EMBO reports

Opposing roles of miR-294 and MBNL1/2 in shaping the gene regulatory network of embryonic stem cells

Da-Ren Wu, Kai-Li Gu, Jian-Cheng Yu, Xing Fu, Xi-Wen Wang, Wen-Ting Guo, Le-Qi Liao, Hong Zhu, Xiao-Shan Zhang, Jingyi Hui, Yangming Wang

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Transaction Report:

(Note: With the exception of the correction of typographical or spelling errors that could be a source of ambiguity, letters and reports are not edited. The original formatting of letters and referee reports may not be reflected in this compilation.)

1st Editorial Decision 14 February 2018

Thank you for the submission of your research manuscript to EMBO reports. I apologize for coming back to you with delay, but originally a third referee had agreed to assess your study, but also after repeated reminders the reviewer never delivered his/her report. I therefore forward to you the reports from the 2 remaining referees that were asked to evaluate your study, which can be found at the end of this email.

As you will see, both referees think the manuscript is of interest, but requires major revision to allow publication in EMBO reports. Both referees have a number of concerns and/or suggestions to improve the manuscript, which we ask you to address in a revised manuscript. As the reports are below, I will not detail them here. However, we think that it will be of great importance to provide further experimental data addressing points 2, 3 and 6 of referee #1, and points 2, 3, 4 and 5 of referee #2. We think it is further important to strengthen the significance of the suggested negative feedback back loop, as indicated by referee #1 (point 5).

Given the constructive referee comments, we would like to invite you to revise your manuscript with the understanding that all referee concerns must be addressed in the revised manuscript and in a point-by-point response. Acceptance of your manuscript will depend on a positive outcome of a second round of review. It is EMBO reports policy to allow a single round of revision only and acceptance or rejection of the manuscript will therefore depend on the completeness of your responses included in the next, final version of the manuscript.

Revised manuscripts should be submitted within three months of a request for revision; they will otherwise be treated as new submissions. Please contact us if a 3-months time frame is not sufficient for the revisions so that we can discuss the revisions further.

I look forward to seeing a revised version of your manuscript when it is ready. Please let me know if you have questions or comments regarding the revision.

REFEREE REPORTS

Referee #1:

In this manuscript, the authors investigated the role of miR-294 in gene regulation in mouse ESCs. They showed that miR-294 regulates a large number of alternative splicing events and can facilitate the inclusion of ESC-specific exons. Concomitantly, miR-294 also regulates the expression of many RNA-binding proteins and splicing factors via both transcriptional and post-transcriptional mechanisms. The authors then focused on one of the RNA-binding proteins Mbnl1/2. They showed that miR-294 represses Mbnl1/2 by targeting its 3'-UTR and by enhancing PRC2 recruitment at their promoters, and Mbnl1/2 is responsible for a significant fraction of miR-294-mediated alternative splicing events, such as that for Mbd2. Finally, the authors showed that Mbnl1/2 counteracts the function of miR-294 by binding and upregulating the expression of key miR-294 targets, forming a negative-feedback loop with miR-294 to regulate genes involved in ESC proliferation, apoptosis and EMT.

While miRNA regulation of alternative splicing and gene expression via RNA-binding proteins have been described in other systems, such regulation in ESCs has not been clearly demonstrated. Therefore, the findings in this manuscript have important implications for stem cell and development biology, and are of potential interest to the readers of the journal. The authors are encouraged to address the following comments and questions to further enhance the manuscript:

1. miR-294 mainly upregulates the expression of RBPs and SFs in ESCs. However, a significant fraction of miR-294-mediated alternative splicing events can be accounted for by the repression of Mbnl1/2 by miR-294. Can the authors comment on the significance of the hundreds of up-regulated RBPs and SFs?
2. Fig 2F-G: It is interesting that miRNAs appear to promote H3K36me3 occupancy the gene body of RBPs and SFs. Does this happen in both RBPs and SFs that are up-regulated by miR-294 and those that are down-regulated by miR-294?
3. Related to the above, what is the H3K4me3 and H3K36me3 occupancy at Mbnl1/2 in WT vs Dicer1 KO ESCs?
4. "For 403 miR-294 rescued alternative splicing events, 241 of them were rescued more than 1/3 by the transfection of siRNAs to Mbnl1/2". While the result suggested that miR-294 regulates many alternative splicing events through Mbnl1/2, it also suggested that Mbnl1/2 are not solely responsible for a large number of AS events (i.e., any AS events that are not fully rescued by Mbnl1/2 KD may be regulated by other RBPs or SFs). It is thus premature to conclude that miR-294 regulate "the majority" of AS via Mbnl1/2.
5. Can the authors comment of the significance of the negative-feedback loop between miR-294 and Mbnl1/2 on the proliferation, apoptosis, EMT genes?
6. Fig 6: The growth and morphology of the Mbnl1/2 OE cells does not resemble that of normal ESCs. Can the OE cells be maintained, or were they quickly lost during passage? When was the expression of pluripotency genes examined (how soon after Mbnl1/2 OE) in Fig S6B?

Referee #2:

Wu and co-workers report a series of experiments revealing the intricate interplay between Mbnl1/2 and miR-294 regulating alternative splicing (AS) in ESCs and differentiation. By performing RNA-seq of ESCs that have been depleted of Dgcr8 and subsequently rescued with miR-294 or a control, they show that the proper splicing pattern of multiple ESC-specific AS events depends on miR-294. Then, the authors analyze multiple layers of gene regulation, showing that (i) miR-294 directly regulates Mbnl post-transcriptionally and also through enhancing PCR2 recruitment, (ii) many of the AS events mis-regulated upon Dgcr8 KO are Mbnl-dependent, and (iii) Mbnl also binds many miR-294 targets, seemingly functionally counteracting the impact of miR-294 downregulation.

Overall, I found the manuscript to be thorough and interesting, and I would be happy to support its publication after relatively minor revisions.

1) The major issue is easy to solve and relates to the accuracy of writing with regards to the direct and indirect roles of miR-290/302 throughout the whole manuscript. It is often said that miR-294 regulates a feature, but it is unclear whether this is a direct effect. For instance, in the Abstract, it is claimed: "miR-294 regulates hundreds of alternative splicing events". It would be more accurate to say that "hundreds of alternative splicing events are dependent on miR-294 levels" or similar. Overall, I recommend the authors to be more careful when writing about the regulatory roles of miR-294, and that they reword the corresponding sections when needed.

2) Along the same lines, it is concluded that miR-294 can promote the splicing of the short form of Mbd2 (in fact, this is a case of alternative polyadenylation, not AS). While conclusive evidence is shown that Mbnl1/2 directly regulates this alternative polyadenylation event, it is unclear whether miR-294 does it directly or through down-regulation of Mbnl1/2. Does the distal last exon have binding sites for miR-294 in the UTR? If so, is it possible that miR-294 binding inhibits polyadenylation site selection and/or triggers isoform-specific degradation? If this cannot be shown conclusively, it would be more appropriate to use a dash line and a question mark in the scheme of Figure 5h.

3) The effect of the Mbnl1/2 knockdown on ESC-specific events respect to that of miR-294 can be analyzed more globally, not just looking at the few events covered by Han et al 2017. For instance, the authors can use the RNA-seq of the knockdown employed in Han et al Nature 2013 to compare it with their own RNA-seq Dgcr8 KO data.

4) Page 8: I did not find this result "unexpected". Most splicing factors and RNA binding proteins are more highly expressed in ESCs, likely due to the higher metabolic/replicating rates of these cells. Therefore, since the depletion of miR-294 causes a more "differentiated" pattern, it will *indirectly* lead to the downregulation of multiple splicing factors. Indeed, the authors' results (Figure 6) are consistent with this idea (reduction of proliferation).

5) Page 9: "these data suggest that miRNAs upregulate the expression of RBPs and SFs majorly through promoting transcriptional elongation." This statement has to be better supported or the authors should lower the tone. This effect is likely indirect and it is therefore misleading.

6) Page 14: the analysis of RIP-qPCR is nice. But they could have done it genome-wide using Wang et al Cell 2012's CLIP-seq data for Mbnl1 in C2C12 cells and crossing all Mbnl1 bound mRNAs with those with predicted miR-294 targets.

7) Figures 1 and 3: it is recommended not to use green-red color schemes for heatmaps.

1st Revision - authors' response 12 March 2018

Referee #1:

In this manuscript, the authors investigated the role of miR-294 in gene regulation in mouse ESCs. They showed that miR-294 regulates a large number of alternative splicing events and can facilitate the inclusion of ESC-specific exons. Concomitantly, miR-294 also regulates the expression of many RNA-binding proteins and splicing factors via both transcriptional and post-transcriptional mechanisms. The authors then focused on one of the RNA-binding proteins Mbnl1/2. They showed that miR-294 represses Mbnl1/2 by targeting its 3'-UTR and by enhancing PRC2 recruitment at their promoters, and Mbnl1/2 is responsible for a significant fraction of miR-294-mediated alternative splicing events, such as that for Mbd2. Finally, the authors showed that Mbnl1/2 counteracts the function of miR-294 by binding and upregulating the expression of key miR-294 targets, forming a negative-feedback loop with miR-294 to regulate genes involved in ESC proliferation, apoptosis and EMT.

While miRNA regulation of alternative splicing and gene expression via RNA-binding proteins have been described in other systems, such regulation in ESCs has not been clearly demonstrated.

Therefore, the findings in this manuscript have important implications for stem cell and development biology, and are of potential interest to the readers of the journal. The authors are encouraged to address the following comments and questions to further enhance the manuscript:

1. miR-294 mainly upregulates the expression of RBPs and SFs in ESCs. However, a significant fraction of miR-294-mediated alternative splicing events can be accounted for by the repression of Mbnl1/2 by miR-294. Can the authors comment on the significance of the hundreds of up-regulated RBPs and SFs?

Answer: We thank the reviewer to raise this insightful question. We believe that at least some of these upregulated RBPs and SFs are responsible for miR-294-dependent alternative splicing events that are barely or only partially rescued by Mbnl1/2 knockdown. As shown in the **Figures 1E and 4E**, among 403 miR-294-dependent alternative splicing events, 241 of them were rescued more than 1/3 by siRNAs against Mbnl1/2. The remaining 162 alternative splicing events are likely regulated through other miR-294 regulated (directly or indirectly) genes, including these miR-294-upregulated RBPs and SFs. Moreover, when using 67% rescue (comparing to miR-294) as a cutoff, there were 229 AS events rescued below this cutoff by Mbnl1/2 KD. We agree with the reviewer's comment in question 4, "any AS events that are not fully rescued by Mbnl1/2 KD may also be regulated by other RBPs or SFs". Therefore, our analyses imply that a large number of AS events could be regulated by both Mbn1/2 and other RBPs or SFs. These RBPs or SFs may act in cooperation with Mbnl1/2. To fully understand the network of miRNA-regulated alternative splicing, more experiments are required. We hope that our future work will lead to more interesting discoveries along this direction. Meanwhile, in our revised manuscript, we include several sentences on Page 18-19 (in the Discussion section) that discuss the potential role of other RBPs and SFs in miRNA-mediated AS regulation (highlighted in yellow).

2. Fig 2F-G: It is interesting that miRNAs appear to promote H3K36me3 occupancy the gene body of RBPs and SFs. Does this happen in both RBPs and SFs that are up-regulated by miR-294 and those that are down-regulated by miR-294?

Answer: As shown below in **Figure R1**, H3K36me3 occupancy on the gene body of RBPs/SFs upregulated by miR-294 (>1.5 fold) was significantly higher in wild type versus *Dicer1* knockout ESCs. In contrast, H3K36me3 occupancy on the gene body of RBPs/SFs downregulated by miR-294 (>1.5 fold) was only slightly higher in wild type versus *Dicer1* knockout ESCs. Consistent with the conclusion in our manuscript, these data suggest that miRNAs globally promote H3K36me3 occupancy on the gene body of RBPs and SFs. These data suggest that miR-294 might upregulate RBPs/SFs through indirectly promoting transcription elongation, and that miR-294 might downregulate RBPs/SFs mainly through directly targeting their mRNAs. We include these data together with data on H3K4me3 occupancy in **Figure EV3** of the revised manuscript. The description for these data was added on Page 8-9 and highlighted in yellow.

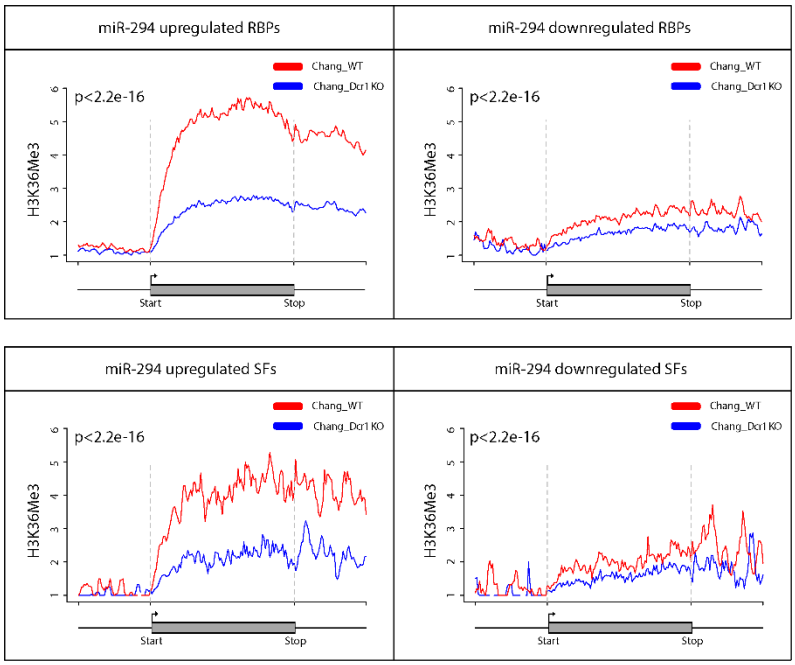

Figure R1. H3K36me3 occupancy on RBPs and SFs that are upregulated or downregulated by miR-294.

3. Related to the above, what is the H3K4me3 and H3K36me3 occupancy at Mbnl1/2 in WT vs Dicer1 KO ESCs?

Answer: The results are shown below in **Figure R2**. H3K4me3 occupancy at Mbnl1/2 promoter is lower in WT vs *Dicer1* KO ESCs, suggesting the transcription initiation is lower for Mbnl1/2 in WT vs *Dicer1* KO ESCs. This is consistent with our results showing that EZH2 binding at Mbnl1/2 promoter is higher in WT vs *Dicer1* KO ESCs, which represses the transcription initiation. In addition, even though the expression of Mbnl1/2 is repressed in WT vs *Dicer1* KO ESCs, H3K36me3 occupancy at Mbnl1/2 gene body is higher in WT vs Dicer1 KO ESCs. The H3K36me3 occupancy at Mbnl1/2 gene body is consistent with the global trend of H3K36me3 occupancy on the gene body of RBPs and SFs in WT vs *Dicer1* KO ESCs. These results and those from our Mbnl1/2 3' UTR reporter assay suggest that miRNAs repress the expression of Mbnl1/2 through both epigenetic (promoting H3K27me3 occupancy and repressing H3K4me3 occupancy) and post-transcriptional (binding to 3'UTR) mechanisms. The epigenetic regulation by miRNAs is likely indirect. We are currently searching for the direct target of miR-294 responsible for epigenetic regulation. We now include the analysis for H3K4me3 and H3K36me3 on Mbnl1/2 in **Appendix Figure 2** and on Page 11 (highlighted in yellow) in revised manuscript.

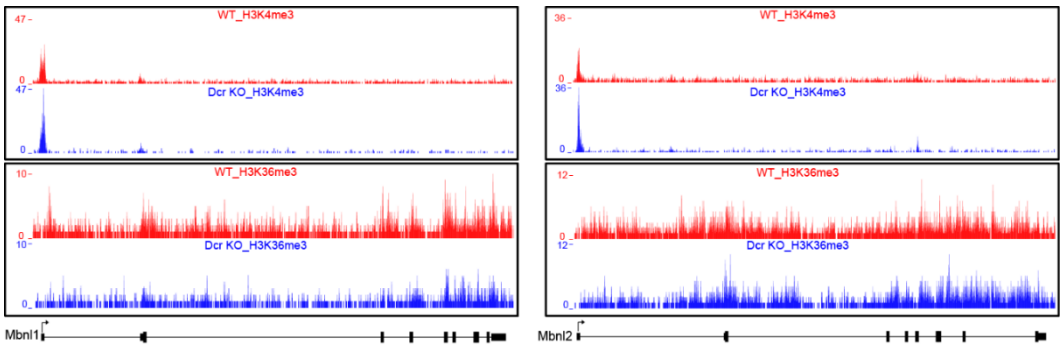

Figure R2. The H3K4me3 and H3K36me3 occupancy at Mbnl1/2 locus.

4. "For 403 miR-294 rescued alternative splicing events, 241 of them were rescued more than 1/3 by the transfection of siRNAs to Mbnl1/2". While the result suggested that miR-294 regulates many alternative splicing events through Mbnl1/2, it also suggested that Mbnl1/2 are not solely responsible for a large number of AS events (i.e., any AS events that are not fully rescued by

Mbnl1/2 KD may be regulated by other RBPs or SFs). It is thus premature to conclude that miR-294 regulate "the majority" of AS via Mbnl1/2.

Answer: Thanks for the comment. We agree with the reviewer. To be more stringent, we changed the sentence on Page 12 "Together, these data demonstrate that miR-294 regulated the majority of alternative splicing events through directly repressing Mbnl1/2" to " Together, these data demonstrate that a large portion of miR-294-dependent alternative splicing events is regulated through the downregulation of Mbnl1/2 by miR-294". In addition, in abstract, the sentence in previous version "Remarkably, ~60% of these splicing events are regulated through repressing Mbnl1/2." is now changed to "Remarkably, ~60% of these splicing events are affected by the depletion of Mbnl1/2". Please also see our response to question 1 on the role of other RBPs and SFs.

5. Can the authors comment of the significance of the negative-feedback loop between miR-294 and Mbnl1/2 on the proliferation, apoptosis, EMT genes?

Answer: Early differentiation of ESCs is accompanied with the decrease in proliferation(Li and Kirschner, 2014) and the increase in apoptosis(Wang et al., 2015) and EMT(Gill et al., 2011). These processes are also actively involved in the induction of pluripotency during reprogramming(Buganim et al., 2013). The function of these processes and related factors in ESC differentiation is only beginning to be revealed. miR-294 family of miRNAs promotes proliferation and inhibits apoptosis and EMT by repressing corresponding targets. By opposing miR-294's repression on these targets, Mbnl1/2 might fine-tunely modulate the differentiation kinetics and/or even the direction of differentiation, which could be important for the establishment of certain lineages. How extensive this type of regulation by MBNL and its functional roles in development, reprogramming and diseases (e.g. mytonic dystrophy) are worthy of future investigation. To answer these important questions, we propose to identify cofactors of Mbnl1/2 in regulating mRNA level versus regulating mRNA splicing. Knocking out their cofactors specific for regulating mRNA level during ESC differentiation may reveal the importance of this regulation and identify specific lineages requiring this type of regulation by Mbnl1/2.

The above are our preliminary thoughts which certainly require extensive experiments to verify. We thank the reviewer for asking the question to stimulate these thoughts. We now include the above contents in the discussion (Page 19-20, highlighted in yellow) of our revised manuscript.

References:
Buganim, Y., Faddah, D.A., and Jaenisch, R. (2013). Mechanisms and models of somatic cell reprogramming. Nature reviews Genetics *14*, 427-439.
Gill, J.G., Langer, E.M., Lindsley, R.C., Cai, M., Murphy, T.L., Kyba, M., and Murphy, K.M. (2011). Snail and the microRNA-200 family act in opposition to regulate epithelial-to-mesenchymal transition and germ layer fate restriction in differentiating ESCs. Stem cells *29*, 764-776.
Li, V.C., and Kirschner, M.W. (2014). Molecular ties between the cell cycle and differentiation in embryonic stem cells. Proceedings of the National Academy of Sciences of the United States of America *111*, 9503-9508.
Wang, E.S., Reyes, N.A., Melton, C., Huskey, N.E., Momcilovic, O., Goga, A., Blelloch, R., and Oakes, S.A. (2015). Fas-Activated Mitochondrial Apoptosis Culls Stalled Embryonic Stem Cells to Promote Differentiation. Current biology : CB *25*, 3110-3118.

6. Fig 6: The growth and morphology of the Mbnl1/2 OE cells does not resemble that of normal ESCs. Can the OE cells be maintained, or were they quickly lost during passage? When was the expression of pluripotency genes examined (how soon after Mbnl1/2 OE) in Fig S6B?

Answer: The Mbnl1/2 OE ESCs can only be maintained for about 4 to 5 passages. As shown in **Figure EV5B** in the revised manuscript (**Figure S6B** in the old version), we examined the expression of pluripotency genes as well as other genes (cell cycle, EMT, etc.) ~48 hours after overexpressing Mbnl1/2. Images for cell morphology were taken and apoptosis was analyzed ~48 hours after overexpression. Cell cycle was analyzed ~24 hours after overexpression. We have added all above information in the Materials and Methods and figure legends (highlighted in yellow).

Referee #2:

Wu and co-workers report a series of experiments revealing the intricate interplay between Mbnl1/2 and miR-294 regulating alternative splicing (AS) in ESCs and differentiation. By performing RNA-seq of ESCs that have been depleted of Dgcr8 and subsequently rescued with miR-294 or a control, they show that the proper splicing pattern of multiple ESC-specific AS events depends on miR-294. Then, the authors analyze multiple layers of gene regulation, showing that (i) miR-294 directly regulates Mbnl post-transcriptionally and also through enhancing PCR2 recruitment, (ii) many of the AS events mis-regulated upon Dgcr8 KO are Mbnl-dependent, and (iii) Mbnl also binds many miR-294 targets, seemingly functionally counteracting the impact of miR-294 downregulation.

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1) The major issue is easy to solve and relates to the accuracy of writing with regards to the direct and indirect roles of miR-290/302 throughout the whole manuscript. It is often said that miR-294 regulates a feature, but it is unclear whether this is a direct effect. For instance, in the Abstract, it is claimed: "miR-294 regulates hundreds of alternative splicing events". It would be more accurate to say that "hundreds of alternative splicing events are dependent on miR-294 levels" or similar. Overall, I recommend the authors to be more careful when writing about the regulatory roles of miR-294, and that they reword the corresponding sections when needed.

Answer: Thanks for the suggestion. We have checked our writing throughout the manuscript and made corresponding corrections on Page 2, 5, 12, and 17 (highlighted in blue).

2) Along the same lines, it is concluded that miR-294 can promote the splicing of the short form of Mbd2 (in fact, this is a case of alternative polyadenylation, not AS).

Answer: Thanks. We have made corrections where applicable, using "alternative last exon usage or alternative polyadenylation".

While conclusive evidence is shown that Mbnl1/2 directly regulates this alternative polyadenylation event, it is unclear whether miR-294 does it directly or through down-regulation of Mbnl1/2. Does the distal last exon have binding sites for miR-294 in the UTR? If so, is it possible that miR-294 binding inhibits polyadenylation site selection and/or triggers isoform-specific degradation? If this cannot be shown conclusively, it would be more appropriate to use a dash line and a question mark in the scheme of Figure 5h.

Answer: In our previous 2015 EMBO Journal paper (cited as reference 28 in the old version of manuscript, also ref 28 in the revised version), we have shown that the long isoform of Mbd2 (Mbd2_L) is a direct target of miR-294 in regulating glycolysis in ESCs and during reprogramming. The original figure from Ref. 28 is shown below, which indicates that Mbd2_L is a direct target of miR-294 (**Figures R3B-E**). We are sorry for this negligence and have now made this clear in the revised manuscript (Page 13).

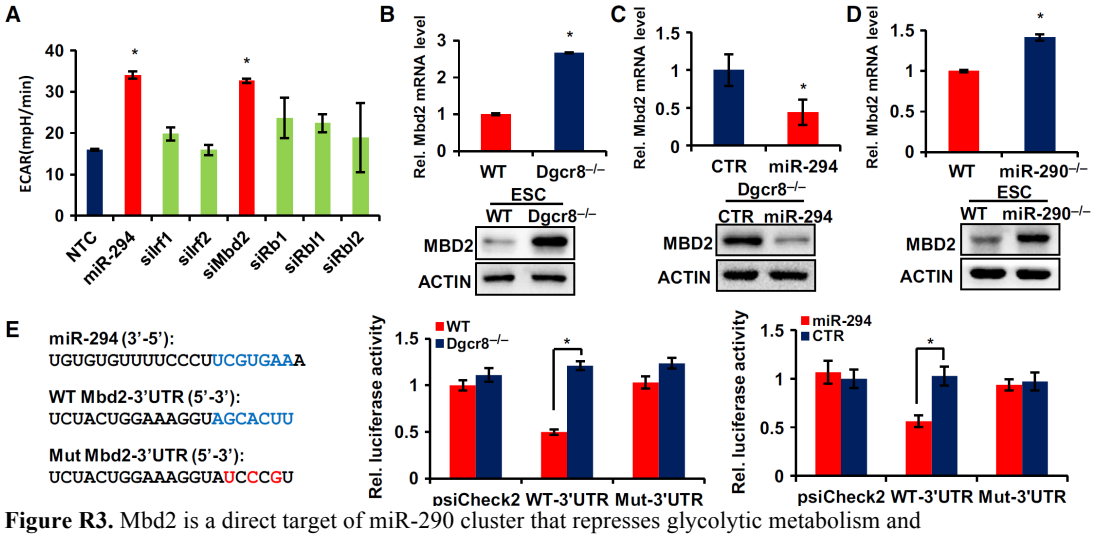

Figure R3. Mbd2 is a direct target of miR-290 cluster that represses glycolytic metabolism and reprogramming. **A**) ECAR screening of *Dgcr8*_/_ ESCs introduced with siRNAs against selected targets. **B**) qRT–PCR and Western blotting analysis of Mbd2 expression in WT and *Dgcr8*_/_ ESCs. **C**) qRT–PCR and Western blotting analysis of Mbd2 expression in *Dgcr8*_/_ ESCs transfected with miR-294 mimic. **D**) qRT–PCR and Western blotting analysis of Mbd2 expression in WT and miR-290 cluster_/_ ESCs. **E**) Luciferase reporter assay for Mbd2 WT and Mut 3' UTRs. *Dgcr8* WT or miR-294 reduced luciferase activity compared to *Dgcr8*_/_ or CTR group, respectively.

(Note: Figure and legends are copied from Figure 4 in Ref. 28: miR-290/371-Mbd2-Myc circuit regulates glycolytic metabolism to promote pluripotency.)

3) The effect of the Mbnl1/2 knockdown on ESC-specific events respect to that of miR-294 can be analyzed more globally, not just looking at the few events covered by Han et al 2017. For instance, the authors can use the RNA-seq of the knockdown employed in Han et al Nature 2013 to compare it with their own RNA-seq Dgcr8 KO data.

Answer: We are sorry for not making this clear. We did compare our results with ESC-specific AS events reported by Han et al Nature 2013 in **Figures 1b, 4b and Supplementary Figure 1a** in our old version of manuscript (**Figures 1B, 4B and Figure EV1A** in the revised manuscript). In Han's paper, they reported 103 ESC-specific AS events in mouse ESCs. We detected 70 of them in our own RNA-seq data. Among them 21 AS events were demonstrated as differentially regulated between WT and *Dgcr8* knockout mouse ESCs. miR-294 and siRNAs to Mbnl1/2 rescued 16 and 9 of them, respectively.

For the siRNA knockdown data in Han's 2013 Nature, C2C12 was the only mouse cell line in which Mbnl1/2 knockdown experiments were performed. Among 103 ESC-specific AS events, they detected a rather small portion of them (41 AS events) in C2C12 cells. In addition, 18 out of 41 detected ESC-specific AS events were changed by siRNAs to Mbnl1/2. Among these 18 events, 14 were detected in our data and 2 were differentially regulated ($|\Delta PSI| > 15$) in WT and *Dgcr8* knockout ESCs, both of which were rescued by miR-294 and siRNAs to Mbnl1/2 in *Dgcr8* knock out ESCs. When we checked against all 41 detected ESC-specific AS events in C2C12, 32 of them were detected in our data. Among these 32, 8 were differentially regulated in WT and *Dgcr8* knockout ESCs. 7 and 4 of these 8 differential AS events were rescued by miR-294 and siRNAs to Mbnl1/2 in *Dgcr8* knockout ESCs, respectively. Since C2C12 cells are more differentiated and likely express higher levels of Mbnl1/2 than ESCs, knockdown of Mbnl1/2 could affect more ESC-specific events in C2C12 than in *Dgcr8* knockout ESCs. In addition, siMbnl1/2 could also cause cell type specific responses in different cells. For example, as shown in Han's 2013 Nature paper, 59 out of 119 detected human ESC specific AS events were changed by siMbnl1/2 in HeLa cells, while only 23 out of 128 detected human ESC specific AS events were changed by siMbnl1/2 in HEK 293T cells.

4) Page 8: I did not find this result "unexpected". Most splicing factors and RNA binding proteins are more highly expressed in ESCs, likely due to the higher metabolic/replicating rates of these cells. Therefore, since the depletion of miR-294 causes a more "differentiated" pattern, it will

**indirectly* lead to the downregulation of multiple splicing factors. Indeed, the authors' results (Figure 6) are consistent with this idea (reduction of proliferation).*

Answer: We removed "unexpected" in the revised manuscript. As pointed out by the reviewer, the *Dgcr8* knockout ESCs indeed grow slower than wild type ESCs (reported in our previous publications, cited as Refs 29 and 43). However, based on the expression levels of pluripotency genes and results from differentiation assay, depletion of *Dgcr8* or miR-294 blocks the differentiation of mouse ESCs (reported in our previous publications, cited as Refs 29 and 32). Therefore, we agree with the reviewer that the downregulation of RBPs and SFs is more likely due to the lower metabolic/replicating rates of *Dgcr8* KO ESCs. Finding the direct target of miR-294 in regulating metabolic rate or replication of ESCs may help build the regulatory network among miRNAs, metabolism/replication and transcriptional regulation of RBPs and SFs.

5) Page 9: "these data suggest that miRNAs upregulate the expression of RBPs and SFs majorly through promoting transcriptional elongation." This statement has to be better supported or the authors should lower the tone. This effect is likely indirect and it is therefore misleading.

Answer: We agree with the reviewer. We have changed this statement to "these data suggest that miRNAs may indirectly upregulate the expression of RBPs and SFs through a transcription elongation-related mechanism".

6) Page 14: the analysis of RIP-qPCR is nice. But they could have done it genome-wide using Wang et al Cell 2012's CLIP-seq data for Mbnl1 in C2C12 cells and crossing all Mbnl1 bound mRNAs with those with predicted miR-294 targets.

Answer: This is a very good suggestion. As shown below in **Figure R4**, we extracted miR-294 predicted targets from TargetScan and searched for genes overlapping with Mbnl1 binding targets in C2C12 cells from Wang et al 2012 Cell paper. Mbnl1 bound a significant portion (760 out of 3201) of predicted targets of miR-294, including many experimentally verified key targets of miR-294. These data suggest that MBNL proteins bind a significant fraction of miR-294 targets.

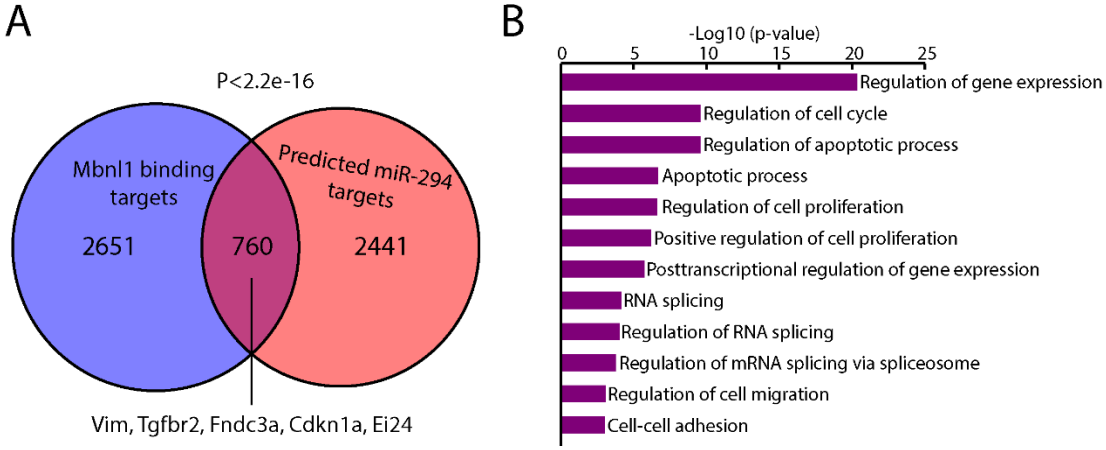

Figure R4. miR-294 and Mbnl1 may share a large number of mRNA targets. **A**) Venn chart shows the overlap between predicted miR-294 targets and Mbnl1 binding targets in C2C12 (Raw data from Wang, ET et al 2012 Cell paper). **B**) Selected GO terms enriched in overlapping targets.

7) Figures 1 and 3: it is recommended not to use green-red color schemes for heatmaps.

Answer: Thanks for the recommendation. We have changed all the heatmaps into midnight blue-scarlet color scheme in the revised manuscript (**Figures 1A, 1B, 4A, 4B and EV2**).

2nd Editorial Decision 28 March 2018

Thank you for the submission of your revised manuscript to our editorial offices. We have now received the reports from the referees that were asked to re-evaluate your study (you will find

enclosed below). As you will see, both referees now support the publication of your manuscript in EMBO reports.

Before we can proceed with formal acceptance, I have the following editorial requests:

Please provide the abstract written in present tense.

The Western blot images sometimes differ very strongly in terms of contrast and brightness (background), even within one panel (see e.g. Fig. 1C). Could you provide in all Western panels as unmodified images possible with similar background intensities? Further, the Western blot panels are of rather low quality and resolution. Please provide higher resolution version of all the Western blot panels (main figures and Appendix figures). See also: http://embopress.org/sites/default/files/EMBOPress_Figure_Guidelines_061115.pdf

As the Western blot panels shown are significantly cropped, we ask you to submit the original source data, also with the aim of making primary data more accessible and transparent to the reader. The source data will be published in a separate source data file online along with the accepted manuscript, and will be linked to the relevant figure. Please submit the source data (scans of entire gels or blots) of all Western blot panels (main figures and Appendix figures) together with the revised manuscript. Please include size markers for scans of entire gels, label the scans with figure and panel number, and send one PDF file per figure.

Could statistical testing be provided for the diagrams shown in Figs. EV4C and EV5?

Please indicate the contribution of author Jian-Cheng Yu in the author contribution section.

Please deposit the RNA-seq data at a public repository, and provide the accession code. See also point 18 of the author checklist.

Please provide the scale bars in the microscopy images (e.g. Fig. 6D) without text and in black. Please indicate the size in the respective figure legend.

Finally, please find attached a word file of the manuscript text with changes we ask you to include in your final manuscript text, and some queries (comments), we ask you to address. Please provide your final manuscript file with track changes, in order that we can see the modifications done.

When submitting your revised manuscript, we will require:
- a Microsoft Word file (.doc) of the revised manuscript text
- editable TIFF or EPS-formatted figure files (main figures and EV figures) in high resolution (of those with adjusted panels or labels).
- the up-dated Appendix
- an updated author checklist (point 18)

In addition I would need from you:
- a short, two-sentence summary of the manuscript
- two to three bullet points highlighting the key findings of your study
- a schematic summary figure (in jpeg or tiff format with the exact width of 550 pixels and a height of about 400 pixels) that can be used as visual synopsis on our website.

I look forward to seeing the final revised version of your manuscript when it is ready. Please let me know if you have questions or comments regarding the revision.

REFEREE REPORTS

Referee #1:

The authors have addressed all my concerns and comments. I have no further questions.

Referee #2:

The manuscript is suitable for publication in EMBO reports without revision.

2nd Revision - authors' response 3 April 2018

The authors have addressed all editorial requests.

EMBO PRESS

YOU MUST COMPLETE ALL CELLS WITH A PINK BACKGROUND ↓

PLEASE NOTE THAT THIS CHECKLIST WILL BE PUBLISHED ALONGSIDE YOUR PAPER

Corresponding Author Name: Dr. Yangming Wang and Dr. Jingyi Hui
Journal Submitted to: EMBO Reports
Manuscript Number: EMBOR-2017-45657V2

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 justified
- 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 χ2 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 statistic testing, we choose sample size based on literatures in the field. All experiments were performed at least in duplicate. No statistical methods were used to predetermine sample size.
1.b. For animal studies, include a statement about sample size estimate even if no statistical methods were used.	NA
2. Describe inclusion/exclusion criteria if samples or animals were excluded from the analysis. Were the criteria pre-established?	NA
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.	NA
For animal studies, include a statement about randomization even if no randomization was used.	NA
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.	NA
4.b. For animal studies, include a statement about blinding even if no blinding was done	NA
5. For every figure, are statistical tests justified as appropriate?	Yes. Page 23, paragraph 4 and also in figure legends.
Do the data meet the assumptions of the tests (e.g., normal distribution)? Describe any methods used to assess it.	NA
Is there an estimate of variation within each group of data?	Yes,they were described in individual figures and figure legends.
Is the variance similar between the groups that are being statistically compared?	Yes

C- Reagents

USEFUL LINKS FOR COMPLETING THIS FORM

http://www.antibodypedia.com
http://1degreebio.org
http://www.equator-network.org/reporting-guidelines/improving-bioscience-research-repo

http://grants.nih.gov/grants/olaw/olaw.htm
http://www.mrc.ac.uk/Ourresearch/Ethicsresearchguidance/Useofanimals/index.htm
http://ClinicalTrials.gov
http://www.consort-statement.org
http://www.consort-statement.org/checklists/view/32-consort/66-title

http://www.equator-network.org/reporting-guidelines/reporting-recommendations-for-tun

http://datadryad.org

http://figshare.com

http://www.ncbi.nlm.nih.gov/gap

http://www.ebi.ac.uk/ega

http://biomodels.net/

http://biomodels.net/miriam/
http://jjj.biochem.sun.ac.za
http://oba.od.nih.gov/biosecurity/biosecurity_documents.html
http://www.selectagents.gov/

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).	Page 21, paragraph 3;Page 22,paragraph 2.
7. Identify the source of cell lines and report if they were recently authenticated (e.g., by STR profiling) and tested for mycoplasma contamination.	Page 21 , paragraph 1. All cells were tested for mycoplasma contamination(Mycoplasma Detection Set,M&C GENE TECHNOLOGY,catalog number COK001)

* 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.	NA
9. For experiments involving live vertebrates, include a statement of compliance with ethical regulations and identify the committee(s) approving the experiments.	NA
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.	NA

E- Human Subjects

11. Identify the committee(s) approving the study protocol.	NA
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.	NA
13. For publication of patient photos, include a statement confirming that consent to publish was obtained.	NA
14. Report any restrictions on the availability (and/or on the use) of human data or samples.	NA
15. Report the clinical trial registration number (at ClinicalTrials.gov or equivalent), where applicable.	NA
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.	NA
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.	NA

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	Page 24,paragraph 3. RNA seq data is deposited. Accession code is GSE111739.
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).	NA
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).	NA
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.	NA

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.	NA