THE EMBO JOURNAL

Manuscript EMBO-2011-78369

RP58 controls neuron and astrocyte differentiation by downregulating the expression of Id1-4 genes in the developing cortex

Shinobu Hirai, Akiko Miwa, Chiaki Ohtaka-Marurama, Masataka Kasai, Shigeo Okabe, Yutaka Hata and Haruo Okado

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Review timeline:

Submission date:	06 June 2011
Editorial Decision:	28 July 2011
Revision received:	16 November 2011
Accepted:	13 December 2011

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 28 July 2011

Thank you for submitting your manuscript for consideration by the EMBO Journal. It has now been seen by three referees, whose comments are shown below. While they find your work of potential interest, they also raised significant concerns that would need to be addressed in a major revision. Their reports are explicit, but I would just highlight the most critical concerns here:

As you will see referees #1 and #2 appreciate the original approach you have taken to show that RP58 regulates neurogenesis via the Id proteins. However, they both list some serious issues about the significance of your findings and suggest experiments to improve and strengthen your conclusions. The main concerns are:
- ChIP to be done in more controlled physiological conditions (ref. #1 point 3 and ref. #2 point 9)
- Investigate further the issue of potential redundancy between Id genes (ref. #1 point 5 and ref. #2 point 7)
- Assess p57 loss of function effect (ref. #1 point 6)
- RP58 presumably regulates other targets; at least some discussion of how might these also contribute to the phenotype would be important (ref. #1 points 1 and 4 and ref. #2 point 4)

Given the overall interest in the study, and providing that you are able and willing to address all referees' comments, we would be happy to consider a revised version of the manuscript for publication in The EMBO Journal within 3 months. We do realize that the necessary revisions will require a considerable amount of work. Should you foresee a problem in meeting this three-month deadline, please let us know and we may be able to extend this up to a maximum of 6 months.

I would like to add that it is EMBO Journal policy to allow only a single round of revision only and

that acceptance or rejection of your manuscript will therefore depend on the completeness of your responses in the final revised version.

When preparing your letter of response to the referees' comments, please note that this will form part of the Review Process File, and will therefore be available online to the community. For more details on our Transparent Editorial Process, please visit our website:
http://www.nature.com/emboj/about/process.html
As a matter of policy, competing manuscripts published during the 3-months period will not negatively impact on our assessment of the conceptual advance presented by your study. However, we request that you contact the editor as soon as possible upon publication of any related work, to discuss how to proceed.

Thank you for the opportunity to consider your work for publication. I look forward to your revision.

Yours sincerely,

Editor
The EMBO Journal

REFEREE REPORTS

Referee #1

The manuscript by Kasai et al. is an interesting piece of work in solving the puzzle of corticogenesis that identifies the transcription factor RF58 as a player at the onset of gliogenesis. A correlation between Id gene expression (all four Id paralogs found upregulated in RP58 KO cells) and enhanced astrocytic differentiation is shown for which phenotypic consequences are consistent with previous observations based on Id overexpression studies. A subsequent link to RP58 indirect regulation of an inhibitor of cell cycle progression (p57, through its negative regulation by Id products) is also put forward as part of the mechanism underlying RP58 KO phenotype. However, authors should address the points below for a better supported conclusions, of which in its present form, some depend on weak experiments.

1. As a transcription factor, RP58 might be expected to regulate many targets. I don't understand that this possibility is maintained out of the study. It's fine that here work focuses on RP58-dependent Id gene repression as a causal role of RP58 KO CNS phenotype, but I feel that a little context would be convenient. GO term analysis or some sort of global assessment of gene expression alterations seen in RYBP KO cells should be indicated.

2. Since enlarged astrocyte populations are detected in caudal, but not rostral, mutant cortex (and being the point that this is a consequence of upregulated Id gene expression) a better, regionalized, characterization of Id gene expression would be required. The analysis would benefit from protein detection, were the appropriate antibodies be available. Are Id genes derepressed in rostral regions? While its (hypothetical) presence in regions devoid of enhanced astrocytic differentiation would not necessarily invalidate the major conclusions, it would expand context and help describing comprehensively RP58 mutant neurogenesis.

3. Trying to determine negative regulation of Id genes by RP58, the authors take a (very) artificial approach by looking at RP58 association to promoter regions in reporter constructs transiently transfected in tissue culture cells. Why the authors decide not to carry out ChIP experiments on wild type neurosphere cultures? In general, and given the far from physiological conditions in transfected cells I wouldn't think the reported ChIP data are meaningful.

4. Experiments in figure 4 are, again, too artificial: whereas overexpression of RP58 and downregulation of Id gene expression is consistent with their upregulation in the absence of Rp58, the perhaps exaggerated levels of ectopic RP58 may cause side effects not related to Id gene expression. Therefore, neurospheres from RP58 KO mouse would also be needed to establish a role in self-renewal. Moreover, claims on self-renewal competence should only be sustained using

clonogenic assays.

5. I wonder whether all four Id genes are required together to mimic RP58 KO phenotype. If this was the case it would a significant finding. Instead, given that concurrent knockdown seems required for phenotype rescue, showing redundancy, it appears that they might be active independently of each other. Their individual overexpression would, perhaps, unveil specific functions for each paralog which would be pertinent to the whole story.

6. I see problematic the interpretation of p57 gain of function experiments: on the one hand, mutant tissues show some decrease of p57 protein levels which might or might not account for aspects of RP58 KO phenotype. I'm not sure what p57 protein levels in p57+/- mice would be and whether they would show defective neurogenesis, but protein levels here are reduced by certainly less than 50%. Differentiation alterations accompanied by p57 overexpression are not necessarily unexpected, but the point here is to link RP58 repression of Id gene expression to core elements of the cell cycle machinery. Obviously, a p57 loss of function approach to see whether Id control of p57 is the key point in RP58 KO phenotype.

Minor points

page 5, line13: reference by Rebecca et al. missing from the reference list.
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page 7, line 1: ISH tells about steady state mRNA levels, not necessarily an indication of "transcription level"
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page 14, line 4: the authors performed Id knock down possibly without relation to "interruption of Id transcription" and therefore some rephrasing is needed.
page 31, panels B-I should be B-E.

Referee #2

The manuscript by Hirai and colleagues investigates the function of the transcription factor Rp58 during cortical development. The authors observe an increase in Sox-2 positive progenitor cells in Rp58-/- mice associated with increased numbers of cyclin-E and GFAP positive cells as compared to age-matched WT control tissues. Due to neonatal lethality, authors next derive cell lines and observe increased propensity of Rp58-/- cells to give rise to GFAP positive progeny. To investigate the mechanism of increased progenitor cells in Rp58-/- mice authors conduct an unbiased screen to identify alterations in gene expression in Rp58-/- cortical tissue and identify repression of the four members of the Id gene family. After appropriate validation of these findings authors use reporter assays and ChIP to demonstrate of Rp58 mediated repression of Id genes. Authors next seek to demonstrate increased self-renewal and observe that Rp58-/- cells produce neurospheres of reduced diameter. To validate the role of Id genes, authors show that electroporation of a cocktail of Id gene vectors increased the percentage of GFAP and Ki67-positive cells in WT mice mimicking the phenotype of Rp58-/- mice and in rescue experiments, authors show that Id knockdown of expression of the cell cycle inhibitor p57 rescues the phenotype of increased progenitor cells in Rp58-/- mice.

Authors present a well-written manuscript highlighting an important finding in a novel mouse model. While the central finding of this report is extremely interesting to a broad audience and supported by earlier literature, there are several instances where interpretations are not convincingly supported by the data presented implying a requirement for additional experiments.

1. A general problem is lack of a convincing compliment of markers to verify specific cell populations. For example, authors conclude that increased numbers of GFAP-positive cells in Rp58-/- cortices results from increased astrogliogenesis. GFAP labels a variety of cells relevant to cortical development including radial glial and some populations of neural progenitor cells. Since defects in these cells might contribute to abnormal cortical development it will be important to be certain that the GFAP-positive cells are not radial glia or a subpopulation of progenitor cells. This could be achieved by co-staining with markers of radial glia, such as BLBP, vimentin or nestin. To eliminate

the possibility that these cells are a subpopulation of progenitor cells co-staining GFAP with Sox-2, other progenitor cell markers or a proliferation marker such as BRDU of Ki67 would be helpful.

2. IHC data is somewhat qualitative. Sample numbers and details regarding morphometric alignment of sections need to be included in all figures. Quantitative analysis would be improved by stereologic cell counting.

3. Culture methods are unclear. On page 5 lines 21-28; are primary cells grown in FBS? To strengthen the claim that Rp58-/- cells more avidly undergo astroglial differentiation cells should be isolated as progenitors using conditions such as those described on page 8 line 6. Then these cells can be subjected to differentiation conditions and all major CNS lineages quantified. Minor point; text refers to Figure 2I-K, data appears in Figure 1.

4. Could an alternative interpretation of microarray data be that loss of Rp58 results in an increase in the percentage of Id expressing lineages rather than a loss of repression? Authors should discuss their microarray methods in more detail. In particular, how were these data normalized in order to be certain that these data do not simply reflect an increased number of Id-positive progenitor cells rather than increased expression in individual cells.

5. Analysis of Id gene expression needs to be accompanied by protein data. Id genes function at the protein level, and as authors allude to, Id protein levels are regulated by the proteasome. It is critical to be certain that alterations in Id gene expression result in changes in protein levels both *in vivo* and *in vitro*.

7. Figure 3 reports data interpreted as indicating that Rp58 directly represses all Id genes. This is a critical part of this manuscript. Are these the only E-boxes in the Id promoters? How did authors choose to mutate this particular E-Box? Luciferase assays and *in vivo* ChIP data are interesting but would be improved if accompanied by data showing that Rp58 will not bind the mutant, and the ChIP experiments were also conducted under physiologic conditions in un-transfected cells.

8. On page 8 line 1; it is unclear what "the self-renewal of NSC proliferation" means. Is this section a study on self-renewal capacity or rate of proliferation? To improve this section self-renewal should be calculated as a percentage of dispersed cells capable of generating new spheres in clonal culture. Reduced sphere diameter could result from slower proliferation or increased cell cycle exit due to NPC differentiation. It will thus be important to know what types of cells make up these smaller spheres and whether their proliferation rate has changed. This can be accomplished by immunocytochemistry or flow cytometry to identify differentiation markers or proliferation markers.

9. Identification of a repressor of an entire gene family is exciting however it is unusual to use a cocktail of four genes (Id1-4) to elicit an effect. Using this approach, it remains unclear whether Id genes are additive or redundant in this context. Are all 4 Id genes over-expressed in every cell? Although admittedly non-trivial, over-expression or knockdown of Id genes 1-4 individually would be very informative.

10. Authors should be certain that these are not tumor cells. Expression of Id, GFAP, Ki67 and Sox2 is associated with brain tumors and authors indicate that RP58 expression has a tumor suppressive effect. Transformation assays could be conducted to determine the oncogenic potential of Rp58-/- cells. For example, do isolated cells form tumors in immunocompromised mice? Are they growth factor independent? Do they grow in soft-agar?

Referee #3

In this manuscript Hirai et al. have nicely shown that using RP58 knockout mice and a number of experimental techniques, RP58 directly inhibits the expression of Id1-4, which negatively regulate neural development. Authors have also shown that p57, a cell cycle inhibitor, is an essential downstream target of Id genes in NSC.

Comments:
1. Authors have cultured Lv-RP58- and Lv-GFP-infected embryonic NSC in EGF alone and

examined their proliferation. However, since bFGF, rather than EGF, is the main mitogen for embryonic NSC, it is important to examine the effect of RP58 overexpression in Lv-RP58- and Lv-GFP-infected embryonic NSC cultured in bFGF alone and a combination of bFGF and EGF.
2. To confirm that RP58 induces cell-cycle exit by inhibiting the expression of Id genes, it is nice to examine whether overexpression of both RP58 and Ids maintains NSC proliferation.
3. Since it is known that the expression of p16/Ink4a is regulated by Id1, authors should check the expression of Ink4 family members in the RP58-null NSC.
4. Authors have used TBR1 to identify the differentiated neurons. To confirm neuronal differentiation in RP58-null brain, it is important to show the expression of other mature neuronal markers, such as neurofilament and GAD.

1st Revision - authors' response 16 November 2011

Referee #1

The manuscript by Kasai et al is an interesting piece of work in solving the puzzle of corticogenesis that identifies the transcription factor RF58 as a player at the onset of gliogenesis. A correlation between Id gene expression (all four Id paralogs found upregulated in RP58 KO cells) and enhanced astrocytic differentiation is shown for which phenotypic consequences are consistent with previous observations based on Id overexpression studies. A subsequent link to RP58 indirect regulation of an inhibitor of cell cycle progression (p57, through its negative regulation by Id products) is also put forward as part of the mechanism underlying RP58 KO phenotype. However, authors should address the points below for a better supported conclusions, of which in its present form, some depend on weak experiments.

Comment 1:
As a transcription factor, RP58 might be expected to regulate many targets. I don't understand that this possibility is maintained out of the study. It's fine that here work focuses on RP58-dependent Id gene repression as a causal role of RP58 KO CNS phenotype, but I feel that a little context would be convenient. GO term analysis or some sort of global assessment of gene expression alterations seen in RYBP KO cells should be indicated.

Reply:
We fully agree with the reviewer regarding the fact that Rp58 may target molecules other than Ids. We have now analyzed the microarray data using Gen MAPP software (Gene MicroArray Pathway Profiler) and included a list of genes that show significantly altered expression in *Rp58* KO mice compared with WT mice in the revised manuscript (Supplemental Tables 1 and 2). We found that the Id genes, which are components of cell cycle-related pathways, were included in the list. In line with this result and the similarity between the phenotypes of Id and Rp58 KO mice, we focused on RP58 Id-mediated gene repression.

We have added the following sentences to the revised manuscript: (page 6, line 11)
"The obtained data were analyzed by MAPP pathway analysis, which indicated that the genes showing significantly altered expression in *Rp58* KO compared with WT were involved in six pathways (Table S1 and S2). First, we focused on the pathways associated with cell cycle progression and astrogenesis. Second, because RP58 acts as a transcriptional repressor (Aoki et al., 1998; Fuks et al., 2002; Takahashi et al., 2008), we noted the genes showing increased expression in the mutant cortex, with particular reference to those that also contained the RP58-binding consensus sequence in human genomic loci corresponding to those in the mouse (Supplementary Figure 5)."

Comment 2:
Since enlarged astrocyte populations are detected in caudal, but not rostral, mutant cortex (and being the point that this is a consequence of upregulated Id gene expression) a better, regionalized, characterization of Id gene expression would be required. The analysis would benefit from protein detection, were the appropriate antibodies be available. Are Id genes derepressed in rostral regions? While its (hypothetical) presence in regions devoid of enhanced astrocytic differentiation would not necessarily invalidate the major conclusions, it would expand context and help describing comprehensively RP58 mutant neurogenesis.

Reply:
In accordance with the reviewer's suggestion, we immunostained sections of caudal and rostral cortex using anti-Id1-4 antibodies. The results showed that, compared with the WT, the four Id proteins were overexpressed only in the caudal cortex, which means that Rp58 works functionally in this region. Id proteins were not increased in the rostral region of the mutant cortex. Therefore, different RP58-dependent or -independent molecular mechanisms may exist for *Id* regulation in the caudal and rostral regions.

We have added the following sentences to the revised manuscript: (page 7, line 6)
"In addition, we used immunohistochemistry to confirm that the expression of Id proteins was increased in the mutant SVZ compared with that in WT SVZ (Supplementary Figure S6Ac–H'c). In the WT cortex, explicit expression of each Id was observed only in the VZ (Figure S6A'c–H'c). In the rostral cortex, few differences in the Id expression patterns were observed between WT and KO mice. Mice of both genotypes showed expression of Id1 and Id4 in VZ / SVZ, Id2 in the total cortical area, and Id3 alone in the VZ (Supplementary Figure S6Ar–Hr)."

Comment 3:
Trying to determine negative regulation of Id genes by RP58, the authors take a (very) artificial approach by looking at RP58 association to promoter regions in reporter constructs transiently transfected in tissue culture cells. Why the authors decide not to carry out ChIP experiments on wild type neurosphere cultures? In general, and given the far from physiological conditions in transfected cells I wouldn't think the reported ChIP data are meaningful.

Reply:
We apologize for the confusion with regard to Figure 3C, D. ChIP analysis using WT neurosphere culture is an appealing idea; however, we performed ChIP experiments using E16.5 cortex as we feel that this better reflects physiological conditions (please see the "Materials and Methods" section). Therefore, to convey the meaning more clearly, we have indicated the sample origin as "E16.5 cortex" in the ChIP experiments described in Figure 3C.

Comment 4:
Experiments in figure 4 are, again, too artificial: whereas overexpression of RP58 and downregulation of Id gene expression is consistent with their upregulation in the absence of Rp58, the perhaps exaggerated levels of ectopic RP58 may cause side effects not related to Id gene expression. Therefore, neurospheres from RP58 KO mouse would also be needed to establish a role in self-renewal. Moreover, claims on self-renewal competence should only be sustained using clonogenic assays.

Reply:
We thank the reviewer for this helpful suggestion. We have examined the self-renewal competency of neurospheres from Rp58 KO mice using clonogenic assays. We passaged the dissociated neurospheres four times and measured variations in sphere diameter. Moreover, to confirm the side effects of Rp58 overexpression, we simultaneously infected neurospheres with lentivirus expressing both RP58 and Id. The reduction in sphere diameter caused by Rp58 infection was partially rescued by Id expression (Supplemental Figure S9). Therefore, we concluded that the reduction in self-renewal competency in Rp58-infected neurospheres is due to reduced Id expression.

We have added the following sentences to the revised manuscript: (page 9, line 6)
"Lv-Rp58-infected neurospheres lost their competency for self-renewal, which was partially rescued by the co-induction of each individual *Id,* or all four *Ids*, along with RP58 (Supplementary Figure 9)."

Comment 5:
I wonder whether all four Id genes are required together to mimic RP58 KO phenotype. If this was the case it would a significant finding. Instead, given that concurrent knockdown seems required for phenotype rescue, showing redundancy, it appears that they might be active independently of each other. Their individual overexpression would, perhaps, unveil specific functions for each paralog which would be pertinent to the whole story.

Reply:

We have already performed rescue experiments using single Id knock down and, as shown in Supplemental Figure S19, could not rescue the phenotypes for increased astrocytes and progenitors in *Rp58* KO mice. Besides, in accordance with the reviewer's suggestion, we tried to mimic the KO phenotype by electroporating single Id genes. Overexpression of single *Id* genes was able to mimic the *Rp58* KO phenotype. These results suggest that all four *Id* genes can play a compensatory role in the proliferation of NSCs and differentiation into astrocytes. In other words, the simultaneous repression of transcription of all four *Id* genes by RP58 is important for the cell cycle exit of NSCs and differentiation into neurons (Supplemental Fig. S18).

We have added the following sentences to the revised manuscript: (page 14, line 28)
"In fact, we found that cortices electroporated with the single *Id* showed similar phenotypes to those electroporated with all four *Ids*. Quantitative analysis of $Ki67^+/GFP^+$ progenitors (13.9% ± 3.7%) and $GFAP^+/GFP^+$ astrocytes (7.4% ± 1.6%) in the control cortices revealed the distribution of electroporated cells. Embryos electroporated with a single *Id* vector showed significantly more $Ki67^+/GFP^+$ progenitors (*Id1*: 49.0% ± 6.5%, *Id2*: 46.3% ± 9.4%, *Id3*: 51.8% ± 8.6%, *Id4*: 45.9% ± 3.6%) and $GFAP^+/GFP^+$ astrocytes (*Id1*: 48.3% ± 2.0%, *Id2*: 41.8% ± 3.3%, *Id3*: 47.0% ± 7.5%, *Id4*: 41.4% ± 10.0%) (Supplementary Figure 18)."

Comment 6:
I see problematic the interpretation of p57 gain of function experiments: on the one hand, mutant tissues show some decrease of p57 protein levels which might or might not account for aspects of RP58 KO phenotype. I'm not sure what p57 protein levels in p57+/- mice would be and whether they would show defective neurogenesis, but protein levels here are reduced by certainly less than 50%. Differentiation alterations accompanied by p57 overexpression are not necessarily unexpected, but the point here is to link RP58 repression of Id gene expression to core elements of the cell cycle machinery. Obviously, a p57 loss of function approach to see whether Id control of p57 is the key point in RP58 KO phenotype.

Reply:
In line with the reviewer's suggestion, we have investigated the effects of functional inhibition of p57 by *in utero* electroporation of truncated *p57* (Cterp57) devoid of the cyclin/CDK binding/inhibitory domains. Tury et al. previously reported that cells overexpressing Cterp57 show attenuated proliferation (Tury et al., 2011). We have used the same procedure to observe whether Cterp57 overexpressing cells differentiate into astrocytes (Fig. S16). The results support the idea that p57 is a main downstream factor of Rp58. The increased levels of astrogenesis and progenitor proliferation observed in *Rp58* KO mice are likely due to decreased levels of p57 caused by the depletion of Rp58.

We have added the following sentences to the revised manuscript: (page 11, line 17)
"Conversely, to determine the effect of astrocyte differentiation observed in the WT cortex on p57 functional inhibition, we used the C-terminal portion of p57 (Cterp57, a Cip/Kip family member, which lacks the cyclin/CDK binding/inhibitory domains and contains unique proline and acidic domains). It is reported that the inhibition of p57 function by Cterp57 enhances cell proliferation (Tury et al., 2011); therefore, we asked whether inhibiting the function of p57 affects astrocyte differentiation. *In utero* electroporation was performed at E15.5, followed by immunostaining of the cortices with GFAP at P9.5. Upon electroporation with a control plasmid, most cells produced differentiated neurons that migrated into the cortical plate over the next few days; however, large numbers of GFAP+/GFP+ cells were observed in Cterp57 electroporated cortices (100.0% ± 0.0% control and 82.74% ± 5.37% Cterp57-induced cells; Supplementary Figure 16). These results further support our conclusion that the decreased expression of p57 in the *Rp58* KO cortex results in increased numbers of progenitors and astrocytes."

Minor points

page 5, line13: reference by Rebecca et al. missing from the reference list.
page 5, line 26: wrong label for panels referred to as Figure 2I-K.
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page 14, line 4: the authors performed Id knock down possibly without relation to "interruption of Id transcription" and therefore some rephrasing is needed.

Reply:
We apologize for these errors and have now corrected them in the revised manuscript.

page 31, panels B-I should be B-E.

Reply:
We have made the appropriate corrections.

page 7, line 1: ISH tells about steady state mRNA levels, not necessarily an indication of "transcription level"

Reply:
As suggested, we have changed "transcription level" to "mRNA levels".

Referee #2

The manuscript by Hirai and colleagues investigates the function of the transcription factor Rp58 during cortical development. The authors observe an increase in Sox-2 positive progenitor cells in Rp58-/- mice associated with increased numbers of cyclin-E and GFAP positive cells as compared to age-matched WT control tissues. Due to neonatal lethality, authors next derive cell lines and observe increased propensity of Rp58-/- cells to give rise to GFAP positive progeny. To investigate the mechanism of increased progenitor cells in Rp58-/- mice authors conduct an unbiased screen to identify alterations in gene expression in Rp58-/- cortical tissue and identify repression of the four members of the Id gene family. After appropriate validation of these findings authors use reporter assays and ChIP to demonstrate of Rp58 mediated repression of Id genes. Authors next seek to demonstrate increased self-renewal and observe that Rp58-/- cells produce neurospheres of reduced diameter.
To validate the role of Id genes, authors show that electroporation of a cocktail of Id gene vectors increased the percentage of GFAP and Ki67-positive cells in WT mice mimicking the phenotype of Rp58-/- mice and in rescue experiments, authors show that Id knockdown of expression of the cell cycle inhibitor p57 rescues the phenotype of increased progenitor cells in Rp58-/- mice.

Authors present a well-written manuscript highlighting an important finding in a novel mouse model. While the central finding of this report is extremely interesting to a broad audience and supported by earlier literature, there are several instances where interpretations are not convincingly supported by the data presented implying a requirement for additional experiments.

Comment 1:
A general problem is lack of a convincing compliment of markers to verify specific cell populations. For example, authors conclude that increased numbers of GFAP-positive cells in Rp58-/- cortices results from increased astrogliogenesis. GFAP labels a variety of cells relevant to cortical development including radial glial and some populations of neural progenitor cells. Since defects in these cells might contribute to abnormal cortical development it will be important to be certain that the GFAP-positive cells are not radial glia or a subpopulation of progenitor cells. This could be achieved by co-staining with markers of radial glia, such as BLBP, vimentin or nestin. To eliminate the possibility that these cells are a subpopulation of progenitor cells co-staining GFAP with Sox-2, other progenitor cell markers or a proliferation marker such as BRDU of Ki67 would be helpful.

Reply:
We agree with the reviewer and have carried out additional experiments to clarify whether GFAP-positive cells are astrocytes. Burns et al. (Glia. 2009 Aug 1; 57(10):1115-29) reported that immature astrocytes sometimes express markers for both radial glial cells and proliferating cells. Raponi et al. also reported that S100β-positive progenitors only differentiate into astrocytes (Raponi et al., 2007). Therefore, we tried to identify astrocytes by double-immunostaining with GFAP and S100β. The results showed that almost all GFAP-positive cells in RP58 mice also expressed S100β. Consequently, we concluded that the GFAP-positive cells in p58 KO mice were immature astrocytes.

We have added the following sentences to the revised manuscript: (page 5, line 18)
"Double-immunostaining with GFAP and the astrocyte marker S100β was performed to confirm that the GFAP-positive cells were astrocytes rather than radial glia (Raponi et al., 2007) (Supplementary Figure 4)."

Comment 2:
IHC data is somewhat qualitative. Sample numbers and details regarding morphometric alignment of sections need to be included in all figures. Quantitative analysis would be improved by stereologic cell counting.

Reply:
Thank you for this helpful suggestion. We have added the information regarding the IHC-stained sections to the Figure legends. We agree that quantitative analysis is a useful tool for the precise evaluation of target protein-expressing cells. We have supplemented the result by counting cells positive for Sox2 and Cyclin-E, which are clearly increased in *Rp58* KO mice compared with WT (Fig. 1 and Fig. S1). It was difficult to distinguish individual cells in some of the tissue sections because of diffuse or overlapping staining patterns (e.g., Tuj1 and GFAP in Fig. 1 and Id1 and Id4 in Fig. S6). We are sorry that we could not fully comply with the reviewer's request.

Comment 3:
Culture methods are unclear. On page 5 lines 21-28; are primary cells grown in FBS? To strengthen the claim that Rp58-/- cells more avidly undergo astroglial differentiation cells should be isolated as progenitors using conditions such as those described on page 8 line 6. Then these cells can be subjected to differentiation conditions and all major CNS lineages quantified. Minor point; text refers to Figure 2I-K, data appears in Figure 1.

Reply:
We thank the reviewer for the suggestion and have included experiments examining the fate of isolated progenitors using the EdU labeling method. Cells isolated from E16.5 cortices were labeled with EdU for 12 hr in FBS-containing medium and the fate of the progenitor cells incorporating EdU was determined (Figure 1J-L). We have described these culture methods in the "Materials and Methods" section of the revised manuscript (page 20, line 27).
As suggested, we have also changed "Figure 2I-K" to "Figure 1J-L".

We have added the following sentences to the revised manuscript: (page 5, line 25)
"Cells, including NSCs from the E16.5 cerebral cortex, were labeled with EdU (a thymidine analog) in the culture medium for 12 hr and incubated for a further 5 days. Fluorescence labeling was then performed for EdU, GFAP and NeuN, a neuronal marker, to confirm whether the increased progenitors observed in the mutant mice could differentiate into GFAP-positive astrocytes. Approximately 40% of mutant cells and 20% of WT cells did differentiate into astrocytes. No significant difference in neuronal differentiation was observed between mutant and WT cells (Figure 1J–L)."

Comment 4:
Could an alternative interpretation of microarray data be that loss of Rp58 results in an increase in the percentage of Id expressing lineages rather than a loss of repression? Authors should discuss their microarray methods in more detail. In particular, how were these data normalized in order to be certain that these data do not simply reflect an increased number of Id-positive progenitor cells rather than increased expression in individual cells.

Reply:
We apologize for the confusion regarding the descriptions. The results of the microarray analysis comprise the "increase in the percentage of Id expressing linages" and "a loss of repression" because we used whole brain samples obtained at E16.5. To solve this problem, we performed PCR analysis on single neurospheres derived from E14.5. The cDNAs synthesized from each single neurosphere derived from the *Rp58* KO and WT cortices were subjected to real time PCR analysis. The results showed that the expression of all four Ids was repressed at the single cell level by normalized to that of GAPDH, indicating that the loss of RP58 repression causes Id overexpression (Fig. S7). We confirmed that there was no effect on the expression of Pax6, a marker for progenitor cells.

We have added the following sentences to the revised manuscript: (page 7, line 14)
"To determine whether the increase in *Id* mRNAs in the *Rp58* KO cortex was not only due to an increased number of progenitors but also to the loss of direct RP58-dependent regulation, we estimated *Id* mRNA expression at the single cell level in WT- and mutant-derived neurospheres by normalizing it to that of *Gapdh."*

Comment 5:
Analysis of Id gene expression needs to be accompanied by protein data. Id genes function at the protein level, and as authors allude to, Id protein levels are regulated by the proteasome. It is critical to be certain that alterations in Id gene expression result in changes in protein levels both in vivo and in vitro.

Reply:
In accordance with the reviewer's suggestion, we performed immunohistochemistry studies using antibodies to Id1-4 and confirmed that the Ids are overexpressed around SVZ, which is similar to the result obtained in the ISH experiments (Supplemental Fig. S6). Because Id2 and Id4 are also expressed in mature neurons, we could not measure their expression levels *in vitro* using biochemical techniques such as immunoblotting. In fact, the microarray and real-time PCR analyses showed no significant differences between the expression levels of Id2 and Id4 in WT and Rp58 KO mice (Supplemental Tables 2 and 3).

We have added the following sentences to the revised manuscript: (page 7, line 6)
"In addition, we used immunohistochemistry to confirm that the expression of Id proteins was increased in the mutant SVZ compared with that in WT SVZ (Supplementary Figure S6Ac–H'c). In the WT cortex, explicit expression of each Id was observed only in the VZ (Figure S6A'c–H'c). In the rostral cortex, few differences in the Id expression patterns were observed between WT and KO mice. Mice of both genotypes showed expression of Id1 and Id4 in VZ /SVZ, Id2 in the total cortical area, and Id3 alone in the VZ (Supplementary Figure S6Ar–Hr)"

Comment 7:
Figure 3 reports data interpreted as indicating that Rp58 directly represses all Id genes. This is a critical part of this manuscript. Are these the only E-boxes in the Id promoters? How did authors choose to mutate this particular E-Box? Luciferase assays and in vivo ChIP data are interesting but would be improved if accompanied by data showing that Rp58 will not bind the mutant, and the ChIP experiments were also conducted under physiologic conditions in un-transfected cells.

Reply:
Many typical E-boxes (CANNTG) exist around the *id*-coding genomic sequences. However, we can confirm that each Id contains only one putative Rp58-binding E-box (ACANCTG). In addition, we apologize for the misleading labels and sentences in the legend to Figure 3. We performed ChIP experiments using the E16.5 cortex, not transfected cells, under physiological conditions (please see the "Materials and Methods" section). To avoid any confusion, we have now indicated the sample origin as "E16.5 cortex" in the ChIP experiments described in Figure 3C.

We have added the following sentences to the legend for Fig.3:
"Besides the typical E-boxes (CANNTG), there is only one pair of Rp58-binding E-boxes (ACANCTG) situated in close proximity to the *id*-encoding genomic sequences."

Comment 8:
On page 8 line 1; it is unclear what "the self-renewal of NSC proliferation" means. Is this section a study on self-renewal capacity or rate of proliferation? To improve this section self-renewal should be calculated as a percentage of dispersed cells capable of generating new spheres in clonal culture. Reduced sphere diameter could result from slower proliferation or increased cell cycle exit due to NPC differentiation. It will thus be important to know what types of cells make up these smaller spheres and whether their proliferation rate has changed. This can be accomplished by immunocytochemistry or flow cytometry to identify differentiation markers or proliferation mark.

Reply:

We understand the reviewer's point but feel that it is necessary to show that the self-renewal of spheres infected with Lv-Rp58 was suppressed under undifferentiated conditions. To do this, we counted the number of newly-generated secondary spheres *per* dissociated cell (Figure 4D).

We have added the following sentences to the revised manuscript: (page 8, line 25)
"The dissociated primary neurospheres were then replated to analyze their self-renewal competency. Few secondary neurospheres were formed from the Lv-*Rp58*-infected neurospheres (5.67±4.04), whereas control Lv-GFP-infected cells formed approximately 200 secondary neurospheres (202.33±17.5; Fig. 4D)."

Comment 9:
Identification of a repressor of an entire gene family is exciting however it is unusual to use a cocktail of four genes (Id1-4) to elicit an effect. Using this approach, it remains unclear whether Id genes are additive or redundant in this context. Are all 4 Id genes over-expressed in every cell? Although admittedly non-trivial, over-expression or knockdown of Id genes 1-4 individually would be very informative.

Reply:
We examined the effects of overexpression of single Id genes on progenitor proliferation and astrocyte differentiation in supplementary experiments and found that Id genes show functional redundancy in terms of progenitor proliferation and astrocyte differentiation (Supplemental Fig. S18). As shown in Figure S19, we have already performed single Id knockdown experiments, which showed that it does not rescue the phenotypes observed in Rp58 KO mice.

We have added the following sentences to the revised manuscript: (page 14, line 28)
"In fact, we found that cortices electroporated with the single *Id* showed similar phenotypes to those electroporated with all four *Ids*. Quantitative analysis of $Ki67^+/GFP^+$ progenitors (13.9% ± 3.7%) and $GFAP^+/GFP^+$ astrocytes (7.4% ± 1.6%) in the control cortices revealed the distribution of electroporated cells. Embryos electroporated with a single *Id* vector showed significantly more $Ki67^+/GFP^+$ progenitors (*Id1*: 49.0% ± 6.5%, *Id2*: 46.3% ± 9.4%, *Id3*: 51.8% ± 8.6%, *Id4*: 45.9% ± 3.6%) and $GFAP^+/GFP^+$ astrocytes (*Id1*: 48.3% ± 2.0%, *Id2*: 41.8% ± 3.3%, *Id3*: 47.0% ± 7.5%, *Id4*: 41.4% ± 10.0%) (Supplementary Figure 18)."

Comment 10:
Authors should be certain that these are not tumor cells. Expression of Id, GFAP, Ki67 and Sox2 is associated with brain tumors and authors indicate that RP58 expression has a tumor suppressive effect. Transformation assays could be conducted to determine the oncogenic potential of Rp58-/- cells. For example, do isolated cells form tumors in immunocompromised mice? Are they growth factor independent? Do they grow in soft-agar?

Reply:
In accordance with the reviewer's suggestion, we have performed soft agar colony formation assays to evaluate the progenitor cells collected from *Rp58* KO mice. We confirmed that mutant derived progenitors were not able to proliferate under no cell anchoring condition (Table 4 and Supplemental Fig. 20).

We have added the following sentences to the revised manuscript: (page 17, line 16)
"However, progenitors derived from the Rp58 KO cortex were unable to form colonies in soft agar, suggesting that Rp58 single KO is insufficient to initiate cancer (Table S4 and Supplementary Figure 20)."

Referee #3

In this manuscript Hirai et al have nicely shown that using RP58 knockout mice and a number of experimental techniques, RP58 directly inhibits the expression of Id1-4, which negatively regulate neural development. Authors have also shown that p57, a cell cycle inhibitor, is an essential downstream target of Id genes in NSC.

Comment 1:

Authors have cultured Lv-RP58- and Lv-GFP-infected embryonic NSC in EGF alone and examined their proliferation. However, since bFGF, rather than EGF, is the main mitogen for embryonic NSC, it is important to examine the effect of RP58 overexpression in Lv-RP58- and Lv-GFP-infected embryonic NSC cultured in bFGF alone and a combination of bFGF and EGF.

Reply:
We thank the reviewer for this interesting suggestion. We cultured NSC with bFGF alone and with a combination of bFGF and EGF, and found that RP58 has similar effects on embryonic NSC proliferation under both conditions. In all cases, RP58 inhibited the proliferation of neurosphere cells (Supplemental Fig. S8).

We have added the following sentences to the revised manuscript: (page 8, line 25)
"The dissociated primary neurospheres were then replated to analyze their self-renewal competency. Few secondary neurospheres were formed from the Lv-*Rp58*-infected neurospheres (5.67±4.04), whereas control Lv-GFP-infected cells formed approximately 200 secondary neurospheres (202.33±17.5; Fig. 4D)."

Comment 2:
To confirm that RP58 induces cell-cycle exit by inhibiting the expression of Id genes, it is nice to examine whether overexpression of both RP58 and Ids maintains NSC proliferation.

Reply:
In accordance with the reviewer's suggestion, we overexpressed both RP58 and Ids in neurospheres and found that they partially rescued the decrease in sphere diameter (Supplemental Fig. S9).

We have added the following sentences to the revised manuscript: (page 9, line 6)
"Lv-Rp58-infected neurospheres lost their competency for self-renewal, which was partially rescued by the co-induction of each individual *Id,* or all four *Ids*, along with RP58 (Supplementary Figure 9)."

Comment 3:
Since it is known that the expression of p16/Ink4a is regulated by Id1, authors should check the expression of Ink4 family members in the RP58-null NSC.

Reply:
In accordance with the reviewer's suggestion, we reanalyzed the results of the microarray experiments and have included data for the Ink4 family (including p16) in Supplemental Table 3. We found no significant differences in the expression of INK4 family members between WT and Rp58 KO mice.

Comment 4:
Authors have used TBR1 to identify the differentiated neurons. To confirm neuronal differentiation in RP58-null brain, it is important to show the expression of other mature neuronal markers, such as neurofilament and GAD.

Reply:
We previously reported the expression of other mature neuronal markers, including neurofilaments, in Rp58 KO mice (Dev. Biol. 331: 140-151). Regarding GAD, our colleagues have already shown that there is no difference in GAD expression patterns between WT and KO mice (Ohtaka-Maruyama et al.; *in preparation*). In the present study, we only used TBR1 to label the neuronal lineage cells.

