- point 5, ref#3- minor point 9). Furthermore, more discussion of the potential role of TruB1 on additional targets (ref#3- point 2), and controls for the pseudouridylation assays requested by referee #3 (point 3), should be added. In addition to these specific experimental points, please also carefully respond to the other issues the reviewers raise and potentially provide data when available.

We recognize that addressing the referees' comments fully would require a significant amount of experimental work and time, likely including experiments with an unforeseeable outcome. However, we realize that such an extensive revision may currently not be feasible due to the COVID-19/SARS-CoV-2 pandemic affecting labs worldwide. While we can extend the reviewing deadlines and have extended our 'scooping protection policy' to cover the period required for a full revision, we can also offer to discuss a potential transfer of the manuscript with referee reports to EMBO reports, which will have different requirements regarding the extent of mechanistic insight that needs to be provided. Independently of which option you choose, we strongly encourage you to contact us, either to discuss a revision plan or a potential transfer to ensure that all issues are addressed at this stage.

REFEREE REPORTS

Referee #1:

In this paper, the authors describe an interesting set of experiments showing an unexpected role of TruB1 in let-7 miRNA maturation. Previous studies have identified the RNA binding protein Lin-28 represses the processing of let-7. In contrast with this, the authors identified TruB1 as a positive regulator of primary let-7 processing. Interestingly, the authors found that TruB1 enhances pri-let-7 processing independently of its enzymatic activity. Moreover, they conducted biochemical approaches such as HITS-CLIP and obtained a set of data showing that the TruB1 specifically binds to pri-let-7 and that an enhanced affinity between microprocessor and pri-let-7 by TruB1. Finally, the authors revealed the biological significance of the TruB1-mediated regulation of pri-let-7 in cell culture.
The experiments in this paper are organized well and the story is clear and outstanding because this manuscript opened a novel link between tRNA modifying enzyme and microRNA biogenesis. So, this reviewer recommends publication of this paper after the following minor points are revised.

Fig. 2B and Fig. 3G: A size marker should be shown.

Page 16, line 1: "microprocesser" should be " microprocessor ".

Referee #2:

Kurimoto and collaborators report here a potential role for the tRNA pseudouridine synthase TruB1 in the maturation and function of let-7 microRNA. A targeted screening strategy based on the overexpression of human RNA binding proteins (RBPs) was used to identify new factors that modulate let-7 production and activity. Among the ones find, TruB1 was the only RBP that specifically affect let-7. HITS-CLIP experiments further support that TruB1 binds to the stem-loop structure of pri-let-7 and in cellulo data suggest that TruB1 suppresses cancer cells proliferation, in part, through its modulation of let-7.

Overall, this is an interesting study that has the potential to provide significant insights into the new role of TruB1 in the regulation of let-7 maturation and function. Unfortunately, the data presented here fail to support most of their claims and to clearly demonstrate how molecularly TruB acts on let-7 maturation. As listed below, there are several issues with the interpretation of the data that must be addressed experimentally. If the authors can address those concerns appropriately, I believe that this study can become extremely interesting for many scientists in the miRNA/RNA field and thus being considered as a strong candidate for publication in EMBO Journal.

-To demonstrate the biological relevance of TruB1 in the regulation and function of let-7, the authors must monitor the effect of knocking down and altering TruB1 on endogenous let-7 targets. Only using a reporter in which let-7 acts as a siRNA is not sufficient.

-It is extremely difficult to understand why TruB1 KD does not affect the pre-let-7 level if TruB1 is

supposed to stimulate the processing of pri-let-7. The methods used to monitor the effect of TRuB1 on let-7 maturation are likely not optimal. For Northern blotting, the authors should use specific probes targeting pre-let-7 sequences as previously used to demonstrate the role of DGCR8 and Drosha in pri-let-7 processing (see Han et al, Cell 2009). Along this line, it will also be necessary to monitor by Northern blots the level of precursor molecules of miRNA that are not affected TruB1 to support its specificity for let-7. Also, the processing and level of mature let-7 detected by Northern blot are quite suboptimal/low to be able to draw convincingly any conclusion (Figures 1B;3D). The authors should use different cells with more let-7/miRNA efficient processing (such as HeLa). Using chemical (using EDC) instead of UV crosslinking will also greatly help to improve the sensitivity of miRNA detection by Northern blotting.

-The data presented in Figure 3 for supporting that TruB1 function on let-7 maturation is independent of pseudouridylation are not extremely convincing. First, the difference in the let-7 level upon TruB1 WT and mutant variants expression is really hard to appreciate with the Northern blot presented in Figure 3D. As mentioned before, a direct detection of pri- to pre-let-7 processing is essential to support their claim. Second, I doubt that with the level of radiolabeled pri-miRNA used to monitor pseudouridylation in vitro (Figure 3F), it will be possible to detect such modification on pri-let-7 considering the low level detected with a tRNA for which, a great amount of RNA substrate has been used. Finally, it is unclear to me how to interpret the data presented in Figure 3G. Some bands are appearing upon CMC treatment of pri-let-7. Why are those bands not considered as pseudouridine sites? More details/explanations are needed here.

-With the data presented in Figure 5A, it is nearly impossible to conclude that either the overexpression of different TruB1 forms (WT and mutant variants) or the knockdown of endogenous TruB1 affected pri-let-7 maturation as it is unclear which band correspond to which miRNA molecule species. Appropriate controls (Dicer and Drosha KD) are needed to clearly indicate which band corresponds to primary, precursor and mature let-7 molecules (adding a molecular weight marker will also be extremely useful as well). As mentioned before, there is still a concern about the type of cells used here as the processing efficiency appears to be quite low.

-To clearly demonstrate the specificity of TruB1 in the recruitment of DGCR8 as well as the competitive binding with Lin28B on pri-let-7, the same experiments should be performed with primary miRNAs that are not affected with TruB1.

-In Figure 6, to demonstrate the functional effect on KRAS regulation, the level of endogenous KRAS protein should be monitored upon ectopic expression of WT and mutants TruB1. The use of a luciferase reporter assay is not sufficient here to support the functional significance of TruB1 in the control of cancer cells proliferation.

-The introduction section has to be significantly improved. Recent studies and reviews about factors affecting the miRNA production and function should be included with emphasis on recent papers about RBP/proteins affecting miRNA production. The authors should also pay attention to publications referenced in this section as several references are inappropriately listed (for example, let-7 is not described in the Lee et al 1993 paper; the Tabara et al 1999 paper never reported anything related to miRNA).

Referee #3:

General summary and opinion about the principle significance of the study, its questions and

findings
In this manuscript, the authors discover and characterize a novel function of the human pseudouridine synthase TruB1 for specific maturation of the let-7 family of microRNAs which is independent of the RNA modification activity of TruB1. The authors have identified this function through a novel let-7 specific screen and conducted the appropriate experiments to verify and further characterize this discovery. This finding is remarkable for two reasons and is therefore in my opinion of general interest to the readership of EMBO Journal.
First, although pseudouridine synthases are known since a long time, their cellular function and conservation remains mysterious as knockouts of these enzyme often show no or little phenotypic effects. Therefore, it is highly interesting that the authors not only identified a function for human TruB1 which is linked to a phenotype (Fig 6), but that they also demonstrate that this function is independent of TruB1's catalytic activity. This finding is only paralleled by the recent report in Nature Chemical Biology by Song J et al. (2019) that Pus10 also plays a role in miRNA processing although this is entirely different from TruB1's function. Hence, it is of general interest that out of the 10 human pseudouridine synthases two have now been linked to microRNA maturation.
Second, this manuscript is of general interest as it reports an effect of TruB1 on miRNA processing that is unique to a subset of miRNAs, in particular the let-7 family of miRNAs. Several factors influencing miRNA maturation in general have been identified previously and also in this screen (Fig. 1), but it is rare to discover a miRNA-specific function. However, as further outlined below, the authors have to discuss more clearly the scope of TruB1's effects on miRNA maturation. I am convinced that this manuscript will trigger further studies to identify the molecular mechanism of TruB1 during let-7 maturation including the structural basis for the high miRNA specificity.

Specific major concerns essential to be addressed to support the conclusions
1. To avoid misunderstandings, the authors need to clearly distinguish between effects of TruB1 on the maturation, the levels and the function of let-7 miRNA in all areas of the manuscript including the title and abstract. Based on their experiments, it is proven that TruB1 affects the maturation of let-7 as pri-miRNAs are accumulating when TruB1 is reduced (Fig. 2E). It is also demonstrated that overexpressing or knock-down of TruB1 affects the levels of mature let-7 miRNA (Fig. 1, 2). However, no experiment in this study shows a direct effect on let-7 miRNA function! The let-7 miRNA behaves the same no matter whether TruB1 is present or not, and all observations can be simply explained by different levels of let-7 miRNA.
Therefore, I strongly recommend removing any expression suggesting that TruB1 affects or regulates let-7 miRNA function which includes altering the title and the last sentence of the abstract.
2. In Fig. 4E, the authors show that HITS-CLIP also identifies other miRNAs bound by TruB1 which are also downregulated upon TruB1 knock down (page 15, top). This finding suggests that TruB1 is not exclusively specific to let-7 miRNAs which needs to be discussed more clearly (e.g. expanding the discussion on page 20). Do these miRNAs bound by TruB1 have a common structure or sequence motif? Why would TruB1 affect some, but not all miRNAs? Do these miRNAs play similar roles for proliferation as let-7 miRNA?
3. Figure 3F: the signals for uridine are very faint when pre-let-7a1 or pri-miR10a are used as substrates in the pseudouridylation assays. Given that the majority of uridines will not be isomerized by site-specific pseudouridine formation, it is therefore not clear whether a low amount of pseudouridylation could be detected in this assay. The authors should repeat the assays with more RNA such that the uridine spots are of similar intensity for the positive control tRNAPhe as well as the two pri-mRNAs to convincingly show that no significant pseudouridine formation by TruB1 occurs.

Minor concerns that should be addressed

1. The authors should also briefly comment on the top five hits where over-expression increases luciferase activity and whether these candidates are likely to directly reduce let-7 miRNA levels.
2. Surprisingly, one of the top-5 candidates identified in the screen, LARP7, could not be validated. Why not?
3. The authors should briefly explain the sequence and structure variation among the different let-7 miRNA family members. Can these differences explain the slight variation in effects observed, e.g. upon knock-down of TruB1 (Fig. 2)? Or are the differences in miRNA expression within the noise of the measurement?
4. page 16, top, subtitle: the authors show that less let-7 pri-miRNA is bound toDGCR-8 when TruB1 is knocked down, but they never measure the dissociation constant describing the affinity of DGCR-8 to let-7 pri-miRNA. Therefore, the subtitle needs to be rephrased, e.g. to "TruB1 enhances the interaction between...".
5. The subtitle on page 17 is incomplete and needs to be corrected: "TruB1 suppresses cell growth through the promoting let-7". A word seems to be missing.
6. The model presented in Fig. 6 which is partly discussed on page 22 is rather superficial. It would be interesting if the authors can at least present a plausible hypothesis how TruB1 can affect the microprocessing step of let-7 maturation. Is it conceivable that TruB1 alters the RNA structure acting as an RNA chaperone which in turn facilitates recognition of this structure by DGCR-8?
7. The description and figure legend for Fig. 6D needs to be improved to better describe the differences between the curves. Which experiments include TruB1 overexpression? What is the difference between "wt + let7 KD" and "let7 KD"?
8. Figure 3B and F: after nuclease P1 digestion of the RNA in the in vitro pseudouridylation assays, the RNA is degraded into nucleotides with a single phosphate. Therefore, the labelling of the top spot as "UTP" is incorrect. Simply labelling this as "U" will be sufficient.
9. Does TruB1 interact directly with DGCR-8? Could TruB1 bind first to pri-let7-miRNA and thereby help to recruit DGCR-8? As the authors have conducted immunoprecipitations, they should check whether DGCR-8 is co-eluted upon TruB1 immunoprecipitation and vice versa (expanding Fig. 5).

Non-essential suggestions for improving the study
• page 4, middle: remove the term "cleavage" to read "DGCR8-mediated processing"
• In the Materials & Methods section, there are a few instances where microgram is incorrectly abbreviated as ug instead of using the appropriate Greek letter. Also, there should always be a space between a number and the corresponding unit. A few times "nN" is written instead of "mM" to describe a buffer concentration.
• Figure legend for Fig. EV3 C (page 69): The figure only shows data for pri-let-7a1, therefore no reference should be made to tRNAPhe in the figure legend.

Response to referees' comments

We would like to take this opportunity to thank the referee's for their comments and suggestions. The review identified aspects of the original manuscript that needed more detailed explanations or additional information. Our responses to the reviewer's criticisms are listed below. Changes in the revised manuscript are marked in red.

We hope that the revisions have resulted in a more complete manuscript that will be ready for publication.

Referee #1 Comments for the Author.

In this paper, the authors describe an interesting set of experiments showing an unexpected role of TruB1 in let-7 miRNA maturation. Previous studies have identified the RNA binding protein Lin-28 represses the processing of let-7. In contrast with this, the authors identified TruB1 as a positive regulator of primary let-7 processing. Interestingly, the authors found that TruB1 enhances pri-let-7 processing independently of its enzymatic activity. Moreover, they conducted biochemical approaches such as HITS-CLIP and obtained a set of data showing that the TruB1 specifically binds to pri-let-7 and that an enhanced affinity between microprocessor and pri-let-7 by TruB1. Finally, the authors revealed the biological significance of the TruB1-mediated regulation of pri-let-7 in cell culture.

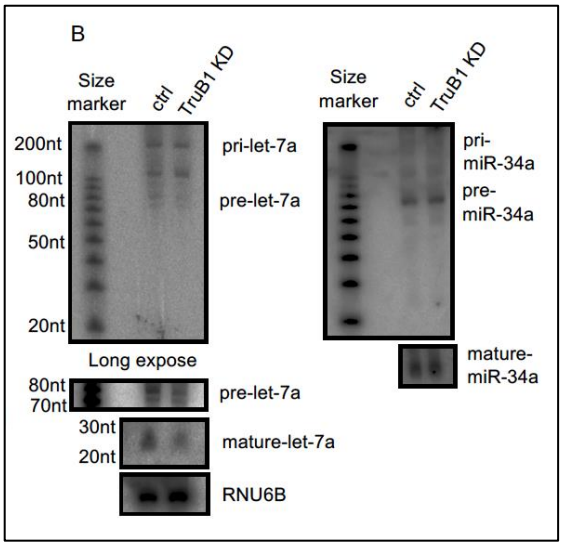
The experiments in this paper are organized well and the story is clear and outstanding because this manuscript opened a novel link between tRNA modifying enzyme and microRNA biogenesis. So, this reviewer recommends publication of this paper after the following minor points are revised.

1. Fig. 2B and Fig. 3G: A size marker should be shown.

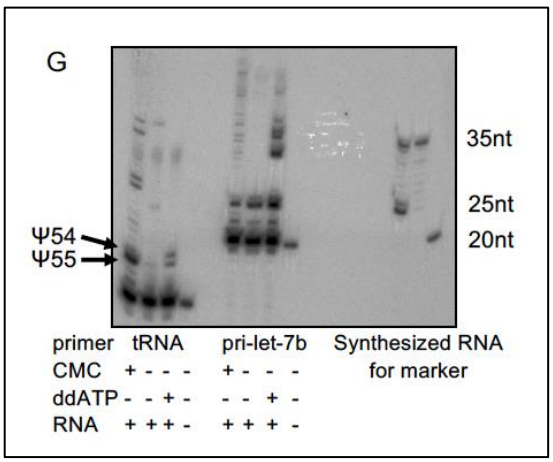
Response

We would like to thank you for this helpful comment. We added a size marker on revised Fig. 2B and Fig. 3G. Decade marker for small RNA was used in Fig. 2B. Synthesized RNAs were used in Fig. 3G.

New Fig.2B



New Fig.3G



2. Page 16, line 1: "microprocesser" should be " microprocessor ".

Response

Thank you for pointing out this error; we have revised it accordingly.

Referee #2 Comments for the Author.

Kurimoto and collaborators report here a potential role for the tRNA pseudouridine synthase TruB1 in the maturation and function of let-7 microRNA. A targeted screening strategy based on the overexpression of human RNA binding proteins (RBPs) was used to identify new factors that modulate let-7 production and activity. Among the ones find, TruB1 was the only RBP that specifically affect let-7. HITS-CLIP experiments further support that TruB1 binds to

the stem-loop structure of pri-let-7 and in cellulo data suggest that TruB1 suppresses cancer cells proliferation, in part, through its modulation of let-7.

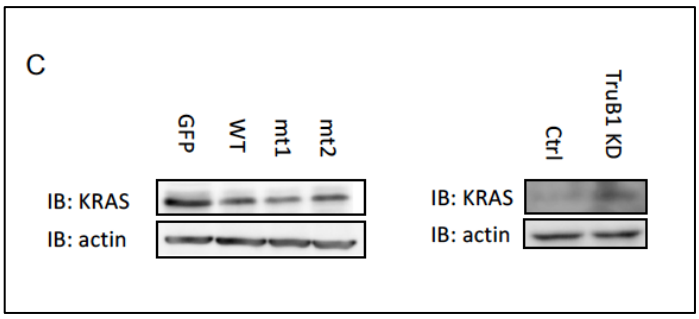
Overall, this is an interesting study that has the potential to provide significant insights into the new role of TruB1 in the regulation of let-7 maturation and function. Unfortunately, the data presented here fail to support most of their claims and to clearly demonstrate how molecularly TruB acts on let-7 maturation. As listed below, there are several issues with the interpretation of the data that must be addressed experimentally. If the authors can address those concerns appropriately, I believe that this study can become extremely interesting for many scientists in the miRNA/RNA field and thus being considered as a strong candidate for publication in EMBO Journal.

1. To demonstrate the biological relevance of TruB1 in the regulation and function of let-7, the authors must monitor the effect of knocking down and altering TruB1 on endogenous let-7 targets. Only using a reporter in which let-7 acts as a siRNA is not sufficient.

Response

We appreciate this referee's critical comment. Therefore we analyzed the effect of KD and overexpression of TruB1 on the endogenous expression of KRAS, which is a main target of the let-7 family, by Western blotting. We found that overexpression of TruB1 wt and mt1 decreased the expression of KRAS protein, whereas TruB1 KD increased it. We added this information in a new figure panel, and to the Results section of the revision manuscript.

New Fig. 6C



(Page 19, Line 8-10)

We also analyzed endogenous expression of KRAS by Western blotting, and found that overexpression of TruB1 wt and mt1 decreased the expression of KRAS protein. TruB1 KD increased the expression of the KRAS protein.

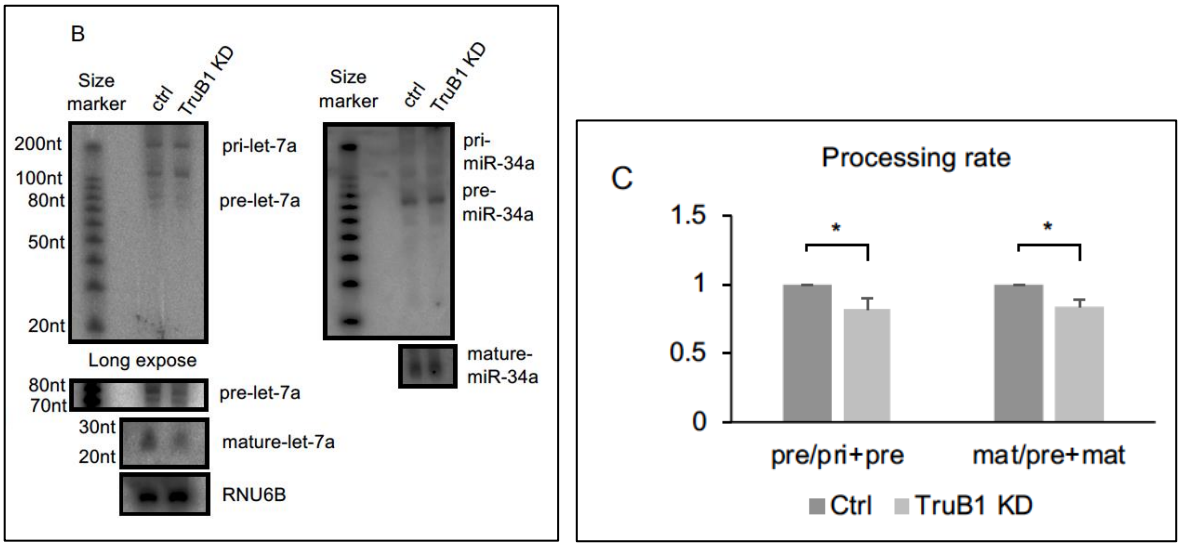
2. It is extremely difficult to understand why TruB1 KD does not affect the pre-let-7 level if TruB1 is supposed to stimulate the processing of pri-let-7. The methods used to monitor the effect of TRuB1 on let-7 maturation are likely not optimal. For Northern blotting, the authors should use specific probes targeting pre-let-7 sequences as previously used to demonstrate the role of DGCR8 and Drosha in pri-let-7 processing (see Han et al, Cell 2009). Along this line, it will also be necessary to monitor by Northern blots the level of precursor molecules of miRNA that are not affected TruB1 to support its specificity for let-7. Also, the processing and level of mature let-7 detected by Northern blot are quite suboptimal/low to be able to draw convincingly any conclusion (Figures 1B;3D). The authors should use different cells with more let-7/miRNA efficient processing (such as HeLa). Using chemical (using EDC) instead of UV crosslinking will also greatly help to improve the sensitivity of miRNA detection by Northern blotting.

Response

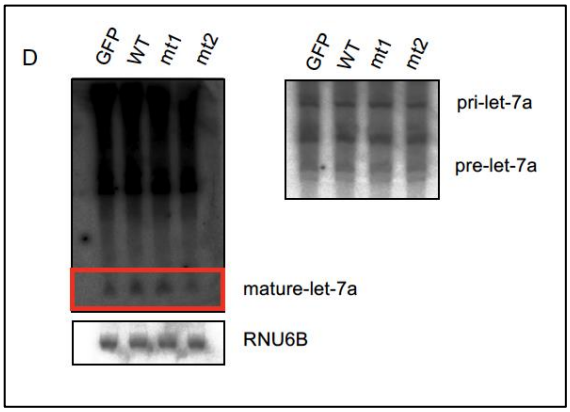
We appreciate this reviewer's critical comment. Northern blot was followed up in the manner indicated. HeLa cells with high expression of let-7 were used. This resulted in a more pronounced decrease in the amount of mature let-7a in knockdown. This trend was not observed for the other microRNA, microRNA34a. With overexpression, the amount of mature let-7 was also increased, and the increase was not seen with mt2.

In addition, using specific pre-let-7a1 probes, we found that the knockdown of TruB1 decreased the level of pre-let-7a1. Furthermore, no change in pre-let-7 was observed upon TruB1 overexpression. When we measured the processing rates directly, we found that there was a significant difference in both pri-to-pre processing and pre-to-mature processing. These results indicate that TruB1 function cannot be explained solely by the enhancement of processing from pri to pre, and that it also affects processing from pre to mature. We have revised the figure and manuscript as follows.

New Fig. 2B, C



New Fig. 3D



(Page 11, Line 9-16)

These data were further confirmed by large scale TaqMan PCR for miRNA and northern botting (NB) in HeLa cells, revealing that almost all endogenous mature let-7 family genes were significantly downregulated upon TruB1 knockdown (Fig 2B-D). We confirmed these findings in A549 cells (Fig EV2B). Thus, large scale qPCR experiments revealed a similar trend for each member of the let-7 family upon TruB1 knock down in three cell types (HEK293FT cells, HeLa cells and A549 cells). Even the members that did not show a statistically significant difference still showed a tendency to decline. TruB1 knockdown also decreased the level of pre-let-7a1 (Fig 2B). In contrast, TruB1 knockdown increased the levels of endogenous, immature, primary-let7 (Fig 2E). When we measured the processing rate, TruB1 knockdown significantly decreased the processing rate of both pri-to-pre and pre-to-mature processing.

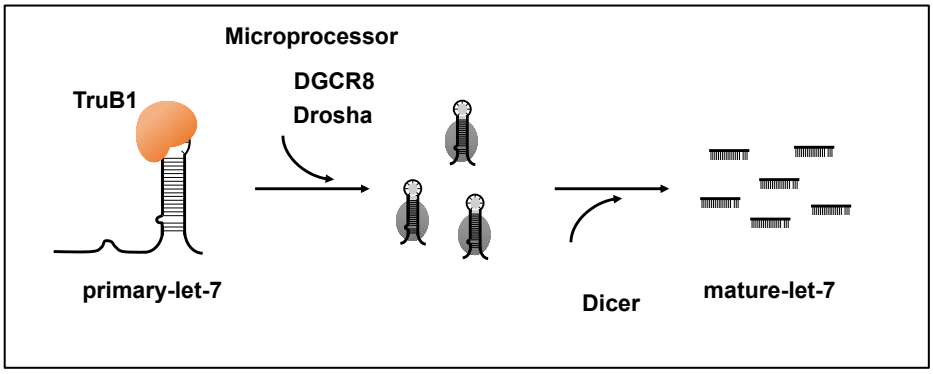
(Page13, Line 8-10)

Although no change in pre-let-7 was observed, the processing rate of pre-to-mature was elevated by overexpression of Wt and mt1.

(Page 17, Line 12-14)

These results suggest that let-7a biogenesis is promoted at the level of conversion from the pri-miRNA to the pre-miRNA and from the pre-miRNA to the mature-miRNA.

New Fig. 6F



3. The data presented in Figure 3 for supporting that TruB1 function on let-7 maturation is independent of pseudouridylation are not extremely convincing.

3-1. First, the difference in the let-7 level upon TruB1 WT and mutant variants expression is really hard to appreciate with the Northern blot presented in Figure 3D. As mentioned before, a direct detection of pri- to pre-let-7 processing is essential to support their claim.

Response

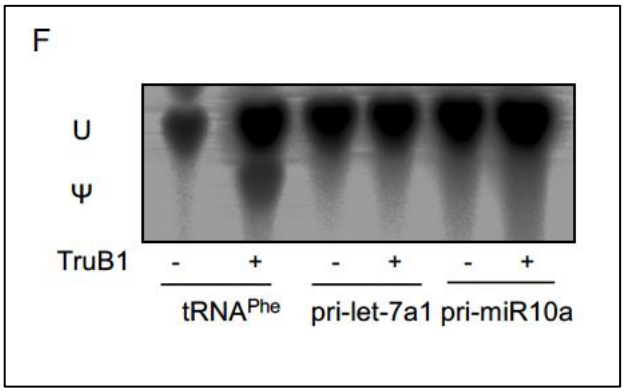
We appreciate the comment. New northern blots have been performed, as noted in the previous comment.

3-2. Second, I doubt that with the level of radiolabeled pri-miRNA used to monitor pseudouridylation in vitro (Figure 3F), it will be possible to detect such modification on pri-let-7 considering the low level detected with a tRNA for which, a great amount of RNA substrate has been used.

Response

We apologize that the RI labeled RNA levels are different. We performed the same reaction with more microRNA. As a result, there is no pseudouridine in vitro as previously reported. We revised the figure as follows.

New Fig. 3F



3-3. Finally, it is unclear to me how to interpret the data presented in Figure 3G. Some bands are appearing upon CMC treatment of pri-let-7. Why are those bands not considered as pseudouridine sites? More details/explanations are needed here.

Response

We apologize for our insufficient explanation of the CMC reaction. CMC binds strongly to pseudouridine and weakly to UTP and GTP. It is also known that even after 4 hours of treatment with alkaline solutions, binding to these non-pseudouridine still remains. Therefore, we monitored the position of the UTP using ddATP using a primer extension assay. The following additional explanation has been added to the text in revised manuscript:

(Page 14, Line 1- 11)

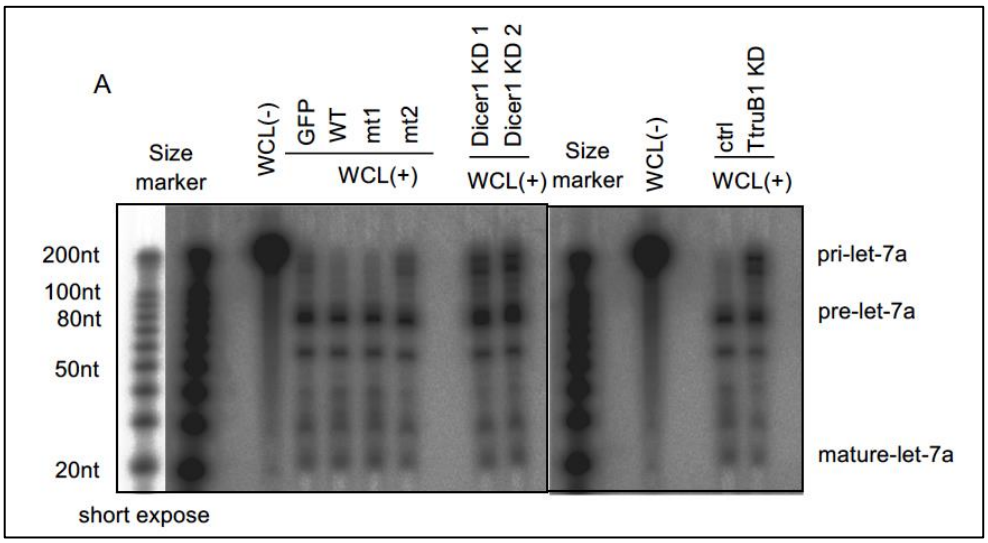
We monitored the position of the UTP using ddATP using a primer extension assay. If a band is found in CMC-treated RNA that does not match the height of this UTP, it can be determined to be non-specific binding. Furthermore, the bands observed in non-CMC-treated RNA cannot indicate the presence of pseudouridine. Based on these conditions, the multiple thin bands found around 35 nt of CMC-treated RNA in let-7 are likely to be non-specific bands. The bands at the height of the UTP was also only of the same intensity as the other non-specific bands in let-7. In contrast, in tRNAs, a dense band of CMC-treated RNA consistent with the height of UTP was observed, indicating the presence of pseudouridine. These results indicate that pseudouridine is not present in endogenous let-7.

4. With the data presented in Figure 5A, it is nearly impossible to conclude that either the overexpression of different TruB1 forms (WT and mutant variants) or the knockdown of endogenous TruB1 affected pri-let-7 maturation as it is unclear which band correspond to which miRNA molecule species. Appropriate controls (Dicer and Drosha KD) are needed to clearly indicate which band corresponds to primary, precursor and mature let-7 molecules (adding a molecular weight marker will also be extremely useful as well). As mentioned before, there is still a concern about the type of cells used here as the processing efficiency appears to be quite low.

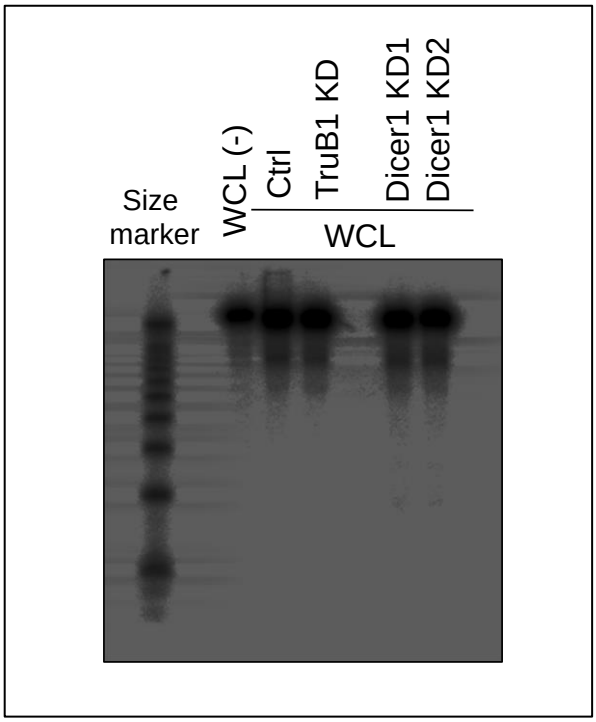
Response

We appreciate the comment. To clarify the size of the microRNA molecules, a size marker (decade marker for small RNA) was used in the in vitro processing assays. In addition, as this referee suggests, we used Dicer KD to clarify the band of pre-let-7. As a result, the pre-let-7 band was observed to be dense around the 70-80nt marker. It is now clear that there is also a pre-let-7a. In the assay using HeLa cells, even at the same scale as 293FT cells, there was not enough processing to the mature miRNA. This trend has been seen in previous reports with whole cell lysate without altering microprocessors (Michlewski et al., 2008; Michlewski et al., 2010). Therefore, we continued using 293FT cells for the in vitro processing assay, which gave the same results. We have revised the figure and manuscript as follows.

New Fig. 5A



(in vitro processing in HeLa cells)



(Page 15, Line 4-15)

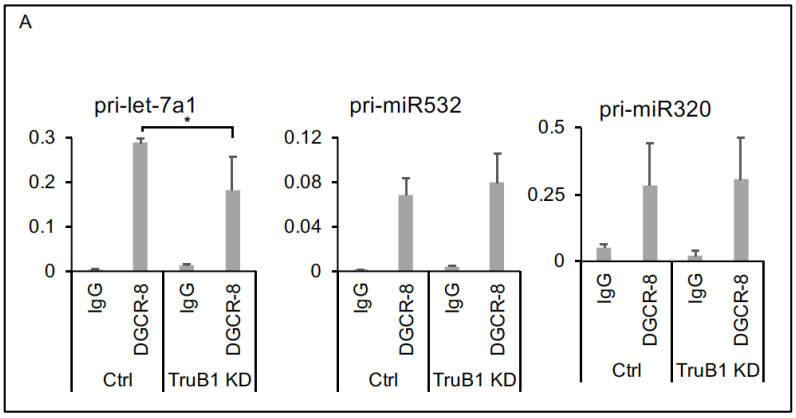
The above results indicated that TruB1 directly binds to pri-let-7a1 under physiological conditions. To elucidate the function of this molecular interaction, we analyzed whether TruB1 was involved in the microprocessing step of miRNA biogenesis using an in vitro processing assay. RI-labeled pri-let-7a1 was incubated with a cell extract obtained from HEK293FT cells overexpressing different forms of TruB1. We used Dicer KD to clarify the band of pre-let-7. We found that the extract from cells overexpressing TruB1 promoted maturation of pri-let-7a1 into pre- / mature-let-7a, whereas the extract from cells overexpressing mutant TruB1(mt2) did not. We also found that knockdown of endogenous TruB1 by siRNA inhibited maturation of pri-let-7a (Fig. 5A, B). These results suggest that let-7a biogenesis is promoted at the level of conversion from the pri-miRNA to the pre-miRNA and from the pre-miRNA to the mature-miRNA.

5. To clearly demonstrate the specificity of TruB1 in the recruitment of DGCR8 as well as the competitive binding with Lin28B on pri-let-7, the same experiments should be performed with primary miRNAs that are not affected with TruB1.

Response

A similar experiment with microRNAs unaffected by TruB1 (i.e., microRNA-532 and microRNA-320) showed that, unlike let-7, affinity with DGCR-8 was unchanged. We added this result as a revised figure to the manuscript.

New Fig. EV5A



(Page 18, Line 3-4)

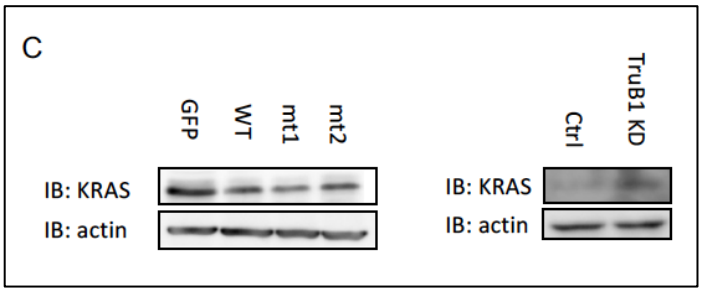
TruB1 KD by siRNA treatment reduced this interaction between only pri-let-7a1 and DGCR-8 (Fig 5C, Fig EV5A), without affecting the expression level of DGCR-8.

6. In Figure 6, to demonstrate the functional effect on KRAS regulation, the level of endogenous KRAS protein should be monitored upon ectopic expression of WT and mutants TruB1. The use of a luciferase reporter assay is not sufficient here to support the functional significance of TruB1 in the control of cancer cells proliferation.

Response

We appreciate this referee's critical comment. We analysed the endogenous expression of KRAS, , as noted in the previous comment. We found that overexpression of TruB1 wt and mt1 decreased the expression of KRAS protein. We added this information in a new figure and to the Results of the revision manuscript.

New Fig. 6C



(Page 19, Line 8-10)

We also analyzed endogenous expression of KRAS by Western blotting, and found that overexpression of TruB1 wt and mt1 decreased the expression of KRAS protein. TruB1 KD increased the expression of the KRAS protein.

7. The introduction section has to be significantly improved. Recent studies and reviews about factors affecting the miRNA production and function should be included with emphasis on recent papers about RBP/proteins affecting miRNA production. The authors should also pay attention to publications referenced in this section as several references are inappropriately listed (for example, let-7 is not described in the Lee et al 1993 paper; the Tabara et al 1999 paper never reported anything related to miRNA).

Response

We apologize for our inappropriate references. We revised the references and add recent reports in the introduction session as follows.

(Page 4, Line 6)

Lin-4 and let-7 were the first reported miRNAs in *C. elegans* (Lee et al., 1993; Reinhart., 2000).

(Page 5, Line 1-6)

and v) incorporation into the RNA-induced silencing complex (RISC) with AGO family proteins to generate the final mature miRNA (Mourelatos et al., 2002). Although the first transcription step for pri-miRNA expression can be driven by transcription factors and RNA Pol-II as well as mRNA, the following maturation steps, ii-v), are also critical and unique to miRNA biogenesis and dynamics (Bartel., 2018; Ha and Kim., 2014).

(Page 5, Line 15- Page 6, Line 4)

Based on these fundamental findings, the factors mediating let-7 multi-step regulation, which may be critical for various biological and pathological events, have been extensively explored by several approaches (Newman et al., 2008; Trabucchi et al., 2009; Treiber et al., 2017). Recently, it has been reported that METTL1 promotes processing of microRNA let-7 by m7G methylation (Pandolfini et al., 2019).

Referee #3 Comments for the Author.

General summary and opinion about the principle significance of the study, its questions and findings

In this manuscript, the authors discover and characterize a novel function of the human pseudouridine synthase TruB1 for specific maturation of the let-7 family of microRNAs which is independent of the RNA modification activity of TruB1. The authors have identified this function through a novel let-7 specific screen and conducted the appropriate experiments to verify and further characterize this discovery. This finding is remarkable for two reasons and is therefore in my opinion of general interest to the readership of EMBO Journal.

First, although pseudouridine synthases are known since a long time, their cellular function and conservation remains mysterious as knockouts of these enzyme often show no or little phenotypic effects. Therefore, it is highly interesting that the authors not only identified a function for human TruB1 which is linked to a phenotype (Fig 6), but that they also demonstrate that this function is independent of TruB1's catalytic activity. This finding is only paralleled by the recent report in Nature Chemical Biology by Song J et al. (2019) that Pus10 also plays a role in miRNA processing although this is entirely different from TruB1's function. Hence, it is of general interest that out of the 10 human pseudouridine synthases two have now been linked to microRNA maturation.

Second, this manuscript is of general interest as it reports an effect of TruB1 on miRNA processing that is unique to a subset of miRNAs, in particular the let-7 family of miRNAs. Several factors influencing miRNA maturation in general have been identified previously and also in this screen (Fig. 1), but it is rare to discover a miRNA-specific function. However, as further outlined below, the authors have to discuss more clearly the scope of TruB1's effects on miRNA maturation. I am convinced that this manuscript will trigger further studies to identify the molecular mechanism of TruB1 during let-7 maturation including the structural basis for the high miRNA specificity.

Specific major concerns essential to be addressed to support the conclusions

1. To avoid misunderstandings, the authors need to clearly distinguish between effects of TruB1 on the maturation, the levels and the function of let-7 miRNA in all areas of the manuscript including the title and abstract. Based on their experiments, it is proven that TruB1 affects the maturation of let-7 as pri-miRNAs are accumulating when TruB1 is reduced (Fig. 2E). It is also demonstrated that overexpressing or knock-down of TruB1 affects the levels of mature let-7 miRNA (Fig. 1, 2).

However, no experiment in this study shows a direct effect on let-7 miRNA function! The let-7

miRNA behaves the same no matter whether TruB1 is present or not, and all observations can be simply explained by different levels of let-7 miRNA.
Therefore, I strongly recommend removing any expression suggesting that TruB1 affects or regulates let-7 miRNA function which includes altering the title and the last sentence of the abstract.

Response

We appreciate this referee's critical comment and have toned down the claim that TruB1 regulates let-7 miRNA function as requested:

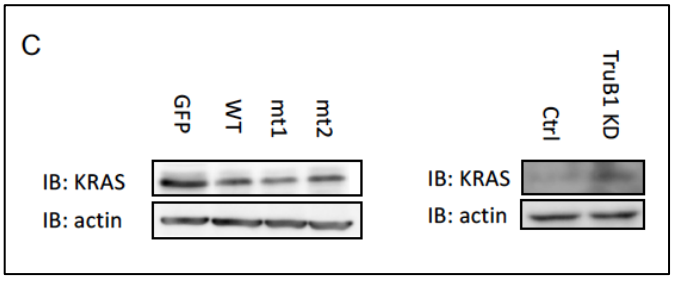
(Page 1, Line 1)
Title: The tRNA pseudouridine synthase TruB1 regulates the maturation of let-7 miRNA

(In Abstract, Page 3, Line 5)
Altogether, we reveal an unexpected function for TruB1 in promoting let-7 maturation.

(In Introduction, Page 6, Line 15-Page 7, Line 1)
We identified a new regulatory mechanism: TruB1, an RNA-modifying enzyme, selectively regulates let-7 levels in an enzymatic activity-independent manner.

In response to a comment from Reviewer 2, we analyzed the endogenous expression of KRAS, which is a main target of the let-7 family, by Western blotting as a first step to monitoring let-7 miRNA function mediated by TruB1. We found that overexpression of TruB1 wt and mt1 decreased the expression of KRAS protein, whereas TruB1 knock down increased it. We have added this information in a new figure and to the Results section of the revised manuscript.

New Fig. 6C



(Page 19, Line 8-10)

We also analyzed endogenous expression of KRAS by Western blotting, and found that overexpression of TruB1 wt and mt1 decreased the expression of KRAS protein. In contrast, TruB1 KD increased the expression of KRAS (Fig 6C).

2. In Fig. 4E, the authors show that HITS-CLIP also identifies other miRNAs bound by TruB1 which are also downregulated upon TruB1 knock down (page 15, top). This finding suggests that TruB1 is not exclusively specific to let-7 miRNAs which needs to be discussed more clearly (e.g. expanding the discussion on page 20). Do these miRNAs bound by TruB1 have a common structure or sequence motif? Why would TruB1 affect some, but not all miRNAs? Do these miRNAs play similar roles for proliferation as let-7 miRNA?

Response

We appreciate the referee's critical comments and expanded the discussion (Page 24, Line 1-13) As follows:

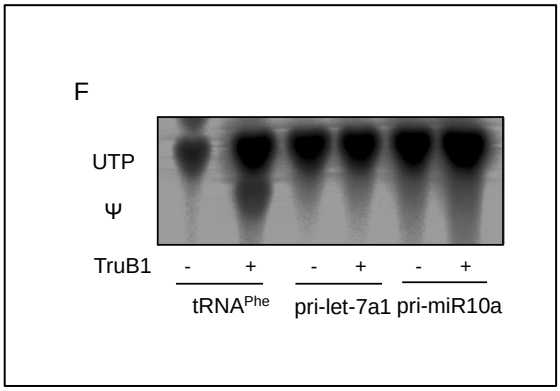
TruB1 also bound to other miRNAs (miR29b, miR107 and miR139), which are also downregulated upon TruB1 knock down in our HITS-CLIP, suggesting a common regulatory mechanism. However, no common sequences in the three loop structures of miR29b, miR107 and miR139 were found. The binding consensus sequence of Lin28A/B, GGAG, was found in a stem-loop in miR107 and miR139 but not in miR29b. However, the regulation of these miRNAs by LIN28A/B was not reported. The size of the loops was uniform, 11-14 nt. Moreover, it has been reported that KSRPs, which bind to the same stem-loop structure of let-7 as TruB1, promotes not only let-7 maturation but also several other microRNAs, including miR20a and miR21 (Trabucchi et al., 2009). However, none of the three miRNAs was altered in KSRP (Trabucchi et al., 2009). Each of these three microRNAs has been reported to suppress tumor proliferation (Sur et al., 2019; Song et al., 2015; Sun et al., 2015). Further studies are needed in order to identify the common regulatory mechanism.

3. Figure 3F: the signals for uridine are very faint when pre-let-7a1 or pri-miR10a are used as substrates in the pseudouridylation assays. Given that the majority of uridines will not be isomerized by site-specific pseudouridine formation, it is therefore not clear whether a low amount of pseudouridylation could be detected in this assay. The authors should repeat the assays with more RNA such that the uridine spots are of similar intensity for the positive control tRNAPhe as well as the two pri-mRNAs to convincingly show that no significant pseudouridine formation by TruB1 occurs.

Response

We apologize that the RI labeled RNA levels are different. We performed the same reaction with more microRNA. As a result, no pseudouridine was observed in vitro as previously reported. We revised the figure as follows.

New Fig. 3F



Minor concerns that should be addressed

1. The authors should also briefly comment on the top five hits where over-expression increases luciferase activity and whether these candidates are likely to directly reduce let-7 miRNA levels.

Response

Among the other candidate genes, SF3A3 (Splicing Factor 3a Subunit 3) was identified as one of the genes binding to the miRNA hairpin of let-7 (Treiber et al., 2017). SF3A3 has a zinc finger domain for mRNA splicing. The detailed mechanism of microRNA regulation by SF3A3 is unknown.

Reportedly, LARP7 binds to a specific U2 snoRNA and inhibits transcriptional elongation by RNA polymerase II. It has been reported that there are miR-302/ 367 clusters in the intragenic region of LARP7, however a microRNA regulatory function for LARP7 has not been reported.

GLTSCR2 is a Glioma tumor-suppressor candidate region gene 2, which is specifically repressed in brain tumors. It has been reported that GLTSCR2 is involved in the stabilization of p53, however there have been no reports showing a direct interaction with microRNAs.

EEF1E1 (Eukaryotic Translation Elongation Factor 1 Epsilon 1), also known as AIMP3, is one of the complexes of aminoacyl tRNA synthetase. It has been reported that AIMP3 induces the activation of

p53. Reportedly, AIM3 is a target of miR-543 and miR-590-3p, however there are no other reports suggesting a direct involvement with microRNAs.

We described the comment about other candidate genes in the Discussion session as follows.

(Page 21, Line 9-12)

Among the other candidate genes, SF3A3 (Splicing Factor 3a Subunit 3) has been identified as one of the genes binding to the miRNA hairpin of let-7 (Treiber et al., 2017). However, the detailed mechanism of microRNA regulation by SF3A3 is unknown. There are no reports suggesting the direct involvement with microRNAs of the three other candidate genes.

2. Surprisingly, one of the top-5 candidates identified in the screen, LARP7, could not be validated. Why not?

Response

Not only was there no difference in LARP7 in the large-scale luciferase reporter assay, there was also only a small difference in endogenous let-7 levels by qPCR. Given this variation in the LARP7 results we think that the screening results may have been a false positive due to the microscale of 384 wells. To confirm this, we would need to repeat the large scale test for the candidates identified in the screen as in this case.

3. The authors should briefly explain the sequence and structure variation among the different let-7 miRNA family members. Can these differences explain the slight variation in effects observed, e.g. upon knock-down of TruB1 (Fig. 2)? Or are the differences in miRNA expression within the noise of the measurement?

Response

Thank you for the comment. It was reported that Lin28 binds in a sequence-dependent manner to let-7 family members, and could not bind to human let-7a-3 (Triboulet et al., 2015). In contrast, the effects of TruB1 had a similar trend on both let-7 families in the present study.

To address this point, we added the following to the results section (Page 11, Line 12-16):

Although there was some variation in the effects observed, the large scale qPCR experiments revealed a similar trend for each member of the let-7 family upon TruB1 knock down in three cell

types (HEK293FT cells, HeLa cells and A549 cells). Even the members that did not show a statistically significant difference still showed a tendency to decline.

4. page 16, top, subtitle: the authors show that less let-7 pri-miRNA is bound to DGCR-8 when TruB1 is knocked down, but they never measure the dissociation constant describing the affinity of DGCR-8 to let-7 pri-miRNA. Therefore, the subtitle needs to be rephrased, e.g. to "TruB1 enhances the interaction between...".

Response

We apologize for our inappropriate subtitle. We have revised it accordingly.

5. The subtitle on page 17 is incomplete and needs to be corrected: "TruB1 suppresses cell growth through the promoting let-7". A word seems to be missing.

Response

Thank you for pointing out this error; we have revised it as follow; "TruB1 suppresses cell proliferation by promoting let-7 maturation"

6. The model presented in Fig. 6 which is partly discussed on page 22 is rather superficial. It would be interesting if the authors can at least present a plausible hypothesis how TruB1 can affect the microprocessing step of let-7 maturation. Is it conceivable that TruB1 alters the RNA structure acting as an RNA chaperone which in turn facilitates recognition of this structure by DGCR-8?

Response

Thank you for raising this point. We have added additional discussion as follows (Page 23, Line 5-16):

It has been reported that the structure of RNA affects microprocessing with accessory proteins. For example, hnRNPA1 binds to the loop of pri-miR-18a and induces a relaxation at the stem. This relaxation at the stem facilitates Drosha-mediated processing of the specific microRNA clusters (Michlewski et al., 2008). The Dicer-TRBP complex has been reported to stabilize the stem structure of pre-let-7 before its cleavage reaction (Liu et al., 2018). These accessory proteins contribute to the

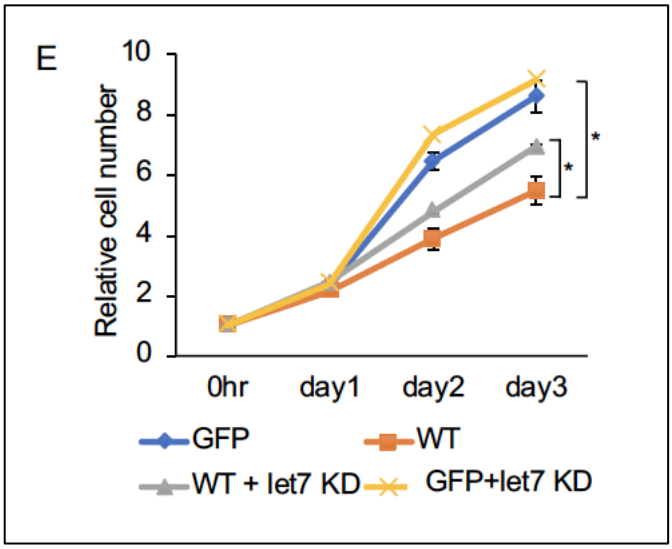
structural changes in RNA and affect its processing. Like these accessory proteins, TruB1 also stabilizes the stem structure of tRNA and affects the enzymatic activity (Hoang and Ferré- D'Amaré., 2001; Keffer-Wilkes et al., 2016). Thus, stabilization of RNA by accessory proteins may assist processors such as DGCR-8. More detailed analyses are needed to determine the structural effects of TruB1 binding to let-7 and how it affects the microprocessing step of let-7 maturation.

7. The description and figure legend for Fig. 6D needs to be improved to better describe the differences between the curves. Which experiments include TruB1 overexpression? What is the difference between "wt + let7 KD" and "let7 KD"?

Response

We apologize for our poor explanation. “WT” means wild type TruB1 overexpression (orange line). “WT + let7 KD” means wild type TruB1 overexpression with let-7 KD (grey line). “let-7 KD” means GFP with let-7 KD (yellow line). We revised this information in the new figure as follows.

New Fig. 6E



New Figure legend. 6E

GFP: GFP without let-7 KD. WT: overexpression of wild type TruB1 without let-7 KD. WT + let7 KD: WT: overexpression of wild type TruB1 with let-7 KD. GFP + let-7KD: GFP with let-7 KD.

8. Figure 3B and F: after nuclease P1 digestion of the RNA in the in vitro pseudouridylation assays, the RNA is degraded into nucleotides with a single phosphate. Therefore, the

labelling of the top spot as "UTP" is incorrect. Simply labelling this as "U" will be sufficient.

Response

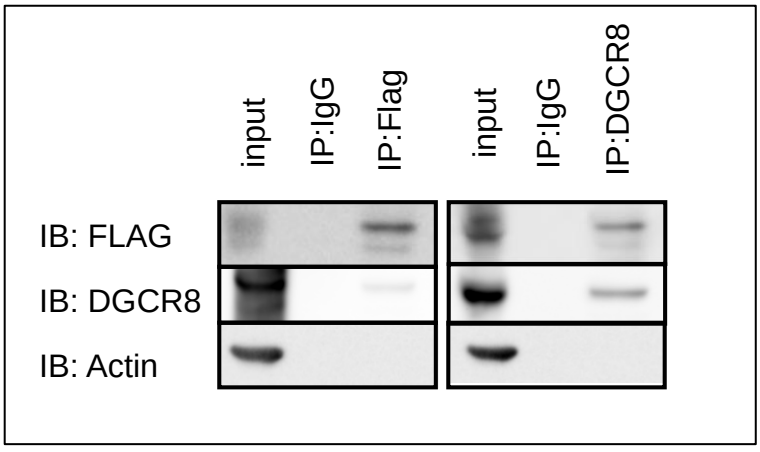
Thank you for pointing out this error; we have revised it accordingly.

9. Does TruB1 interact directly with DGCR-8? Could TruB1 bind first to pri-let7-miRNA and thereby help to recruit DGCR-8? As the authors have conducted immunoprecipitations, they should check whether DGCR-8 is co-eluted upon TruB1 immunoprecipitation and vice versa (expanding Fig. 5).

Response

According to your suggestion, we performed immunoprecipitation in TruB1-Flag cells. When immunoprecipitated with anti-Flag antibody, a slight DGCR-8 band was found in the immunoblot with anti-DGCR-8 antibody. Moreover, when immunoprecipitated with anti-DGCR-8 antibody, immunoblotting with anti-Flag antibody detected TruB1. These results suggest that TruB1 and DGCR-8 are bound to each other. The order of binding is not clear, however, the results indicate that they form a complex. We added this information in a new figure and to the Results of the revision manuscript.

New Fig. EV5B



(Page 18, Line 4-9)

Next, we performed immunoprecipitation in TruB1-Flag cells. When immunoprecipitated with an anti-Flag antibody, a slight DGCR-8 band was detectable by immunoblot with anti-DGCR-8.

Moreover, when immunoprecipitated with an anti-DGCR-8 antibody, immunoblotting with an anti-Flag antibody detected TruB1 (Fig. EV5B). Thus, TruB1 and DGCR-8 were bound to each other.

Non-essential suggestions for improving the study

• page 4, middle: remove the term "cleavage" to read "DGCR8-mediated processing"

Response

Thank you for pointing out this error; we have revised it accordingly.

• In the Materials & Methods section, there are a few instances where microgram is incorrectly abbreviated as ug instead of using the appropriate Greek letter. Also, there should always be a space between a number and the corresponding unit. A few times "nN" is written instead of "mM" to describe a buffer concentration.

Response

Thank you for pointing out these errors; we have revised accordingly.

• Figure legend for Fig. EV3 C (page 69): The figure only shows data for pri-let-7a1, therefore no reference should be made to tRNAPhe in the figure legend.

Response

Thank you for pointing out this error; we have revised it accordingly.

2nd Editorial Decision 5th Aug 2020

Thank you for submitting your revised manuscript for our consideration. Please apologize the delay in communicating this decision to you, which was due to a delayed referee report on account of the current pandemic situation as well as the high number of new submission we are currently receiving. We now have the reports from the original referees (see comments below). I am pleased to say that all referees now support publication. Referee # 2 points out some minor textual issues that can be resolved in a final revised version. In this version, I would also ask you to please address a number of editorial issues that are listed in detail below. Please make any changes to the manuscript text in the attached document only using the "track changes" option. Once these remaining issues are resolved, we will be happy to formally accept the manuscript for publication.

Thank you again for giving us the chance to consider your manuscript for The EMBO Journal. I look forward to receiving your final revision. Please feel free to contact me if you have further questions regarding the revision or any of the specific points listed below.

REFEREE REPORTS

Referee #1:

The authors revised the manuscript according to the comments by this reviewer. The quality and impact of the presented data are sufficient to merit publication of the manuscript in EMBO J.

Referee #2:

I appreciate the thorough revision the authors made: they did an excellent job answering my comments adequately. I feel that this manuscript is significantly improved and now deserves being published in The EMBO Journal. For the final version of the manuscript, it will be important to use appropriate microRNA and genes nomenclature throughout. For example:

-microRNA names should be miR-X and not microRNAX or miRX.

-The terminology "mature-miR-x" is not really used. When we say miR-X, we always refer to its mature form.

-DGCR8 and not DGCR-8

Referee #3:

Kuirmoto and co-workers present a significantly revised manuscript that includes substantial new data of high quality to support their claims as well as a more careful discussion of the scope and mechanism of TruB1's effect on let-7 miRNA maturation. In general, the comments by all three referees have been appropriately addressed such that the revised manuscript is suitable for publication in EMBO Journal.
As outlined in my original referee report, this study is of high interest to the life science community in general as the authors uncover a cellular function of the pseudouridine synthase TruB1 that is independent of its catalytic activity and that is specific to the let-7 miRNA family. Both the unusual function of this RNA modifying enzyme and the specificity to selected miRNAs is highly unusual and will gain significant attention in the research community.
In revising the manuscript, I appreciate that the authors have added new experimental data such as western blotting of endogenous KRAS, a let-7 miRNA target, upon TruB1 overexpression and knock-down, as well as a qualitatively improved pseudouridylation assay. In addition, through careful analysis and enhanced experiments the authors now demonstrate that TruB1 affects not only pri- to pre-miRNA processing, but also processing from pre-miRNA to mature miRNA which is an important clarification.
The authors have also carefully revised the manuscript text clarifying several experimental interpretations and discussions. Importantly, they now stress clearly that TruB1 affects let-7 miRNA maturation (and thus levels).

2nd Authors' Response to Reviewers 7th Aug 2020

The Authors have made the requested editorial changes.

Accepted 8th Aug 2020

Thank you again for submitting the final revised version of your manuscript. I am pleased to inform you that we have now accepted it for publication in The EMBO Journal. However, please remember to make the datasets related to the study publicly accessible as soon as possible.

Your article will be processed for publication in The EMBO Journal by EMBO Press and Wiley, who will contact you with further information regarding production/publication procedures and license requirements.

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YOU MUST COMPLETE ALL CELLS WITH A PINK BACKGROUND ↓

PLEASE NOTE THAT THIS CHECKLIST WILL BE PUBLISHED ALONGSIDE YOUR PAPER

Corresponding Author Name: hiroshi Asahara
Journal Submitted to: The EMBO Journal
Manuscript Number: EMBOJ-2020-104708R

Reporting Checklist For Life Sciences Articles (Rev. June 2017)

This checklist is used to ensure good reporting standards and to improve the reproducibility of published results. These guidelines are consistent with the Principles and Guidelines for Reporting Preclinical Research issued by the NIH in 2014. Please follow the journal's authorship guidelines in preparing your manuscript.

A- Figures

1. Data

The data shown in figures should satisfy the following conditions:

- ➔ the data were obtained and processed according to the field's best practice and are presented to reflect the results of the experiments in an accurate and unbiased manner.
- ➔ figure panels include only data points, measurements or observations that can be compared to each other in a scientifically meaningful way.
- ➔ graphs include clearly labeled error bars for independent experiments and sample sizes. Unless justified, error bars should not be shown for technical replicates.
- ➔ if n< 5, the individual data points from each experiment should be plotted and any statistical test employed should be iustified
- ➔ Source Data should be included to report the data underlying graphs. Please follow the guidelines set out in the author ship guidelines on Data Presentation.

2. Captions

Each figure caption should contain the following information, for each panel where they are relevant:

- ➔ a specification of the experimental system investigated (eg cell line, species name).
- ➔ the assay(s) and method(s) used to carry out the reported observations and measurements
- ➔ an explicit mention of the biological and chemical entity(ies) that are being measured.
- ➔ an explicit mention of the biological and chemical entity(ies) that are altered/varied/perturbed in a controlled manner.

- ➔ the exact sample size (n) for each experimental group/condition, given as a number, not a range;
- ➔ a description of the sample collection allowing the reader to understand whether the samples represent technical or biological replicates (including how many animals, litters, cultures, etc.).
- ➔ a statement of how many times the experiment shown was independently replicated in the laboratory.
- ➔ definitions of statistical methods and measures:
  - common tests, such as t-test (please specify whether paired vs. unpaired), simple $\chi 2$ tests, Wilcoxon and Mann-Whitney tests, can be unambiguously identified by name only, but more complex techniques should be described in the methods section;
  - are tests one-sided or two-sided?
  - are there adjustments for multiple comparisons?
  - exact statistical test results, e.g., P values = x but not P values < x;
  - definition of 'center values' as median or average;
  - definition of error bars as s.d. or s.e.m.

Any descriptions too long for the figure legend should be included in the methods section and/or with the source data.

In the pink boxes below, please ensure that the answers to the following questions are reported in the manuscript itself. Every question should be answered. If the question is not relevant to your research, please write NA (non applicable). We encourage you to include a specific subsection in the methods section for statistics, reagents, animal models and human subjects.

B- Statistics and general methods

Please fill out these boxes ↓ (Do not worry if you cannot see all your text once you press return)

1.a. How was the sample size chosen to ensure adequate power to detect a pre-specified effect size?	For all assays, experiments were typically performed in triplicate or with more higher replicate numbers, which is indicated throughout the text. For only cell-based screen and HITS-CLIP, experiments were performed in duplicate or single, respectively.
1.b. For animal studies, include a statement about sample size estimate even if no statistical methods were used.	N/A
2. Describe inclusion/exclusion criteria if samples or animals were excluded from the analysis. Were the criteria pre-established?	Data were not excluded. In cell-based screen (Fig 1D), We only included genes that did not change the luciferase activity of the let-7 sequence minus the reporter (0.5 <, > 2.0) for analysis (results are shown in Supplementary Table 1). All raw data of screen were included in Supplementary Table 1.
3. Were any steps taken to minimize the effects of subjective bias when allocating animals/samples to treatment (e.g. randomization procedure)? If yes, please describe.	N/A
For animal studies, include a statement about randomization even if no randomization was used.	N/A
4.a. Were any steps taken to minimize the effects of subjective bias during group allocation or/and when assessing results (e.g. blinding of the investigator)? If yes please describe.	N/A
4.b. For animal studies, include a statement about blinding even if no blinding was done	N/A
5. For every figure, are statistical tests justified as appropriate?	Yes, all data were statistical tests as appropriate as described at Materials and Methods.
Do the data meet the assumptions of the tests (e.g., normal distribution)? Describe any methods used to assess it.	Yes, all data meet the assumptions of the test. For qPCR, all data were checked by the dissociation curve. The intensity of band in WB, NB and in vitro assay were represented with unprosessed original data.
Is there an estimate of variation within each group of data?	We showed the error bar at all presented data. Moreover, all attempts at replication were successful.

USEFUL LINKS FOR COMPLETING THIS FORM

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http://oba.od.nih.gov/biosecurity/biosecurity_documents.html
http://www.selectagents.gov/

Is the variance similar between the groups that are being statistically compared?	Yes, the variance between the groups is almost similar.

C- Reagents

6. To show that antibodies were profiled for use in the system under study (assay and species), provide a citation, catalog number and/or clone number, supplementary information or reference to an antibody validation profile. e.g., Antibodypedia (see link list at top right), 1DegreeBio (see link list at top right).	We described the detail of these information at Reagent tool table.
7. Identify the source of cell lines and report if they were recently authenticated (e.g., by STR profiling) and tested for mycoplasma contamination.	All cell lines were purchased commercially. No commonly misidentified cell lines were used. Cells were periodically tested with PCR or DAPI staining for mycoplasma contamination and confirmed negative.

* for all hyperlinks, please see the table at the top right of the document

D- Animal Models

8. Report species, strain, gender, age of animals and genetic modification status where applicable. Please detail housing and husbandry conditions and the source of animals.	N/A
9. For experiments involving live vertebrates, include a statement of compliance with ethical regulations and identify the committee(s) approving the experiments.	N/A
10. We recommend consulting the ARRIVE guidelines (see link list at top right) (PLoS Biol. 8(6), e1000412, 2010) to ensure that other relevant aspects of animal studies are adequately reported. See author guidelines, under 'Reporting Guidelines'. See also: NIH (see link list at top right) and MRC (see link list at top right) recommendations. Please confirm compliance.	N/A

E- Human Subjects

11. Identify the committee(s) approving the study protocol.	N/A
12. Include a statement confirming that informed consent was obtained from all subjects and that the experiments conformed to the principles set out in the WMA Declaration of Helsinki and the Department of Health and Human Services Belmont Report.	N/A
13. For publication of patient photos, include a statement confirming that consent to publish was obtained.	N/A
14. Report any restrictions on the availability (and/or on the use) of human data or samples.	N/A
15. Report the clinical trial registration number (at ClinicalTrials.gov or equivalent), where applicable.	N/A
16. For phase II and III randomized controlled trials, please refer to the CONSORT flow diagram (see link list at top right) and submit the CONSORT checklist (see link list at top right) with your submission. See author guidelines, under 'Reporting Guidelines'. Please confirm you have submitted this list.	N/A
17. For tumor marker prognostic studies, we recommend that you follow the REMARK reporting guidelines (see link list at top right). See author guidelines, under 'Reporting Guidelines'. Please confirm you have followed these guidelines.	N/A

F- Data Accessibility

18: Provide a "Data Availability" section at the end of the Materials & Methods, listing the accession codes for data generated in this study and deposited in a public database (e.g. RNA-Seq data: Gene Expression Omnibus GSE39462, Proteomics data: PRIDE PXD000208 etc.) Please refer to our author guidelines for 'Data Deposition'. Data deposition in a public repository is mandatory for: a. Protein, DNA and RNA sequences b. Macromolecular structures c. Crystallographic data for small molecules d. Functional genomics data e. Proteomics and molecular interactions	All the data required to reproduce this study are included in this published article and supplementary information. The raw data of TaqMan array and HITS-CLIP were deposited in the GEO under accession number GSE143508 and GSE143510, respectively. Further information and requests for resources and reagents should be directed to and will be fulfilled by Hiroshi Asahara (asahara@scripps.edu or asahara.syst@tmd.ac.jp).
19. Deposition is strongly recommended for any datasets that are central and integral to the study; please consider the journal's data policy. If no structured public repository exists for a given data type, we encourage the provision of datasets in the manuscript as a Supplementary Document (see author guidelines under 'Expanded View' or in unstructured repositories such as Dryad (see link list at top right) or Figshare (see link list at top right).	All the data required to reproduce this study are included in this published article and supplementary information. The raw data of TaqMan array and HITS-CLIP were deposited in the GEO under accession number GSE143508 and GSE143510, respectively.
20. Access to human clinical and genomic datasets should be provided with as few restrictions as possible while respecting ethical obligations to the patients and relevant medical and legal issues. If practically possible and compatible with the individual consent agreement used in the study, such data should be deposited in one of the major public access-controlled repositories such as dbGAP (see link list at top right) or EGA (see link list at top right).	N/A
21. Computational models that are central and integral to a study should be shared without restrictions and provided in a machine-readable form. The relevant accession numbers or links should be provided. When possible, standardized format (SBML, CellML) should be used instead of scripts (e.g. MATLAB). Authors are strongly encouraged to follow the MIRIAM guidelines (see link list at top right) and deposit their model in a public database such as Biomodels (see link list at top right) or JWS Online (see link list at top right). If computer source code is provided with the paper, it should be deposited in a public repository or included in supplementary information.	N/A

G- Dual use research of concern

22. Could your study fall under dual use research restrictions? Please check biosecurity documents (see link list at top right) and list of select agents and toxins (APHIS/CDC) (see link list at top right). According to our biosecurity guidelines, provide a statement only if it could.	No, our study did not fall dual use research restrictions.