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MicroRNA-independent functions of DGCR8 are essential for neocortical development and TBR1 expression

Federica Marinaro, Matteo J. Marzi, Nadin Hoffmann, Hayder Amin, Roberta Pelizzoli, Francesco Niola, Francesco Nicassio, and Davide De Pietri Tonelli

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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 26 June 2016

Thank you for the submission of your research manuscript to EMBO reports. We have now received reports from the three referees that were asked to evaluate your study, which can be found at the end of this email.

As you will see, all three referees acknowledge the potential interest of the findings. However, all three referees have raised a number of concerns and suggestions to improve the manuscript or to strengthen the data and the conclusions drawn, which need t be addressed. In particular, referees #1 and #2 note that more evidence for the proposed targeting of conserved hairpins in the Tbr1 mRNA by Dgcr8 needs to be provided (see second specific comment by referee #1 and point 5 by referee #2). I fully agree and also think that it is essential to substantiate in particular this part of the manuscript with further experiments (e.g. with those suggested by the referees).

Given these constructive comments, we would like to invite you to revise your manuscript with the understanding that all referee concerns (as detailed in their reports) must be fully addressed in a complete 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.

REFEREE REPORTS

Referee #1:

In this manuscript, Marinaro and colleagues investigate a possible role for non-canonical functions of DGCR8 in neocortical development. The experimental approach is based on an exhaustive phenotypic comparison of conditional Dgcr8 and Dicer Kos in the cornea, which revealed that depletion of DGCR8 results in differentiation phenotypes. Of importance, these phenotypes are exclusive to DGCR8 depletion but are not seen with depletion of DICER. Mechanistically, these results can be explained, at least in part, by a DGCR8-mediated cleavage of a stem-loop in the Tbr1 transcript. This is most likely due to non-canonical functions of the Microprocessor (Drosha-DGCR8).

First, the authors carried out conditional deletion of Dgcr8 or Dicer genes in mouse apical progenitor cells (Aps) before the onset of neurogenesis. This experiments revealed a critical role for Dgcr8 during corticogenesis. The authors went on to show that Dgcr8 depletion led to loss of cortical architecture, whereas Dicer ablation resulted in the reduction of neuronal layers. Another interesting phenotype observed associated with the loss of DGCR8 expression was the increased generation of Tbr1+ neurons. The authors followed up on this observation and ruled out that Neurogenin (Ngn2) expression was responsible for the upregulation of Tbr1 in newborn cortical neurons. Ngn2 was an interesting candidate since it was previously shown to be destabilized by DROSHA.

Finally, Marinaro and co-authors demonstrated that DGCR8 regulates the stability of Tbr1 in a miRNA-independent manner.

In summary, this is an excellent paper with very interesting findings, which very elegantly confirms the existence of miRNA-independent functions of DGCR8. In principle, this study is suitable for publication in EMBO reports.

Specific comments:

- On Fig. 1B, there seems to be still some considerable expression of Dgcr8 RNA in KO cells. Please explain

- The authors present experiments suggesting a role for Drosha-DGCR8 in the regulation of the stability of Tbr1 in a miRNA-independent manner. The assay used shows this in an indirect manner, with siRNA-mediated depletion of DGCR8 in N2A cells leading to upregulation of Luciferase reporters harbouring 5 individual predicted hairpins. The way this experiment was done is only suggestive of a cleavage effect by Drosha, but does not demonstrate this directly. This is the weakest part of this study. Ideally, the authors should show Drosha-mediated cleavage of Tbr1 mRNA in vitro. At a minimum, they should show levels of endogenous Tbr1 mRNA and/or protein upon Drosha and/or DGCR8 depletion.

Minor comments:

- References on the first paragraph of page 3 are wrongly formatted.

- Again, on page 3, when referring to the "direct modulation of RNA transcription/stability by the microprocessor through miRNA-independent", the authors may also wish to refer to a paper by the Proudfoot lab, showing that Drosha regulates gene expression independently of RNA cleavage (Gromak et al. (2013) Cell reports)

Referee #2:

The manuscript by Marinaro et al. describes genetic evidence indicating that Dgcr8 has non-miRNA functions in early neurogenesis. By comparing the embryonic brains of Dgcr8 and Dicer conditional knockout mice, they observed a more severe phenotype in Dgcr8-/- than that in Dicer-/-, including loss of cortical architecture, increased production of neurons and altered proliferation and balance of

apical and basal progenitor cells. By comparing the small RNA reads from next generation sequencing of the wild-type and knockout embryonic neocortex, the authors classify miRNAs based on their Dgcr8- and Dicer-responsiveness. Finally, they propose that Dgcr8 confers miRNA-independent function in corticogenesis by targeting conserved "miRNA precursor-like" hairpins in Tbr1 mRNA. I think this manuscript would be interesting and worth publishing if everything the authors say is true. However, there are a number of issues that need to be addressed for the story to be convincing.

1. The Dgcr8-/- only decreases the Dgcr8 expression by about 50% relative to the wild type. It is important to perform immunostaining of the Dgcr8 protein and correlate with patterns of Tbr1, Tbr2 and Pax6, as well as BrdU and activated caspase signals. The data shown in Fig. 1B and S3 do not provide this information.

2. It is quite possible that the Dgcr8-/- cells have undergone more apoptosis than the Dicer-/- cells. As such, one may argue that the increased neurons are ways of compensating the loss of other cell types.

3. It is surprising that the miRNAs did not change as much in Dgcr8-/- than in Dicer-/- or in previous conditional Dgcr8 knockout studies (for example, Yi et al PNAS 2009 in the reference list). It is not clear whether the miRNAs remaining are less responsive to Dgcr8 ablation, or they simply come from cells that still express Dgcr8. Because of these complications, I am not sure if the classification really means that much.

4. The Tbr1+ cells appear to have mislocalized to VZ and SVZ. How does this evidence fit in the model?

5. The evidence for Dgcr8 to target conserved hairpins in Tbr1 mRNA is weak for several reasons. (a) There is no evidence indicating that Dgcr8 directly binds these hairpins. (b) To show that the hairpins are the true targets, one should mutate the hairpin and show a loss of regulation. Importantly, it is common to use compensatory mutagenesis to show that the regulation can be restored by combining compensatory mutations that are disruptive individually. (c) The evidence comes from a reporter-based assay in which the hairpins are inserted in the 3'UTR. It is not known whether similar effects happen to the endogenous transcript, in which the hairpin resides in the coding regions. (d) It is not clear what "targeting" means. I assume it means that Dgcr8 binding to the hairpin results in cleavage by Drosha. If it is the case, a Drosha knockdown would be important.

Referee #3:

This interesting, clearly-written manuscript describes striking, novel findings that significantly broaden our understanding of cortical developmental mechanisms. It concerns a molecule involved in miRNA generation, Dgcr8. The authors compare cortex that is conditionally deleted for Dgcr8 or Dicer and find convincingly stronger effects of Dgcr8 deletion. This is a remarkable finding, given how powerful an effect Dicer loss has. They investigate the underlying mechanisms and first show that Dgcr8 loss causes progenitors to differentiate prematurely, producing too many Tbr1 positive neurons. They then study whether this is likely to be miRNA dependent but find that depletion of miRNAs is far greater following Dicer loss than Dgcr8 loss. They postulate that the effects of Dgcr8 loss are probably miRNA-independent and that Tbr1 mRNA might be an important Dgcr8 target.

I have two comments for revision:

1. I don't understand how they use one way ANOVA to test significance where they have 2 variables, e.g. age and genotype. Surely they should do two way ANOVA, report the significance of the effect of each variable and the interaction, and then apply post hoc tests. It is important to get the statistics right - they have done a commendable amount of quantification.
2. The fact that depletion of miRNAs is much greater after Dicer loss is striking, but isn't it possible

that Dgcr8 loss causes loss of some particularly critical miRNAs that Dicer loss does not? There could be complex reasons why loss of some miRNAs might affect the Tbr1/ neurogenesis system, perhaps directly but also indirectly. Could the authors discuss more clearly whether this is possible, and if they think it's unlikely, why?

1st Revision - authors' response 25 November 2016

Referee #1:

In this manuscript, Marinaro and colleagues investigate a possible role for non-canonical functions of DGCR8 in neocortical development. The experimental approach is based on an exhaustive phenotypic comparison of conditional Dgcr8 and Dicer Kos in the cornea, which revealed that depletion of DGCR8 results in differentiation phenotypes. Of importance, these phenotypes are exclusive to DGCR8 depletion but are not seen with depletion of DICER. Mechanistically, these results can be explained, at least in part, by a DGCR8-mediated cleavage of a stem-loop in the Tbr1 transcript. This is most likely due to non-canonical functions of the Microprocessor (Drosha-DGCR8).

First, the authors carried out conditional deletion of Dgcr8 or Dicer genes in mouse apical progenitor cells (Aps) before the onset of neurogenesis. This experiments revealed a critical role for Dgcr8 during corticogenesis. The authors went on to show that Dgcr8 depletion led to loss of cortical architecture, whereas Dicer ablation resulted in the reduction of neuronal layers. Another interesting phenotype observed associated with the loss of DGCR8 expression was the increased generation of Tbr1+ neurons. The authors followed up on this observation and ruled out that Neurogenin (Ngn2) expression was responsible for the upregulation of Tbr1 in newborn cortical neurons. Ngn2 was an interesting candidate since it was previously shown to be destabilized by DROSHA. Finally, Marinaro and co-authors demonstrated that DGCR8 regulates the stability of Tbr1 in a miRNA-independent manner.

In summary, this is an excellent paper with very interesting findings, which very elegantly confirms the existence of miRNA- independent functions of DGCR8. In principle, this study is suitable for publication in EMBO reports.

Authors' Answer: We thank the reviewer for his/her positive comments and strong support of this study. In particular we are grateful that the reviewer acknowledges that "*experiments revealed a critical role for Dgcr8 during corticogenesis*", "*this is an excellent paper with very interesting findings" and* that "*very elegantly confirms the existence of miRNA- independent functions of DGCR8"*. We have addressed the concerns you raised. The revised text is highlighted in red in the MS. The point-by-point response follows. We sincerely hope that we have addressed all your concerns appropriately.

Reviewer's specific comment 1: "*On Fig. 1B, there seems to be still some considerable expression of Dgcr8 RNA in KO cells. Please explain".*

Authors' Answer to comment 1: We agree with your comment. In the revised version of our MS we now show by western blotting (new panel B in Fig 1) that endogenous DGCR8 protein is totally depleted in E13.5 cortices of DGCRO cKO embryos compared to controls. Regarding residual *Dgcr8* transcript in *Dgcr8* cKO cortices, we believe it could be due to "contamination" of mRNA extracted from cortical cells bearing normal Dgcr8 expression, that emerge due to the high sensitivity of qRT-PCR. Indeed, *Emx1* drives recombination in cortical progenitors of the dorsolateral cortex, but not in ventral progenitors, which give rise to interneurons also present in cortices at E13.5, or blood vessels. For qRT-PCR analysis we used previously published primers. In particular, used by Narry Kim in Han J. et al., 2009 (proven to recognize the DGCR8 allele deleted in our mouse model: i.e. the EX4, encoding for the WW protein-protein binding domain is floxed in our mouse model). We also tested 4 other primer pairs. In our hands all primer pairs gave similar results. Finally, we would respectfully remark that the residual DGCR8 mRNAs levels detected upon conditional knockdown of DGCR8 in our model are not dissimilar to those published by others. Indeed in most, if not all, the studies in which DGCR8 was knockout, the transcript was never totally depleted in cKO cells/tissues.

Reviewer's specific comment 2: *The authors present experiments suggesting a role for Drosha-DGCR8 in the regulation of the stability of Tbr1 in a miRNA- independent manner. The assay used shows this in an indirect manner, with siRNA-mediated depletion of DGCR8 in N2A cells leading to upregulation of Luciferase reporters harbouring 5 individual predicted hairpins. The way this experiment was done is only suggestive of a cleavage effect by Drosha, but does not demonstrate this directly. This is the weakest part of this study. Ideally, the authors should show Drosha-mediated cleavage of Tbr1 mRNA in vitro. At a minimum, they should show levels of endogenous Tbr1 mRNA and/or protein upon Drosha and/or DGCR8 depletion.*

Authors' Answer to comment 2: We agree with this comment. Following your suggestions, we performed knockdown of DROSHA and repeated the Luciferase assays. Our results show that all the Tbr1 hairpins that previously responded to DGCR8 knockdown also responded to DROSHA knockdown, confirming that *Tbr1* Hairpins can be regulated by DROSHA cleavage activity (see new fig 7 of the revised MS). We are aware that this experiments is only suggestive of a microprocessor/dgcr8 dependent cleavage of Tbr1. However, we respectfully remark that the main scope of this study is not to dissect the direct mechanism of DGCR8-dependent control of Tbr1 gene expression. We thank you for this comment and we agree that this part of our MS might be misunderstood. Therefore, we have now rephrased several sentences related to this point in the revised MS.

With respect to Dgcr8 dependent control of endogenous Tbr1 expression: we respectfully argue that Dgcr8 cKO leads to increase in the endogenous TBR1 levels *in vivo* (see immunofluorescence staining in Fig3 and Fig EV3). To further corroborate this finding, we now overexpress DGCR8 in murine N2A cells and found that DGCR8 overexpression leads to the decrease of endogenous TBR1 protein level in these cells (Fig 7B in the revised MS). We have also obtained similar results *in vivo* upon *in utero* electroporation of *Dgcr8* in embryonic mouse cortices (Fig 1 for reviewers [data not included in the Peer Review Process File], preliminary data that we will not include in the present manuscript as part of a study that is currently being prepared for submission elsewhere). This data consistently show that endogenous TBR1 protein expression is reduced upon overexpression of *Dgcr8*.

Following your suggestions, we also tried to check levels of endogenous Tbr1 upon knockdown of Dgcr8 or Drosha in N2A. After 48 hours, we extracted proteins and checked for TBR1 protein levels. Unfortunately we did not manage to get significant upregulation of endogenous Tbr1 in these cells. We speculate that the well-known feedback response of Dgcr8 to Drosha knockdown (See Fig 7 of the revised MS, and references Triboulet R, et al 2009; Han J et al 2009 references in the MS) or insufficient time or knockdown of Dgcr8/Drosha could account for this result.

In sum, our results (Fig 3, fig EV3 and new figure 7 and fig 1 for reviewers [data not included in the Peer Review Process File]) together with the evidence of altered Ngn2 level could not account for the increased expression of endogenous TBR1 in DGCR8 cKO cortices (present study and Fig 1 for reviewers [data not included in the Peer Review Process File]), strongly indicate that DGCR8 regulates endogenous TBR1 expression by a post-transcriptional miRNA-independent mechanism. We favor the hypothesis that such mechanism could involve cleavage of Tbr1 hairpins by the microprocessor.

Reviewer's Minor comments:

References on the first paragraph of page 3 are wrongly formatted.

Answer: We fixed it, thank you.

Again, on page 3, when referring to the "direct modulation of RNA transcription/stability by the microprocessor through miRNA-independent", the authors may also wish to refer to a paper by the Proudfoot lab, showing that Drosha regulates gene expression independently of RNA cleavage (Gromak et al. (2013) Cell reports)

Answer: We added this reference, thank you.

Referee #2:

The manuscript by Marinaro et al. describes genetic evidence indicating that Dgcr8 has non-miRNA functions in early neurogenesis. By comparing the embryonic brains of Dgcr8 and Dicer conditional knockout mice, they observed a more severe phenotype in Dgcr8-/- than that in Dicer-/-, including loss of cortical architecture, increased production of neurons and altered proliferation and balance of apical and basal progenitor cells. By comparing the small RNA reads from next generation sequencing of the wild-type and knockout embryonic neocortex, the authors classify miRNAs based on their Dgcr8- and Dicer-responsiveness. Finally, they propose that Dgcr8 confers miRNA-independent function in corticogenesis by targeting conserved "miRNA precursor-like" hairpins in Tbr1 mRNA. I think this manuscript would be interesting and worth publishing if everything the authors say is true. However, there are a number of issues that need to be addressed for the story to be convincing.

Authors' Answer: We thank the reviewer for his/her positive comments and constructive criticism on our study. In particular, we are grateful that the reviewer acknowledges that "*this manuscript would be interesting and worth publishing...".* The revised text is highlighted in red in the MS. The point-by-point response follows. We sincerely hope that we have addressed all your concerns appropriately.

Reviewer's specific comment 1*: The Dgcr8-/- only decreases the Dgcr8 expression by about 50% relative to the wild type. It is important to perform immunostaining of the Dgcr8 protein and correlate with patterns of Tbr1, Tbr2 and Pax6, as well as BrdU and activated caspase signals. The data shown in Fig. 1B and S3 do not provide this information.*

Authors' Answer to comment 1: We acknowledge your positive criticism, thus in the revised version of our MS we now show by western blotting (new panel B in Fig 1) that endogenous DGCR8 protein is totally depleted in E13.5 cortices of DGCRO cKO embryos compared to controls. Regarding residual *Dgcr8* transcript in *Dgcr8* cKO cortices, we believe it could be due to "contamination" of mRNA extracted from cortical cells bearing normal Dgcr8 expression, that emerge due to the high sensitivity of qRT-PCR method. See also answer to Reviewer #1, point #1. Given that our new data indicate that DGCR8 protein is totally depleted in Dgcr8 cKO we feel the suggested experiment is no longer crucial. Moreover, it would be very challenging, since currently there is no good antibodies anti-DGCR8 suitable for immunofluorescence (we tried 4 different antibodies and none of them showed enough specificity for immunofluorescence assay).

***Reviewer's specific comment* 2:** *It is quite possible that the Dgcr8-/- cells have undergone more apoptosis than the Dicer-/- cells. As such, one may argue that the increased neurons are ways of compensating the loss of other cell types.*

Authors' Answer to comment 2: We agree with your comment. As you requested, we performed this experiment and found that more cells undergo apoptosis in Dgcr8 cKO cortices compared to Dicer cKO (figure 2 for reviewers [data not included in the Peer Review Process File]). However, our new data show that overexpression of DGCR8 in developing cortices by *in utero* electroporation leads to a decrease in the proportion of Tbr1+ neurons (Fig 1 for reviewers [data not included in the Peer Review Process File], preliminary data that we will not include in the present manuscript as part of a study that is currently being prepared for submission elsewhere). Moreover, we now show that overexpression of *Dgcr8* leads to the decrease of Tbr1 protein level in N2A cells (see fig 7 in the revised MS). These new data are consistent with our model and therefore further support the crucial role of DGCR8 for the proper control of neuronal production during corticogenesis.

Reviewer's specific comment 3. *It is surprising that the miRNAs did not change as much in Dgcr8-/- than in Dicer-/- or in previous conditional Dgcr8 knockout studies (for example, Yi et al PNAS 2009 in the reference list). It is not clear whether the miRNAs remaining are less responsive to Dgcr8 ablation, or they simply come from cells that still express Dgcr8. Because of these complications, I am not sure if the classification really means that much.*

Authors' Answer to comment 3: We thank the reviewer for this important comment. Indeed we were also "surprised" to see so many residual miRNAs in Dgcr8 cKO cortices. However in light of our new experiments we can now argue that depletion of DGCR8 protein in *Dgcr8* cKO cortices is complete (See new Figure 1B of the revised MS). This new finding thus reinforces our analysis (that was already performed with stringent criteria, see material and methods of the revised MS). Moreover, the evidence that 8 of the 39 non-canonical miRNAs identified in our study were already classified as Dgcr8-independent in previous publications (in ES cells or in post-mitotic neurons, Fig EV4 and Table EV1 of the revised MS and enclosed references) corroborates the validity of our classification. In light of these considerations, we can speculate that the impact of alternative miRNA biogenesis pathways and miRNA-independent functions of the microprocessor have been so far underestimated in corticogenesis. Thus, we strongly believe that our study paves the way to further comparative investigations of *Dgcr8* Vs. *Dicer* mutants in the developing nervous system.

Reviewer's specific comment 4: *The Tbr1+ cells appear to have mislocalized to VZ and SVZ. How does this evidence fit in the model?*

Authors' Answer to comment 4: We acknowledge your positive criticism, thus in the revised version of our MS we now performed a more detailed analysis of the architecture of the VZ and in the telencephalon of our mutants (see new Fig EV3 of the revised MS). In *Dgcr8* cKO cortices, we found short and disorganized apical progenitors as revealed by immunostaining for radial glial cells markers Nestin (Fig EV3M, 3N of the revised MS) and Glutamate Transporter GLAST (data available upon request). This result suggests that VZ derangement is the main cause of Tbr1+ cells misplacement upon depletion of DGCR8.

***Reviewer's specific comment* 5:** *The evidence for Dgcr8 to target conserved hairpins in Tbr1 mRNA is weak for several reasons. (a) There is no evidence indicating that Dgcr8 directly binds these hairpins. (2) To show that the hairpins are the true targets, one should mutate the hairpin and show a loss of regulation. Importantly, it is common to use compensatory mutagenesis to show that the regulation can be restored by combining compensatory mutations that are disruptive individually. (c) The evidence comes from a reporter-based assay in which the hairpins are inserted in the 3'UTR. It is not known whether similar effects happen to the endogenous transcript, in which the hairpin resides in the coding regions. (d) It is not clear what "targeting" means. I assume it means that Dgcr8 binding to the hairpin results in cleavage by Drosha. If it is the case, a Drosha knockdown would be important.*

Authors' Answer to comment 5: We agree with this comment. As related to "targeting" meaning: our hypothesis is that DGCR8, by binding to *Tbr1* hairpins resembling microRNA precursors, could mediate its cleavage by the microprocessor. Following your logic, we performed knockdown of *Drosha* and repeated the Luciferase assays. Our results show that all the Tbr1 hairpins that previously responded to DGCR8 knockdown, also responded to *Drosha* knockdown, confirming that hairpins of *Tbr1* mRNA can be post-transcriptionally regulated by microprocessor cleavage activity (see new fig 7 of the revised MS), thus reinforcing our hypothesis.

We acknowledge that the experiments suggested would be a better way to investigate the direct mechanism of DGCR8-*Tbr1* mRNA binding. However, we respectfully remark that the aim of our study is not to dissecting the direct mechanism of DGCR8-dependent *Tbr1* gene expression control. Indeed, we realize that this part of our MS might be misunderstood. Therefore, we have now rephrased several sentences related to this point in the revised MS.

With respect to *Dgcr8*-dependent control of endogenous Tbr1 expression: we respectfully argue that *Dgcr8* cKO leads to increase in the endogenous TBR1 levels *in vivo* (see immunofluorescence staining in Fig3 and Fig EV3). To further corroborate this finding, we overexpressed *Dgcr8* in murine N2A cells and found that it leads to a decrease of endogenous TBR1 protein level in these cells (Fig 7B in the revised MS). We have also obtained similar results *in vivo* upon *in utero* electroporation of *Dgcr8* in embryonic mouse cortices (Fig 1 for reviewers [data not included in the Peer Review Process File], preliminary data that we will not include in the present manuscript as part of a study that is currently being prepared for submission elsewhere). This data consistently show that endogenous TBR1 protein expression is reduced upon overexpression of *Dgcr8*.

In sum, our results (Fig 3, fig EV3 and new figure 7 and fig 1 for reviewers [data not included in the Peer Review Process File]) together with the evidence of altered Ngn2 level could not account for the increased expression of endogenous TBR1 in DGCR8 cKO cortices (present study and Fig 1 for reviewers [data not included in the Peer Review Process File]), strongly indicate that DGCR8 regulates endogenous TBR1 expression by a post-transcriptional miRNA-independent mechanism. We favor the hypothesis that such mechanism could involve cleavage of Tbr1 hairpins by the microprocessor (See also answer to Reviewer#1, comment 2).

Referee #3:

This interesting, clearly-written manuscript describes striking, novel findings that significantly broaden our understanding of cortical developmental mechanisms. It concerns a molecule involved in miRNA generation, Dgcr8. The authors compare cortex that is conditionally deleted for Dgcr8 or Dicer and find convincingly stronger effects of Dgcr8 deletion. This is a remarkable finding, given how powerful an effect Dicer loss has. They investigate the underlying mechanisms and first show that Dgcr8 loss causes progenitors to differentiate prematurely, producing too many Tbr1 positive neurons. They then study whether this is likely to be miRNA dependent but find that depletion of miRNAs is far greater following Dicer loss than Dgcr8 loss. They postulate that the effects of Dgcr8 loss are probably miRNA-independent and that Tbr1 mRNA might be an important Dgcr8 target.

Authors' Answer: We thank the reviewer for the positive comments and strong support of this study. In particular, we are grateful that the reviewer acknowledges that "*interesting, clearly-written manuscript describes striking, novel findings that significantly broaden our understanding of cortical developmental mechanisms..".* The revised text is highlighted in red in the MS. The point-by-point response follows. We sincerely hope that we have addressed all your concerns appropriately.

Reviewer's specific comment 1.
I don't understand how they use one way ANOVA to test significance where they have 2 variables, e.g. age and genotype. Surely they should do two way ANOVA, report the significance of the effect of each variable and the interaction, and then apply post hoc tests. It is important to get the statistics right - they have done a commendable amount of quantification.

Authors' Answer to comment 1. Thanks for your suggestion. We repeated statistical analysis of the experiments involving 2 variables. In particular, for the quantification of the cortical thickness we found that the population means of the three different genotypes (WT, *Dgcr8* cHET, and *Dgcr8* cKO) are significantly different only at E12.5 but not at E11.5. Significant differences were determined by two-way ANOVA followed by Tukey's post-hoc test. Furthermore, the interaction between genotypes and embryonic ages is not significantly different.

For the quantification of the ventricle length we found that the ventricle length is significantly lower at E13 in *Dgcr8* cKO genotype in comparison to WT and *Dgcr8* HET genotypes, as determined by two-way ANOVA followed by Tukey's post-hoc test. Furthermore, the interaction between genotypes and embryonic ages factors is not significantly different.

For the quantification of activated-caspase 3+ cells and pyknotic nuclei we found that the proportion of cells positive of the marker activated-caspase 3 is significantly higher at E12.5 and E13.5 in *Dgcr8* cKO in comparison to WT and *Dgcr8* HET, as determined by two-way ANOVA followed by Tukey's post-hoc test. Furthermore, we tested the interaction between genotypes and embryonic age factors, and we found a significant interaction between WT and *Dgcr8* cHET, that is likely due to the absence of cellular death in these genotypes over all embryonic ages.

Reviewer's specific comment 2.
The fact that depletion of miRNAs is much greater after Dicer loss is striking, but isn't it possible that Dgcr8 loss causes loss of some particularly critical miRNAs that Dicer loss does not? There could be complex reasons why loss of some miRNAs might affect the Tbr1/ neurogenesis system, perhaps directly but also indirectly. Could the authors discuss more clearly whether this is possible, and if they think it's unlikely, why?

Authors' Answer to comment 2. We thank the reviewer his/her constructive criticism, and agree with this suggestion. We now analyzed the expression levels of the experimentally-supported miRNAs known to target *Tbr1* mRNA (new Fig 7A of the revised MS), or that are predicted to target *Tbr1* (new Appendix Fig S4). We found that all these miRNAs were more downregulated in *Dicer* cKO compared to *Dgcr8* cKO cortices, thus excluding loss of this subset of miRNAs as cause of increased Tbr1 expression. We mentioned this hypothesis in the results section of the revised MS.

2nd Editorial Decision 10 January 2017

Thank you for the submission of your revised manuscript to our editorial offices. We have now received the reports from the three referees that were asked to re-evaluate your study that you will find enclosed below. As you will see, all three referees support the publication of your manuscript in EMBO reports. However, referee #2 has a final majr concern and some further minor suggestions to improve the manuscript, we ask you to address in a final revised version. Further, I have a few editorial.

Please change the format of the Appendix. Please add a table of contents to the first page (below the title), listing the items displayed in the Appendix with page numbers. Then move the legends such that they follow their respective figures. Please upload the tables EV1-EV3 as separate files (.doc) including title and legend and remove them from the main manuscript file. It seems Table EV3 has no callout in the manuscript text. Please adjust this.

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:

Overall, the authors have performed a reasonable revision and have either addressed or attempted to address the concerns raised during the first round of reviews. It is my view that they have done a good job and that the revised manuscript is improved. I think that this paper is now acceptable for publication in EMBO reports.

Referee #2:

In this revision, the authors have addressed most of my concerns. My only remaining major issue concerns the identification of "non-canonical miRNAs." Some of the miRNAs listed in Table EV1 as bona fide non-canonical miRNAs are known to originate from canonical miRNA hairpins. This will be confusing. While such a finding will be very interesting, it takes more experiments to prove it. It seems that overall the decrease in miRNA expression is more modest than previous studies. It is possible that substantial amount of DGCR8 protein remains, just below detection limit of the anti-DGCR8 immunoblot. Alternatively, maybe there are contaminating miRNAs from other cells. Without further experimental proof, I believe that the conclusion has to be qualified to reflect an appropriate degree of uncertainty.

Some minor suggestions:

1. In Figure 7C, EV4 (panels A and B), Table EV1, and main text line 262, I believe that the "fold changes" are actually log2 of fold changes.
2. Figure 8C', the secondary structures need to be drawn more clearly. Additionally, are these

structures supported by compensatory mutations in sequence alignments?
3. The first letter in Microprocessor should be capital, to distinguish from microprocessors used in computer.
4. Lines 45-47, this sentence needs to be rephrased.
5. Figure 7A, numbers should be in the same format.
6. Line 333, delete "of."

Referee #3:

I think the authors have done a thorough revision in response to my comments. I have no further suggestions.

2nd Revision - authors' response 16 January 2017

Referee #1: *Overall, the authors have performed a reasonable revision and have either addressed or attempted to address the concerns raised during the first round of reviews. It is my view that they have done a good job and that the revised manuscript is improved. I think that this paper is now acceptable for publication in EMBO reports.*

Authors' Answer: We thank the reviewer for his/her strong support to our study.

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Authors' Answer: We thank the reviewer for his/her strong support to our study.

Referee #2: *In this revision, the authors have addressed most of my concerns. My only remaining major issue concerns the identification of "non-canonical miRNAs." Some of the miRNAs listed in Table EV1 as bona fide non-canonical miRNAs are known to originate from canonical miRNA hairpins. This will be confusing. While such a finding will be very interesting, it takes more experiments to prove it. It seems that overall the decrease in miRNA expression is more modest than previous studies. It is possible that substantial amount of DGCR8 protein remains, just below detection limit of the anti-DGCR8 immunoblot. Alternatively, maybe there are contaminating miRNAs from other cells. Without further experimental proof, I believe that the conclusion has to be qualified to reflect an appropriate degree of uncertainty.*

Authors' Answer: We thank the reviewer for the thorough reading of our study and the very constructive feedback. In particular, we are grateful that the reviewer acknowledges, *"the authors have addressed most of my concerns".* We estimate having addressed all your comments and sincerely hope that it can now be accepted for publication in *EMBO reports*. The revised text is highlighted in BLUE in the MS.

With respect to the identification of non-canonical miRNAs, as you suggested, we have introduced the following sentence in the discussion of the revised MS: *" This potentially remarkable finding however warrants future validation, given that the incomplete depletion of DGCR8 protein (e.g., below detection limit of the anti-DGCR8 immuno-blotting, see Fig. 1) might account in part for the residual miRNAs observed in Dgcr8 cKO mice."* (Lines 369-373). Contamination of miRNAs from "not-recombined" cells seems less likely, given that in the telencephalon of E13.5 mice the proportion of Emx1-Cre-driven Tomato+ cells is typically >95%, as revealed by FACS.

The point-by-point response to Minor suggestions follows:

1. *"In Figure 7C, EV4 (panels A and B), Table EV1, and main text line 262, I believe that the "fold changes" are actually log2 of fold changes".*

Authors' Answer: Thank you for pointing this out. We have added Log2 in panel C of Fig. 6; in Panels A, B in Fig. EV4 and amended text/legends accordingly.

2. *"Figure 8C', the secondary structures need to be drawn more clearly. Additionally, are these structures supported by compensatory mutations in sequence alignments? "*

Authors' Answer: We have increased sizes of the hairpins structures shown in Fig 7C. Regarding compensatory mutations: RNA secondary structures were drawn by RNA fold program using the "default" parameters *see* (http://rna.tbi.univie.ac.at/cgi-bin/RNAWebSuite/RNAfold.cgi).

3. *"The first letter in Microprocessor should be capital, to distinguish from microprocessors used in computer."*

Authors' Answer: we have corrected this typo, thanks

4. *"Lines 45-47, this sentence needs to be rephrased."*

Authors' Answer: we have rephrased this sentence, thanks

5. *"Figure 7A, numbers should be in the same format."*

Authors' Answer: We have checked fonts in the indicated figure, thanks.

6. *"Line 333, delete "of""*

Authors' Answer: we have deleted "of", thanks.

3rd Editorial Decision 19 January 2017

I am very pleased to accept your manuscript for publication in the next available issue of EMBO reports. Thank you for your contribution to our journal.

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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: Davide De Pietri Tonelli
Journal Submitted to: EMBO Reports
Manuscript Number: EMBOR-2016-42800V1

Reporting Checklist For Life Sciences Articles (Rev. July 2015)

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.

Please ensure that the answers to the following questions are reported in the manuscript itself. We encourage you to include a specific subsection in the methods section for statistics, reagents, animal models and human subjects.

In the pink boxes below, provide the page number(s) of the manuscript draft or figure legend(s) where the information can be located. Every question should be answered. If the question is not relevant to your research, please write NA (non applicable).

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 the sake of reproducibility and consistency, we collected at least 3 repeated trials performed in different preparations and from at least 3 different experiments.
1.b. For animal studies, include a statement about sample size estimate even if no statistical methods were used.	At least 3 repeated measured were performed from different litters and from at least 3 different animals.
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.	No randomization procedure has been done.
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	No blinding procedure has been done.
5. For every figure, are statistical tests justified as appropriate?	Yes
Do the data meet the assumptions of the tests (e.g., normal distribution)? Describe any methods used to assess it.	Yes. Turkey's post hoc testing was conducted.
Is there an estimate of variation within each group of data?	No estimate has been done. Variation among population means was computed and expressed as standard error of the mean.
Is the variance similar between the groups that are being statistically compared?	Equal variance has not been considered for statistical tests.

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).	DGCR8 Antibody (Rabbit Polyclonal, Proteintech 10996-1-AP). Drosha Antibody (Rabbit mAb, Cell Signaling, D28B1). TBR1 Antibody (Rabbit, Abcam, ab31940). Tuj1 Antibody (Covance, A488-435L). Sox2 Antibody (Millipore, AB5603). Activated Caspase 2 Antibody (Cell Signaling, Asp175, 5A1E). Pax6 Antibody (Covance, PRB-278P). Tbr2 Antibody (Abcam, ab23345). Tbr1 Antibody (Abcam, ab31940). BrdU Antibody (Abcam, ab6326). Mcm2 Antibody (BD Biosciences, 610700). Ngn2 Antibody (R&D Systems, MAB3314). Ctip2 Antibody (Abcam, ab28448) . Sox5 Antibody (Abcam, ab94396). All antibodies were reactive against mouse proteins.
7. Identify the source of cell lines and report if they were recently authenticated (e.g., by STR profiling) and tested for mycoplasma contamination.	N2A (Neuro-2A) cells were purchased from ATCC (https://www.lgcstandards-atcc.org/products/all/CCL-131.aspx?geo_country=gb).

* 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.	Mice were housed under standard conditions at Istituto Italiano di Tecnologia (IIT), Genoa, Italy. All experiments and procedures were approved by the Italian Ministry of Health (Permits No. 057/2013 and 214/2015-PR) and IIT Animal Use Committee, in accordance with the Guide for the Care and Use of Laboratory Animals of the European Community Council Directives. Drs. G Hannon (Cold Spring Harbor Laboratory, MA, USA), E. Fuchs (Rockefeller Univ. NY, USA) and S. Itohara (RIKEN, Wako, Japan) kindly providED Dicer-flox, Dgcr8-flox and Emx1-Cre mouse lines, reispectively.
9. For experiments involving live vertebrates, include a statement of compliance with ethical regulations and identify the committee(s) approving the experiments.	Italian Ministry of Health (Permits No. 057/2013 and 214/2015-PR) and IIT Animal Use Committee approved the experiments, in accordance with the Guide for the Care and Use of Laboratory Animals of the European Community Council Directives.
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.	Dicer-flox mouse line: Murchison EP, Partridge JF, Tam OH, Cheloufi S, Hannon GJ (2005) Characterization of Dicer-deficient murine embryonic stem cells. Proc Natl Acad Sci U S A 102: 12135–12140. Dgcr8-flox mouse line: Yi R, Pasolli HA, Landthaler M, Hafner M, Ojo T, Sheridan R, Sander C, O'Carroll D, Stoffel M, Tuschl T, et al. (2009) DGCR8-dependent microRNA biogenesis is essential for skin development. Proc Natl Acad Sci U S A 106: 498–502. Emx1-Cre mouse line: Iwasato T, Datwani A, Wolf AM, Nishiyama H, Taguchi Y, Tonegawa S, Knöpfel T, Erzurumlu RS, Itohara S (2000) Cortex-restricted disruption of NMDAR1 impairs neuronal patterns in the barrel cortex. Nature 406: 726–731.

E- Human Subjects

USEFUL LINKS FOR COMPLETING THIS FORM

http://www.antibodypedia.com	Antibodypedia
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http://www.mrc.ac.uk/Ourresearch/Ethicsresearchguidance/Useofanimals/index.htm	MRC Guidelines on animal use
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http://www.consort-statement.org/checklists/view/32-consort/66-title	CONSORT Check List
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http://www.selectagents.gov/	List of Select Agents

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 accession codes for deposited data. See author guidelines, under '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	The deep sequencing data from this publication have been submitted to the NCBI GEO database and assigned the identifier GSE82069.
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. As far as possible, primary and referenced data should be formally cited in a Data Availability section. Please state whether you have included this section. Examples: **Primary Data** Wetmore KM, Deutschbauer AM, Price MN, Arkin AP (2012). Comparison of gene expression and mutant fitness in Shewanella oneidensis MR-1. Gene Expression Omnibus GSE39462 **Referenced Data** Huang J, Brown AF, Lei M (2012). Crystal structure of the TRBD domain of TERT and the CR4/5 of TR. Protein Data Bank 4O26 AP-MS analysis of human histone deacetylase interactions in CEM-T cells (2013). PRIDE PXD000208	NA
22. 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

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