Oxidative stress regulates progenitor behavior and cortical neurogenesis

Angela Chui, Qiangqiang Zhang, Qi Dai and Song-Hai Shi
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MS TITLE: Oxidative stress regulates neocortical progenitor behavior and neurogenesis transition

AUTHORS: Angela Chui and Songhai Shi

I have now received all the referees' reports on the above manuscript, and have reached a decision. The referees' comments are appended below, or you can access them online: please go to BenchPress and click on the 'Manuscripts with Decisions' queue in the Author Area.

As you will see, the referees express considerable interest in your work, but have some significant criticisms and recommend a substantial revision of your manuscript before we can consider publication. In particular, there is concern that the data as presented lack novelty, due to prior work related to the main finding of the paper. Adding new information on the effect on specific populations of progenitors, and nailing the role of RG would add substantial novelty, if you are willing to experimentally address these points. If you are able to revise the manuscript along the lines suggested, which will involve further experiments, I will be happy receive a revised version of the manuscript. Your revised paper will be re-reviewed by one or more of the original referees, and acceptance of your manuscript will depend on your addressing satisfactorily the reviewers' major concerns. Please also note that Development will normally permit only one round of major revision.

Please attend to all of the reviewers' comments and ensure that you clearly highlight all changes made in the revised manuscript. Please avoid using 'Tracked changes' in Word files as these are lost in PDF conversion. I should be grateful if you would also provide a point-by-point response detailing how you have dealt with the points raised by the reviewers in the 'Response to Reviewers' box. If you do not agree with any of their criticisms or suggestions please explain clearly why this is so.

Reviewer 1

Advance summary and potential significance to field

The manuscript by Chui and Shi, investigates the effect of oxidative stress on corticogenesis. The authors used RNAseq and immunostaining to suggest that oxidative stress pathways are controlled

during cortical development in VZ progenitors. They analysed neurogenesis and progenitor proliferation parameters in a conditional Emx1:Cre;PRDM16 mutant. They showed that precocious cell cycle exit results in defects of deep and superficial layer neuron production. They report of differences between the dorsal and lateral cortex and of the presence of heterotopia in the latter one. Some of these defects are suppressed by expression of mitochondrially targeted Catalase supporting the authors' conclusions that abnormal deep to superficial transition is influenced by cellular ROS levels.

I have no main concerns on the quality of the data. The images and quantifications are very convincing. However, the manuscript lack novelty. As mentioned by the authors, PRDM16 was already shown to regulate ROS and lineage progression in Drosophila. A basically identical conditional KO was analysed by Baizabal et al. (2018) which described the involvement of PRDM16 in cell cycle exit, neuronal differentiation and heterotopia in the cerebral cortex. Moreover, Inoue et al. (2017) previously reported of the dynamics of ROS activity in cortical development and on the control by PRDM16 of PGC1alpha and mtROS.

The possible novelty in the submitted data are in the different behaviour between dorsal and lateral progenitors. The differential effects might only be due to the differentiation gradient normally observed in the cortex (indeed the reported results at E13.5 and E15.5 for Pax6 and Tbr2 support this conclusion as the decrease in Tbr2+ cells is first seen in lateral and later in the dorsal cortex). The heterotopia and the analysis of the rescue of specific defects with catalase has not been reported and expanding on what cortical defects are or not rescued could significantly increase the impact of the manuscript.

Comments for the author

I have some comments mainly concerning the layout of the data and the description of the up and down-regulated genes by RNAseq.

1) The analysis of RNAseq data is very succinct and should be expanded. It is very hard to appreciate what these data are indicating as similar GO term are found up and down-regulated and repeated within the two categories. The authors explain in Figure legend 2 that the genes are not the same. However, since which gene sets are considered part of each GO term are not provided (RNAseq data sets are not included or their accession number), this entire part is superficial and quite meaningless and confusing. An in depth analysis of the RNAseq data could give important novel insights onto what can mediate the phenotype.

2) Comparison of the submitted and published results using Nex:Cre, Emx1:Cre and Nes:Cre PRDM16 cKOs should give the authors important cues of what the differences might depend on in the lateral versus the dorsal cortex. For example, the authors nicely show in Suppl Fig.1 that VZ progenitors of the ventral/lateral pallium are not undergoing recombination.

3) The authors report differences in the lateral versus dorsal cortex in several experiments but not all. They should compare these two regions for all experiments (for example in the NexCre (Suppl Figure 8) as this might help them to explain interesting differences especially regarding the rescue with mCat.

4) In the RNAseq one GO term also coming up is the retinoic acid pathway which has been shown to regulate corticogenesis. The phenotype observed in the mutant of precocious differentiation and depletion of the progenitor pool could also be related to alterations of this pathway. Is there a relationship with the ROS pathway and could this help explaining the dorsal/lateral differences in the cKO mutant and upon mCat rescue?

Minor Comments:
1) The background in Fig. 3A should be the same for WT and cKO.
2) In my modest opinion the lack of cell death in the mutant should be moved to after Fig. 10C-E to improve the rational as you expect maybe cell death to explain a loss of RGPs at E15.5 not before.

Reviewer 2

Advance summary and potential significance to field

In their manuscript Chui and Shi study the role of oxidative stress in radial glial (RG) development in the cerebral cortex. To accomplish this the authors use a number of approaches to test oxidative stress responses during cortical development, the role of Prdm16 in the oxidative stress response and rescue of Prdm16 oxidative stress related phenotypes via crossing with mCAT overexpressing transgenic mice.

The strengths of this study are: 1) the experiments are extremely well executed, 2) the data are very clearly presented 3) and the manuscript is well written. The putative link between oxidative stress and Prdm16 in cortical development is also novel and an additional strength lies in the use of a combination of approaches including conditional KOs and RNA-seq analyses.

Despite this, there are two caveats:
1) The conclusions as to the specific role of oxidative stress on RG cells as opposed to a putative role in the other non-SVZ populations is overstated. This claim appears to be based on the specificity of dissections of the VZ, the phenotype in Prdm16 conditional mutants, and putative rescue of cortical phenotypes with mCat. While the data strongly support a critical role for oxidative stress and Prdm16 in cortical development, the proposed role for oxidative stress specifically in RG cells as opposed to other cells in the cortex is not fully supported.
2) With regard to the role of Prdm16 in the cortex, similar deficits were found in a recent study (Baizabal et at., Neuron 2018). Thus, while this analysis here is very well conducted, and somewhat extends these recently published results, this somewhat reduces novelty

However despite these caveats, the quality and depth of the experimental approaches and the novelty of a putative link RG/cortical development and oxidative stress together suggest that this study may be foundational in setting the stage for future studies of ROS as a critical pathway in cortical development.

Comments for the author

In addition to addressing the above caveats, the following should be considered:

Figs 2, 3: It is unclear from the methods how VZ tissue was isolated from SVZ/MZ cells. This is important as Fig 1 also shows developmentally regulated increases in oxidative stress responses not just in the VZ, but throughout the SVZ and mantle.

Fig 2. Although perhaps acting upstream, it would be interesting to know if Prdm16 was also identified in the screen (it is suggested that a Table of all screen results be included in supplementary data)

Fig 3. Neurospheres might not be the best readout of changes in oxidative stress in RG cells in Prdm16 mutants in vivo, therefore it is recommended that this figure be moved to supplementary and then perhaps moving Supplementary Fig 9 to a regular figure as the findings shown in this figure are central to the study.

Fig 8. The analysis/utility of the mCAT transgenic rescue is a bit superficial/unclear and does entirely resolve the mechanism of Prdm16 function through ROS. As the overexpression is global it does not directly point to a role in the VZ/RG population especially as the phenotype is not completely rescued.

Reviewer 3

Advance summary and potential significance to field

The manuscript by Angela Chui and Song-Hai Shi investigates the role of elevated reactive oxygen species (ROS) and oxidative stress in radial glia progenitor (RGP) behaviour during mouse cerebral

cortex development. The authors show that oxidative stress increases over the course of embryonic development in ventricular zone (VZ) areas and this regulates temporal fate transitions in RGPs. They also show that the transcriptional and epigenetic regulator Prdm16 regulates ROS levels and RGP behaviour: Prdm16 cortical specific cKO leads to increased ROS levels, oxidative stress and decreased ratio of upper vs lower neuron generation, a phenotype that is partially rescued by mitochondrial overexpression of the catalase enzyme CAT, which should reduce ROS.

While Prdm16 cortical specific cKO has already been described, the focus on Prdm16 regulation of ROS and variations in ROS/oxidative stress during the course of cortical development is definitely an advance in the field and helps understand how the interplay between transcriptional/epigenetic factors and metabolic processes shapes fate transition in the cortex.

Comments for the author

The manuscript is interesting, well written and the data are clearly presented. I would recommend it for publication. I summarize below some comments and suggestions that could strengthen the paper:

•As a general comment, I wonder whether the authors think that it is the oxidative stress to DNA, lipid, proteins (and thus a certain level of damage) that drives signaling and phenotype or is the physiological redox signaling triggered by variations in ROS and use oxidative stress just as a readout of increased ROS levels?

•In relation to previous comment, how do ROS levels change in RGP during the course of cortical development? Data in figure 1 show increased oxidative stress on DNA and lipid, but the intensity quantification uses arbitrary units, which vary very little and perhaps weakens appreciation of the effect. Since these data are leading the whole paper, would the authors consider using another method to strengthen and complement their results? For example, increased ROS in Prdm16 cKO in figure 3A looks great, could not they the use same method, CellROX, either on isolated progenitors or on ex vivo sections taken from different embryonic stages to show ROS variations during developmental time?

Minor
•Graphs in figure 2 often show same ontology terms up- and down-regulated. I understand there might be different genes within the same term that go up or down, but then I wonder whether there is a better way to visualize variations in metabolic processes or could they show only one time point and enlarge some of the categories to show the genes that go up and down..?

•Could the authors please specify in the text or figure legend what they use for GO analysis in figure 2? In figure 3 they consider top 500 differentially expressed genes, is it the same for figure 2?

•Figure 3G-H: qPCR for Ptx3 and Cyp26b1: just out of clarity, could the authors please specify what is the expression relative to, e.g. what is =1? It might be E11.5 wt for Ptx3, but I cannot guess for Cyp26b1.

•Since Cyp26b1 is known to generate ROS via monooxygenate reactions (see Hrycay, Bandiera, Involvement of Cytochrome P450 in Reactive Oxygen Species Formation and Cancer, Advances in Pharmacology, 2015), could the increase in this gene mediate or strongly contribute to Prdm16 cKO phenotype? Would it be possible to reduce its expression in vivo in Prdm16 cKO? It would help to link the parts of the paper.

•Cyp26b1 does not particularly vary in a time dependent manner in wt, so I wonder whether the genes that instead vary over the course of development (identified in fig 2, like Prdx2, Prdx6, Sod1, Gpx3, Gpx8 and Nos) are regulated by Prdm16? How do they vary in Prdm16 cKO?

•As the overall number of neurons and cortical area/size does not change between WT and Prdm16 cKO (eg it kind of balances out), I wonder how much of the effect at E15 is a secondary effect of the role at E13? Would conditional deletion of Prdm16 at E15 lead to the same increase in cell cycle exit and reduction in upper layer neurons? If they cannot do the experiment, perhaps they can speculate on this in results or discussion.

•in the RNA seq of wt vs Prdm16 cKO, do the authors see variations in migration genes that could explain the heterotopia (like in Baizabal 2018)? This would help clarify how Prdm16 leads to heterotopia, since they do not observe defects in post mitotic migration (eg are migratory genes dysregulated or it is indeed a primary defect in RGP specification/differentiation?)

•in Fig 6, Pax6 domain in Prdm16 cKO does not look expanded as it does in supplementary figure 9F (same stage). is this just variability? Do they have a better picture to show in fig 6?

•I am not sure about figure number/manuscript size limits, but it would be great to see some of the data in supplementary fig 9 and 10 (Pax6, Tbr2 and pHH3 analyses) to be moved to the main part of the manuscript. They are quite interesting and they have not been shown before in previous characterization of this cKO.

•What is the effect of mCAT overexpression on ROS levels, especially in Prdm16 cKO? Could they use CellROX to show at least partial reduction in ROS?

•As the authors talk about temporal transition in fate, I wonder whether increased ROS in Prdm16 cKO might also impact astrocytes that are produced later in development? Would they be decreased? Perhaps the authors could add a sentence in the discussion?

First revision

Author response to reviewers' comments

Reviewer 1

The manuscript by Chui and Shi, investigates the effect of oxidative stress on corticogenesis. The authors used RNAseq and immunostaining to suggest that oxidative stress pathways are controlled during cortical development in VZ progenitors. They analysed neurogenesis and progenitor proliferation parameters in a conditional Emx1:Cre;PRDM16 mutant. They showed that precocious cell cycle exit results in defects of deep and superficial layer neuron production. They report of differences between the dorsal and lateral cortex and of the presence of heterotopia in the latter one. Some of these defects are suppressed by expression of mitochondrially targeted Catalase supporting the authors' conclusions that abnormal deep to superficial transition is influenced by cellular ROS levels.

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Moreover, Inoue et al. (2017) previously reported of the dynamics of ROS activity in cortical development and on the control by PRDM16 of PGC1alpha and mtROS.

The possible novelty in the submitted data is in the different behaviour between dorsal and lateral progenitors. The differential effects might only be due to the differentiation gradient normally observed in the cortex (indeed the reported results at E13.5 and E15.5 for Pax6 and Tbr2 support this conclusion as the decrease in Tbr2+ cells is first seen in lateral and later in the dorsal cortex). The heterotopia and the analysis of the rescue of specific defects with catalase have not been reported and expanding on what cortical defects are or not rescued could significantly increase the impact of the manuscript.

Response: We thank the reviewer for considering "the images and quantifications are very convincing", and for providing valuable and constructive comments to improve our study.

Reviewer 1 Comments for the Author:

I have some comments mainly concerning the layout of the data and the description of the up and down-regulated genes by RNAseq.

1) The analysis of RNAseq data is very succinct and should be expanded. It is very hard to appreciate what these data are indicating as similar GO term are found up and down-regulated and repeated within the two categories. The authors explain in Figure legend 2 that the genes are not the same. However, since which gene sets are considered part of each GO term are not provided (RNAseq data sets are not included or their accession number), this entire part is superficial and quite meaningless and confusing. An in depth analysis of the RNAseq data could give important novel insights onto what can mediate the phenotype.

Response: Following the reviewer's suggestion, we performed additional analyses of the RNA- seq data. We provided the list of genes that were used in the GSEA for each GO term **(Table 1)**. We also submitted the RNA-seq data (**GEO Accession number: GSE142684**).

Notably, the key ROS pathway-related genes identified in the WT and *Prdm16* cKO RNA-seq analysis (Fig. 3) largely overlaps the genes reflecting the progressive increase in ROS level and oxidative stress in RGPs during normal cortical development (**Fig. 2**). We also highlighted several ROS-related genes, including *Prdx2, Prdx6, Sod1, Gpx3, Gpx8*, and *Nos1*, and discussed their roles in regulating ROS level (**pages 6-7**).

2) Comparison of the submitted and published results using Nex:Cre, Emx1:Cre and Nes:Cre PRDM16 cKOs should give the authors important cues of what the differences might depend on in the lateral versus the dorsal cortex. For example, the authors nicely show in Suppl Fig.1 that VZ progenitors of the ventral/lateral pallium are not undergoing recombination.

Response: We compared and contrasted the differences between the *Emx1:Cre* and *Nex:Cre Prdm16* cKO brains, as well as the previously published *Nestin:Cre Prdm16 cKO* brain (**page 19-20**).

The main reason underlying the phenotype difference is likely due to distinct spatial and temporal expression of Cre recombinase. *Emx1:Cre* drives Cre expression in RGPs of the dorsal cortex with a strong medial to weak lateral gradient starting at ~E9.5 (Gorski et al., 2002). *Nex:Cre* drives Cre expression in postmitotic neurons in the dorsal cortex (Goebbels et al., 2006). *Nestin:Cre* drives Cre expression in neural progenitor cells broadly starting at ~E10.5 (Tronche et al., 1999). In addition, the dorsal versus later cortical phenotype difference is likely related to PRDM16 expression pattern, which exhibits a weak medial to strong lateral gradient **(Fig. S3)**.

3) The authors report differences in the lateral versus dorsal cortex in several experiments but not all. They should compare these two regions for all experiments (for example in the NexCre (Suppl Figure 8) as this might help them to explain interesting differences especially regarding the rescue with mCat.

Response: Following the reviewer's suggestion, we systematically analyzed the dorsal versus lateral cortex for all the key experiments (**Fig. 4, 5, 6, 7, 8, 9, 10 and Fig. S6, S8, S9**). We also included additional discussion on the phenotypic difference between the lateral and dorsal cortex (**page 20-22**).

4) In the RNAseq one GO term also coming up is the retinoic acid pathway which has been shown to regulate corticogenesis. The phenotype observed in the mutant of precocious differentiation and depletion of the progenitor pool could also be related to alterations of this pathway. Is there a relationship with the ROS pathway and could this help explaining the dorsal/lateral differences in the cKO mutant and upon mCat rescue?

Response: We provided additional discussion on the retinoic acid pathway and its role in ROS regulation as following **(page 19-20)**:

"Interestingly, upon PRDM16 removal at E15.5 and E17.5, there is an upregulation of genes negatively regulating the retinoic acid pathway, which regulates early neurogenesis by RGPs, suppresses ROS

level, and can induce apoptosis (Haushalter, Asselin, Fraulob, Dolle, & Rhinn, 2017; Jeong & Joo, 2016). One of these crucial regulators is *Cyp26b1*, which controls the level of retinoic acid by catalyzing a multistep reaction to oxidize retinoic acid and thereby preventing abnormal cell signaling via this pathway (Rhinn & Dolle, 2012). Future efforts to assess the precise function of CYP26B1 in cortical RGPs will provide new insight into the regulation of the retinoic acid pathway and its influence on ROS level and RGP behavior."

Minor Comments:

1) The background in Fig. 3A should be the same for WT and cKO.

Response: The entire experiment of WT and *Prdm16* cKO was conducted in parallel. The images were also acquired using the same parameters and analyzed under the same condition. The background may appear to be different, because the fluorescence intensity of the CellROX staining for the WT neurosphere is not as strong as that of the mutant. This difference actually reflects the higher ROS level in the *Prdm16* cKO neurosphere than that in the WT control neurosphere.

We adjusted the image display.

2) In my modest opinion the lack of cell death in the mutant should be moved to after Fig. 10C-E to improve the rational as you expect maybe cell death to explain a loss of RGPs at E15.5 not before.

Response: Following the reviewer's suggestion, we changed the order of the presentation and move the cell death data to the last **(Fig. S10)**.

Reviewer 2

In their manuscript Chui and Shi study the role of oxidative stress in radial glial (RG) development in the cerebral cortex. To accomplish this the authors use a number of approaches to test oxidative stress responses during cortical development, the role of Prdm16 in the oxidative stress response and rescue of Prdm16 oxidative stress related phenotypes via crossing with mCAT overexpressing transgenic mice.

The strengths of this study are: 1) the experiments are extremely well executed, 2) the data are very clearly presented 3) and the manuscript is well written. The putative link between oxidative stress and Prdm16 in cortical development is also novel and an additional strength lies in the use of a combination of approaches including conditional KOs and RNA-seq analyses.

Response: We thank the reviewer for considering our experiments, data, and manuscript "extremely well executed, very clearly presented, and well written", and our findings of oxidative stress in RGPs controls upper and deep layer patterning "novel", and for providing valuable and constructive comments to improve our study.

Despite this, there are two caveats:

1) The conclusions as to the specific role of oxidative stress on RG cells as opposed to a putative role in the other non-SVZ populations is overstated. This claim appears to be based on the specificity of dissections of the VZ, the phenotype in Prdm16 conditional mutants, and putative rescue of cortical phenotypes with mCat. While the data strongly support a critical role for oxidative stress and Prdm16 in cortical development, the proposed role for oxidative stress specifically in RG cells as opposed to other cells in the cortex is not fully supported.

Response: To address the reviewer's concern, we performed new experiments to demonstrate that the vast majority (~80-90%) of cells in the isolated VZ tissues for RNA-seq analyses are RGPs expressing PAX6, a transcription factor highly expressed in cortical RGPs **(Fig. S2)**. We prepared acute dissociated cultures of the isolated VZ tissues and stained them with an antibody against PAX6 to assess their cell identity.

2) With regard to the role of Prdm16 in the cortex, similar deficits were found in a recent study

(Baizabal et at., Neuron 2018). Thus, while this analysis here is very well conducted, and somewhat extends these recently published results, this somewhat reduces novelty

However despite these caveats, the quality and depth of the experimental approaches and the novelty of a putative link RG/cortical development and oxidative stress together suggest that this study may be foundational in setting the stage for future studies of ROS as a critical pathway in cortical development.

Response: We thank the reviewer for recognizing that our study "may be foundational in setting the stage for future studies of ROS as a critical pathway in cortical development". The focus of our study on the regulation of oxidative stress and RGP behavior is a new direction, distinct from the recent study (Baizabal et al., Neuron 2018).

Reviewer 2 Comments for the Author:
In addition to addressing the above caveats, the following should be considered:

Figs 2, 3: It is unclear from the methods how VZ tissue was isolated from SVZ/MZ cells. This is important as Fig 1 also shows developmentally regulated increases in oxidative stress responses not just in the VZ, but throughout the SVZ and mantle.

Response: We isolated the VZ based on the comparison with the fluorescence reporter brain in which RGPs in the VZ were marked in red fluorescence (e.g., *Emx1-Cre/Ai9*). To further address the reviewer's concern, we performed new experiments to demonstrate that the vast majority (~80-90%) of cells in the isolated VZ tissues are RGPs expressing PAX6, a transcription factor highly expressed in cortical RGPs (**Fig. S2**).

Fig 2. Although perhaps acting upstream, it would be interesting to know if Prdm16 was also identified in the screen (it is suggested that a Table of all screen results be included in supplementary data)

Response: Following the reviewer's suggestion, we provided the information on the differentially expressed genes identified in our analyses (**Tables 1, 2, 3**). We also submitted the RNA-seq data (**GEO Accession number: GSE142684**).

Fig 3. Neurospheres might not be the best readout of changes in oxidative stress in RG cells in Prdm16 mutants in vivo, therefore it is recommended that this figure be moved to supplementary and then perhaps moving Supplementary Fig 9 to a regular figure as the findings shown in this figure are central to the study.

Response: Following the reviewer's suggestion, we moved Fig. S9 to Fig. 5.

We agree with the reviewer that neurosphere is not the best readout of oxidative stress of RGPs in vivo. In fact, we had made many attempts to measure oxidative stress level in the embryonic brain in vivo using different methods (e.g., Flow cytometry, whole brain live imaging); however, none of them allowed reliable estimation of the oxidative stress level.

Given that the analysis of ROS level in live RGPs is a critical experiment and that the neurosphere preparation is the best available preparation, we wish to keep the data in Fig. 3A,B.

Fig 8. The analysis/utility of the mCAT transgenic rescue is a bit superficial/unclear and does entirely resolve the mechanism of Prdm16 function through ROS. As the overexpression is global it does not directly point to a role in the VZ/RG population especially as the phenotype is not completely rescued.

Response: We agree with the reviewer that the mCAT expression is not specific for RGPs. To address the reviewer's concern, we performed new experiments to show that mCAT expression partially suppresses the elevated oxidative stress in the VZ/RGPs caused by PRDM16 removal **(Fig. S9 and 10)**. These results are well in line with the partial rescue of cortical defects of PRDM16 removal by mCAT expression **(Fig. 9)**.

Reviewer 3

The manuscript by Angela Chui and Song-Hai Shi investigates the role of elevated reactive oxygen species (ROS) and oxidative stress in radial glia progenitor (RGP) behaviour during mouse cerebral cortex development. The authors show that oxidative stress increases over the course of embryonic development in ventricular zone (VZ) areas and this regulates temporal fate transitions in RGPs. They also show that the transcriptional and epigenetic regulator Prdm16 regulates ROS levels and RGP behaviour: Prdm16 cortical specific cKO leads to increased ROS levels, oxidative stress and decreased ratio of upper vs lower neuron generation, a phenotype that is partially rescued by mitochondrial overexpression of the catalase enzyme CAT, which should reduce ROS.

While Prdm16 cortical specific cKO has already been described, the focus on Prdm16 regulation of ROS and variations in ROS/oxidative stress during the course of cortical development is definitely an advance in the field and helps understand how the interplay between transcriptional/epigenetic factors and metabolic processes shapes fate transition in the cortex.

Response: We thank the reviewer for considering our study "an advance in the field...helps understand the interplay between transcriptional/epigenetic factors and metabolic processes shapes fate transition in the cortex...interesting...well written...and clearly presented", and recommending it for publication, and for providing valuable and constructive comments to improve our study.

Comments for the Author:

The manuscript is interesting, well written and the data are clearly presented. I would recommend it for publication. I summarize below some comments and suggestions that could strengthen the paper:

As a general comment, I wonder whether the authors think that it is the oxidative stress to DNA, lipid, proteins (and thus a certain level of damage) that drives signaling and phenotype or is the physiological redox signaling triggered by variations in ROS and use oxidative stress just as a readout of increased ROS levels?

Response: To measure ROS level and oxidative stress in the *Prdm16* cKO RGPs, we used two well-established oxidative stress markers, 8-OHDG and HNE, which are well-established markers for oxidative DNA and lipid damage, respectively. Based on these experiments and our RNA-seq analysis of ROS related genes in the WT and *Prdm16* cKO RGPs, we think that alterations in ROS related genes lead to elevated ROS levels and consequently the defects in cortical development observed in the mutant animal.

In relation to previous comment, how do ROS levels change in RGP during the course of cortical development? Data in figure 1 show increased oxidative stress on DNA and lipid, but the intensity quantification uses arbitrary units, which vary very little and perhaps weakens appreciation of the effect. Since these data are leading the whole paper, would the authors consider using another method to strengthen and complement their results? For example, increased ROS in Prdm16 cKO in figure 3A looks great, could not they the use same method, CellROX, either on isolated progenitors or on ex vivo sections taken from different embryonic stages to show ROS variations during developmental time?

Response: Following the reviewer's suggestion, we used the same method (neurosphere and CellROX Green staining) and examined ROS level in RGPs at different embryonic stages and showed that ROS level progressively increases in RGPs in the VZ during cortical development (**Fig. S1**). This is consistent with our results using oxidative stress markers, 8-OHDG and HNE, showing the progressive increase in oxidative stress in the VZ as development proceeds (**Fig. 1**).

Minor

Graphs in figure 2 often show same ontology terms up- and down-regulated. I understand there might be different genes within the same term that go up or down, but then I wonder whether

there is a better way to visualize variations in metabolic processes or could they show only one time point and enlarge some of the categories to show the genes that go up and down..?

Response: Following the reviewer's suggestion, we provided a table (**Table 1**) showing the list of genes used in the GSEA for each GO term. We also highlighted a number of ROS related genes including *Prdx2, Prdx6, Sod1, Gpx3, Gpx8, Nos1*, and discussed their roles in regulating ROS level (**pages 6-7**).

Could the authors please specify in the text or figure legend what they use for GO analysis in figure 2? In figure 3 they consider top 500 differentially expressed genes, is it the same for figure 2?

Response: Following the reviewer's suggestion, we specified in the figure legend of Fig. 2 on the genes used for GO analysis **(Table 1)**. We also provide the Tables/Excel Sheets with all of the genes used in the GSEA for Fig. 2 and 3 **(Table 1 and 2)**.

Figure 3G-H: qPCR for Ptx3 and Cyp26b1: just out of clarity, could the authors please specify what is the expression relative to, e.g. what is =1? It might be E11.5 wt for Ptx3, but I cannot guess for Cyp26b1.

Response: The housekeeping gene HPRT was used to normalize the gene expression. Fold change in expression was calculated using the ΔCt method. We included the related information in the Materials and Methods section **(page 27)**.

Since Cyp26b1 is known to generate ROS via monooxygenate reactions (see Hrycay, Bandiera, Involvement of Cytochrome P450 in Reactive Oxygen Species Formation and Cancer, Advances in Pharmacology, 2015), could the increase in this gene mediate or strongly contribute to Prdm16 cKO phenotype? Would it be possible to reduce its expression in vivo in Prdm16 cKO? It would help to link the parts of the paper.

Response: We provide in-depth discussion about *Cyp26b1* **(page 19-20)**. Besides its role in generating ROS via monooxygenate reaction as the reviewer suggested, *Cyp26p1* is also known as encoding a retinoic acid (RA)-degrading enzyme in RA signaling (Rhinn and Dolle, 2012). Therefore, the function of *Cyp26b1* is likely more complex during cortical development.

Based on our RNA-seq analysis, multiple ROS related genes and pathways were either upregulated or downregulated. Therefore, it is rather hard to say that *Cyp26b1* strongly contributes to the *Prdm16* cKO phenotype. It is likely multiple genes/pathways function in concert, leading to elevated ROS level in the *Prdm16* cKO RGPs. Future efforts to assess the precise functions of CYP26B1 as well as other key players in cortical RGPs will likely provide new insight into the regulation of the retinoic acid pathway and its influence on ROS level and RGP behavior.

Cyp26b1 does not particularly vary in a time dependent manner in wt, so I wonder whether the genes that instead vary over the course of development (identified in fig 2, like Prdx2, Prdx6, Sod1, Gpx3, Gpx8 and Nos) are regulated by Prdm16? How do they vary in Prdm16 cKO?

Response: We did find that most of the genes that the reviewer referred to are regulated by PRDM16 except *Prdx2* and *Sod1*. For example, the expression of *Gpx3* is significantly increased, the expression of *Prdx6* and *Nos1* are drastically decreased in *Prdm16* cKO at E15.5, and the *Gpx8* expression is significantly increased in *Prdm16* cKO at E17.5 (**Fig. 3 E,F and Fig. S4B**). We stated these observations (**page 7**).

As the overall number of neurons and cortical area/size does not change between WT and Prdm16 cKO (eg it kind of balances out), I wonder how much of the effect at E15 is a secondary effect of the role at E13? Would conditional deletion of Prdm16 at E15 lead to the same increase in cell cycle exit and reduction in upper layer neurons? If they cannot do the experiment, perhaps they can speculate on this in results or discussion.

Response: Following the reviewer's suggestion, we clarified and provided additional discussion on this (**page 19**). We observed an over-proliferation of RGPs at E13.5, when the deep layer neurons

are produced. In contrast, we observed a decrease in RGPs and an accelerated cell cycle exit at E15.5, when the superficial layer neurons are produced. Therefore, the phenotypes that we observed at these two embryonic time points coincide well with the adult phenotype. Based on these observations, we speculate that there will be similar increase in cell cycle exit and reduction in upper layer neurons with condition removal of PRDM16 at E15.

In the RNA seq of wt vs Prdm16 cKO, do the authors see variations in migration genes that could explain the heterotopia (like in Baizabal 2018)? This would help clarify how Prdm16 leads to heterotopia, since they do not observe defects in post mitotic migration (eg are migratory genes dysregulated or it is indeed a primary defect in RGP specification/differentiation?)

Response: Using the list of migratory genes in Baizabal et al., 2018, we assessed our RNA-seq data of the top 500 differentially expressed genes between WT and *Prdm16* cKO. Only very few of the same genes appeared in our dataset. We provided a list of those genes (**Table 3**).

We did not observe any heterotopia or postmitotic migration defect in the *Nex-Cre; Prdm16* cKO cortex, indicating the heterotopia is unlikely due to PRDM16 function in postmitotic neurons. .

In Fig 6, Pax6 domain in Prdm16 cKO does not look expanded as it does in supplementary figure 9F (same stage). is this just variability? Do they have a better picture to show in fig 6?

Response: We provided better images in Fig. 6 to show the expansion of PAX6 domain.

I am not sure about figure number/manuscript size limits, but it would be great to see some of the data in supplementary fig 9 and 10 (Pax6, Tbr2 and pHH3 analyses) to be moved to the main part of the manuscript. They are quite interesting and they have not been shown before in previous characterization of this cKO.

Response: We moved the original Fig. S9 and S10 to Fig.5 and 7, respectively.

What is the effect of mCAT overexpression on ROS levels, especially in Prdm16 cKO? Could they use CellROX to show at least partial reduction in ROS?

Response: We stained the embryonic brain sections of WT, *Prdm16* cKO, *Prdm16 cKO;mCAT* with the well-established oxidative stress markers, 8-OHDG and HNE, to measure ROS level and oxidative stress, and found that mCAT expression partially suppresses the elevated ROS level and oxidative stress caused by PRDM16 removal **(Fig. S9 and 10)**.

As the authors talk about temporal transition in fate, I wonder whether increased ROS in Prdm16 cKO might also impact astrocytes that are produced later in development? Would they be decreased? Perhaps the authors could add a sentence in the discussion?

Response: Following the reviewer's suggestion, we added a sentence in the discussion on the regulation of glial cell generation by ROS level and PRDM16 as following "Furthermore, it would be interesting to assess whether the generation of astrocytes and/or oligodendrocytes by RGPs was altered upon PRDM16 removal and ROS level change." **(page 22)**.

Second decision letter

MS ID#: DEVELOP/2019/184150

MS TITLE: Oxidative stress regulates progenitor behavior and cortical neurogenesis
AUTHORS: Angela Chui, Qiangqiang Zhang, and Songhai Shi
ARTICLE TYPE: Research Article

I am happy to tell you that your manuscript has been accepted for publication in Development, pending our standard ethics checks. Where referee reports on this version are available, they are appended below.

Reviewer 1

Advance summary and potential significance to field

The authors have responded satisfactorily to my minor criticisms. They have nevertheless decided not to deepen their analysis of the phenotype rescued/not rescued by mCAT OE which represent the most novel part of the paper and could have significantly increase the impact of the manuscript.

Comments for the author

The authors have responded satisfactorily to my minor criticisms. They have nevertheless decided not to deepen their analysis of the phenotype rescued/not rescued by mCAT OE which represent the most novel part of the paper and could have significantly increase the impact of the manuscript.

Reviewer 2

Advance summary and potential significance to field

With new data and analysis the authors have addressed my original comments. This is an excellent study that should contribute significantly to the field.

Comments for the author

I have a few remaining minor comments/suggestions:
1) Important information regarding the RNA seq screen remains lacking, specifically:
The logic for choosing the top 500 genes as the cut off i is not clearly laid out. Does this correspond to a specific log fold change?
P26 Materials and Methods. the approach to isolate VZ tissue for the RNA seq screen is still needs to be better described; was VZ manually dissected from Emx1cre;Ai9 mice or FACs sorted?
2) Please check grammar and tense consistency throughout the manuscript(e.gs P11; “Compare” should be ‘compared’, defect “is” should be defects “are”, P12; at the embryonic stage, should be ‘stages’

Reviewer 3

Advance summary and potential significance to field

The authors have improved the manuscript and addressed most comments. I am happy to see the cellrox in FigS1 worked well in line with the other data. I still have a suggestion to the authors (below), but overall, it is a novel and interesting work that relates ROS and oxidative stress to cortical development.

Comments for the author

I would still recommend to change the pictures they have selected for Figure 6. So far, they have only separated the Pax6+ picture from the merged, which definitely helps to see the Pax6 domain, but it does not scream ‘drastic increase in RGPs’, as they claim in the text (page 13) and which is a bit more evident in figure 5.