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Cyclin D2 in the basal process of neural progenitors is linked to non-equivalent cell fates

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

1st Editorial Decision 20 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 enclosed. As you will see, all three reviewers express interest in your proposal that asymmetric inheritance of cyclinD2 regulates cell fate in neuronal progenitors. However, referees 2 and 3 raise a number of critical concerns with the study that would need to be addressed before we could consider publication of your work.

The most critical point here is how you can be sure which daughter cell is inheriting the basal process - and hence cyclinD2 - and what its subsequent fate will be. Currently, your data here are largely correlative and make major assumptions as to the relationship between position, basal process inheritance and fate. Referee 2 in particular finds this to be a major problem, and I have to say that I agree with many of his/her points (#2). He/she argues that the best way of addressing this would be by live imaging, and again I do agree here. I would therefore strongly encourage you to undertake live imaging experiments (in slice cultures) to analyse mitosis, basal process inheritance, cell position and - as far as is possible - subsequent fate. I realise that there are significant technical challenges here, and if you are unable to do this, please get in touch so that we can discuss how to proceed.

In addition, referees 2 and 3 highlight several other issues that need to be dealt with experimentally. Revising your manuscript accordingly will entail a large amount of work, but given the potential interest here, we would like to invite you to submit a revised version of the manuscript, if you are able to take this on. I should add that it is EMBO Journal policy to allow only a single round of revision, and acceptance of your manuscript will therefore depend on the completeness of your responses in this revised version. When preparing your letter of response to the referees' comments, please bear in mind 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

We generally allow three months as standard revision time. As a matter of policy, competing manuscripts published during this 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. I can imagine that - in order to do the live imaging work - you may need longer than this normal three months, and we should be able to grant you an extension. I suggest you just contact me closer to the official 3 month deadline to let me know your progress.

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

This work addresses an important question - the molecular control of asymmetrically fated divisions in the vertebrate CNS - and delivers very interesting new data that suggests asymmetric inheritance of CyclinD2 RNA via the basal process of the progenitor may well regulate the fate of one daughter. Several recent papers have suggested that inheritance of the basal process of neural progenitors may be important for regulating daughter cell fate, and this work suggests a molecular mechanism for this. The weakness of the paper is that because the work relies on analysis of fixed samples it is very difficult to be confident of the precise fate of the daughter cells in which the authors analyse CyclinD2 distribution (e.g. in Figure 5). Nonetheless I think the data raises interesting ideas and will be an important paper in the field. Because of the doubts about precise daughter cell fate, I think the authors should soften their conclusions and admit that their data suggests that unequal inheritance of CyclinD2 via the basal can influence daughter cell fate, rather than claiming that they have actually proven this point. I think the title should be adjusted also to cover this - perhaps something like "Unequal distribution of CyclinD2 via the basal process of neural progenitors may bias daughter cell fate".

Referee #2

In this manuscript, Tsunekawa et al. reported that asymmetric localization and local translation of Cyclin D2 mRNA in the basal process of radial glial progenitor cells regulates their asymmetric division in the developing neocortex. While this is an intriguing idea and the manuscript is well written, I found that the data do not convincingly support the major conclusion of the study.

Major concerns:

1. While the authors provided some evidence of the cD2 Ab specificity, the staining pattern of cD2 was fundamentally different from the previous studies (e.g. Glickstein et al., J. Neurosci. 2009). In the previous study, cD2 is shown to be mostly expressed in the SVZ cells. However, in the current study, cD2 is suggested to be expressed mostly in the VZ radial glial progenitor cells. Overall, the cD2 staining data in this study were not very consistent (Fig. 1E,G, I; Fig. 4 A-M and Fig. 5) and therefore not very convincing. For example, what were the cells with strong staining in Fig. 1I above the basal membrane? Why did EGFP and cD2 staining have a similar fragmentation pattern in Fig. 1 M-N? If cD2 expresses in the basal processes of most VZ radial glial progenitors (even if not expressed at the cell body), should the authors expect to observe more basal process staining Fig. 1 K-N? What is the strong staining at the VZ surface in Fig. 5 B-C?

2. Fig. 4 and 5 represented the core set of data that directly supported the author's major conclusion of the polarized distribution and inheritance of cD2 mRNA/protein. However, the authors made many assumptions in the interpretation of the data:
a. In most of the images, it is hard to reconstruct the entire morphology of the cells of interest. Particularly, it is hard to know which cell possesses the basal process. The authors always assume the upper (i.e. basally-positioned) cell is the cell possessing the basal process and adopting renewing radial glial progenitor cell fate. While this might be the case when two daughter cells are just born at the ventricular zone surface (Konno et al., 2008), the relative basal/apical position of two daughter cells could change as development proceeds, as the cell bodies of both cells migrate radially with different speed (Tsai J.-W. et al., Nat Neurosci 2010). For example, based on the images it is impossible to figure out which cell possesses the basal and apical process in Fig. 4 K-N'. Similarly, in Fig. 4 G-J', one keeps wondering how it could be possible that the authors know with which cell the broken pieces of basal process associate.
b. The real key experiment is to examine the inheritance of cD2/the basal process in dividing progenitor cells and then correlate the inheritance pattern with fate specification. However, the authors just glossed over the M-phase analysis, and simply used phospho-Vimentin staining (Fig. 4 C-F'). Even in these images, it is unclear where the high-magnification images (Fig. E-F') were from.
Given that the authors were able to achieve single cell fluorescence labeling using lentivirus, to fundamentally address the issue the authors should perform time-lapse imaging to trace the mitosis of individual progenitor cells and then carry out cD2 staining and/or cell fate marker staining to directly link cD2 inheritance and asymmetric cell fate specification. Furthermore, given that the authors had the cD2-/- mice, they should perform the same analysis in the mutant mice to address the functional significance of asymmetric inheritance of cD2.

Minor concerns:
1. Fig. 6: It is unclear why the authors combined SVZ and IZ in quantification. Given that these two regions harbor different types of cells, this is rather uncommon in the field.
2. Fig. 7: It is not convincing that cD2 is expressed in the basal processes of the oSVZ cells. Overall the human cortex part is a bit over-stretched.
3. Page 21: line 18 "site" instead of "sight"?

Referee #3

Tsunekawa et al. investigate the role of cyclinD2 (cD2) in mouse neural development. The authors provide a detailed analysis of cD2 mRNA localization and protein expression showing that cD2, in contrast to other D-cyclins, mRNA is present not only in the soma of neural progenitors but also in their pial endfeet. In vivo electroporation of GFP fusion constructs with cD2 3'UTRs allowed the identification of a minimal domain essential for the correct mRNA distribution while overexpression and RNAi in vivo experiments show that cD2 is important to control neurogenesis. The authors propose that the localization of cD2 mRNA is important to guarantee its asymmetric distribution upon division of a mother cell to only one daughter progenitor cell, the one inheriting the basal enndfoot and self-renewing.

To be precise, showing i) transport of cD2 mRNA, ii) a correlation between cD2 inheritance and neurogenesis, and ii) perturbation of neurogenesis upon manipulation cD2 is not formally proving the authors' hypothesis because G1-cyclins may still influence neurogenesis independently from endfoot localization of their mRNAs. Perhaps the authors should discuss this and moderate some of their perhaps too strong conclusions. Nevertheless, this is a very novel hypothesis and interesting piece of work which elegantly integrates asymmetric distribution of a cell cycle regulator with asymmetric cell division and fate.

While similar mechanisms were described in invertebrates, most notably drosophila, to the best of my knowledge, this is the first work clearly showing the specific subcellular distribution of a mRNA that is important for cell fate change and its asymmetric inheritance in mammalian neural stem cells (perhaps Schwamborn et al., 2009 being an exception that the authors may wish to discuss). This work is also very timely and of broad interest because an increasing literature is being produced indicating that cell cycle length and, specifically G1 (which is regulated by cD1 and 2), controls differentiation of neural and also other somatic stem cells. Here Tsunekawa et al. can provide a first

insight into this process at the cellular and mechanistic level. With a few exceptions, the manuscript is also well written and the experiments well performed.

Altogether, while formal prove of this hypothesis may still require several years of work, I believe that this manuscript contains enough intellectual novelty and robust evidence to deserve prompt attention and diffusion and I am very confident that many scientists in various fields will find the information provided valuable.

Yet, I also think that this manuscript requires a few major improvements before publication in a prestigious journal is being considered.

1) The key novelty of this work is that basal-cD2 mRNA may be critical for cell fate change. RNAi of cD2 shows increased neurogenesis. This is consistent with the authors hypothesis but has already been shown by others. The missing link is that the authors have not shown that RNAi can deplete the basally localized mRNA of endogenous cD2. Panels in Fig S7 are cut at the level of the IZ and in situ hybridizations are not shown. It is in principle possible that shRNAs, which are efficient in depleting cD2 in the VZ, could sill not reach the cell's endfoot several microns away and that basal-cD2 mRNA is unperturbed. This can disprove the authors' hypothesis and should be addressed.

Interpreting overexpression data becomes even more difficult. The authors state that cD2 is present in G2-M (depicted in Fig 4P) but this cannot be the case because G1-cyclins (D/E) are known to be degraded prior to S. Frankly, while controls for this antibody are very good (Fig S1), I have problems to define nuclei/soma of cells in Fig 4B and F as positive. I tend to believe instead that the threshold used by the authors is simply too low and these may be negative cells. importantly, if this should be true, it may even strengthen the authors' hypothesis because it would emphasize the role of the basal-cD2 that is "protected away" from ubiquitination and S degradation.

A number of questions about overexpression also arise. Is the overexpressed cD2 mRNA also localized to the endfoot? Is cD2 protein in the cell body/nucleus also degraded in S phase as the endogenous cD2 is? These two points are very important because a given combination of answers may invalidate the authors' hypothesis and addressing them only requires a few stainings. Also, it is unclear to me whether the electroporated cD2 construct contains its 3'UTR. Even with 3'UTR, ISH should be shown to demonstrate proper localization.

2) It is important that the authors make some point clear. For examples, cD2 is said to be actively transported to the endfoot. I believe that this is convincingly demonstrated but a superficial reader may ask why is passive diffusion excluded. Differences between GFP and RFP in Fig 2 and cD1/3 and cD2 in Fig S2 may still be due to differences in expression levels. This is clearly not the case because deletion experiments show a clear specificity of the 3'UTR domain (Fig S3). However, these data are relegated to the supplement and diffusion is not even mentioned in the manuscript.

Moreover, cD2 mRNA may be accumulated to the endfoot because of a miRNA titrating down cD2 mRNA in the soma. This can be very interesting and the authors may comment on that. Did they check the cD2 3'UTR for mouse miRNAs?

More importantly, a substantial set of data are based on the identification of cell in G1/S/G2/M. The authors write "A transfection protocol was employed to express EGFP in the basal process and immunostaining for Cyclin D2 was conducted to detect protein distribution at different stages of the cell cycle (Fig. 4)". What is this protocol about? How could the authors be sure that a given cell is in G2 rather than G1? Did they do BrdU/EdU experiments to corroborate their criteria? Is the proportion of cells in G1/S/G2/M representing the relative length of these phases known from the literature? Without convincing evidence about the validity of the authors' criteria, data in this figure cannot be interpreted. Moreover, it seems form Fig 4 that the authors find many single cells after 24 hours but all infected cells should have divided at least once so they should not find any single cell...

3) I cannot understand why have the authors merged SVZ and IZ in their analysis of cell fate (Fig 6). These two layers contain very different cell types, dividing intermediate progenitors and migrating postmitotic neurons. By mixing them the authors may lose significance. Splitting may show, for example, a more substantial increase in intermediate progenitors and even stronger inhibition of neurogenesis. Very similar experiments making this distinction were described in

Lange et al., 2009. I feel that the authors may be missing an important occasion to detect an even stronger phenotype.

Minor comments.

4) Introduction: since this is a work in mouse, it would be nice to correlate the steps in corticogenesis with gestation days i.e. onset of neurogenesis (E11) and so forth.

5) Throughout the manuscript we often find "endfoot of neuroepithelial cells" while at the stages being analyzed one should refer to them more as radial glia.

6) The authors point out an inconsistency about cD2 being in intermediate progenitors or apical progenitors (Glickstein versus current work). While this is not a major point, quantifications of cD2 and Tbr2/Sox2 may corroborate the explanation they provide.

7) The authors state that BrdU data upon overexpression of cD2 (Fig S8) suggest that "neurons may have been forced to divide". First, BrdU immunolabeling is no evidence for mitosis and, second, despite all the manipulations performed in the history of science neurons were never seen to complete a mitosis. Thus, this ectopic BrdU is more likely due to delamination of progenitors and/or increase in the abundance of the so-called outer-radial glia (oRG) which, while very modest, has also been described in mouse (Wang et al 2011). These cells preserve a basal endfoot... perhaps containing cD2 mRNA.

8) References are not always optimal: division of intermediate progenitors in the SVZ was described by Noctor, Haubensak, and Miyata in 2004, and certainly not by the subsequent Kosodo, Attardo or Kowalczyk works (see intro). Some references are also out-dated. Taking asymmetric cell division and cell cycle as examples one may think that the relatively old reviews by Fishell & Kriegstein and Ohnuma & Harris 2003 should be complemented (or replaced) by more recent reviews such as Kosodo & Huttner 2009 and Salomoni and Calegari 2010 (and/or Lange & Calegari 2010 concerning cyclins in stem cell differentiation). Later in discussion, Fietz et al. and Hansen et al. 2010 should be accompanied by the independent and equally nice report on ferrets by Reillo at al. 2011 (but published online in 2010). Again, these three works seems very incomplete without the following up paper in mouse by Wang et al. 2011. Perhaps, Schwambron et al. 2009 may be discussed in the frame of asymmetric inheritance and m(i)RNAs.

9) Figures: the nuclei in the top part of Fig 1I are confusing, the brain should finish with the basal lamina like the other panels. Fig 2 should include data from S3 because these are too important to burry in the supplement (discussed above). This figure may then become too crowded. Perhaps the diagram of electroporation (which is a copy-and-paste from the previous Inoue & Osumi 2001 without proper reference!) could go to the supplement. I believe that the readers from this field are fully aware of this technique, which the Osumi lab has pioneered. A little inset showing basal lamina and endfeet should be shown in all figures including Fig 6 and S7.

1st Revision - authors' response 02 January 2012

Referee #1:

This work addresses an important question - the molecular control of asymmetrically fated divisions in the vertebrate CNS - and delivers very interesting new data that suggests asymmetric inheritance of CyclinD2 RNA via the basal process of the progenitor may well regulate the fate of one daughter. Several recent papers have suggested that inheritance of the basal process of neural progenitors may be important for regulating daughter cell fate, and this work suggests a molecular mechanism for this. The weakness of the paper is that because the work relies on analysis of fixed samples it is very difficult to be confident of the precise fate of the daughter cells in which the authors analyse CyclinD2 distribution (e.g. in Figure 5). Nonetheless I think the data raises interesting ideas and will be an important paper in the field. Because of the doubts about precise daughter cell fate, I think the authors should soften their conclusions and admit that their data suggests that unequal inheritance of CyclinD2 via the basal can influence daughter cell fate, rather than claiming that they have actually proven this point. I think the title should be adjusted also to cover this - perhaps

something like "Unequal distribution of CyclinD2 via the basal process of neural progenitors may bias daughter cell fate".

Response: We appreciate the supportive comments of this Referee in favor of acceptance but have asked for a more balanced title to reflect our data and conclusions. In response, we now change our title to:
"Cyclin D2 in the basal process of neural progenitors is linked to non-equivalent cell fates"

Referee #2:

1) While the authors provided some evidence of the cD2 Ab specificity, the staining pattern of cD2 was fundamentally different from the previous studies (e.g. Glickstein et al., J. Neurosci. 2009). In the previous study, cD2 is shown to be mostly expressed in the SVZ cells. However, in the current study, cD2 is suggested to be expressed mostly in the VZ radial glial progenitor cells. Overall, the cD2 staining data in this study were not very consistent (Fig. 1E,G, I; Fig. 4 A-M and Fig. 5) and therefore not very convincing. For example, what were the cells with strong staining in Fig. 1I above the basal membrane? Why did EGFP and cD2 staining have a similar fragmentation pattern in Fig. 1 M-N? If cD2 expresses in the basal processes of most VZ radial glial progenitors (even if not expressed at the cell body), should the authors expect to observe more basal process staining Fig. 1 K-N? What is the strong staining at the VZ surface in Fig. 5 B-C?

Response: We do not perceive there to be major differences between our observations and those of Glickstein et al. (2009). We too observe Cyclin D2 localization in basal progenitors in the subventricular zone (SVZ) (arrowheads in the old Fig. 1I), but the point of difference is our focus on the basal processes of VZ cells. The cells with strong staining for Cyclin D2 above the basement membrane belong to the cranial mesenchyme outside the neuroepithelium. To avoid confusion, we now provide a new figure to focus expression within radial glia (Fig. 1I). Fig. 1M and Fig. 1N are from the same sample, showing that Cyclin D2 protein is localized in the basal process that is also labeled with EGFP-lentivirus. The fragmented staining referred to by the Referee is due to discontinuous basal processes captured in confocal Z-stacks of only 1 mm distance. In contrast, Fig. 4A is a z-stuck confocal image of 20 mm distance showing continuous staining for Cyclin D2 in the basal process. In response to the Referee's query on Fig. 5B-C, the strong staining is from Cyclin D2 present in apical endfeet of radial glial cells. We are pleased with the Referee's criticisms because in response, we provide better quality images in Figs. 1, 4, 5 to illustrate our points, and add description about apical staining (p.35, line 1).

2) Fig. 4 and 5 represented the core set of data that directly supported the author's major conclusion of the polarized distribution and inheritance of cD2 mRNA/protein. However, the authors made many assumptions in the interpretation of the data:

a) In most of the images, it is hard to reconstruct the entire morphology of the cells of interest. Particularly, it is hard to know which cell possesses the basal process. The authors always assume the upper (i.e. basally-positioned) cell is the cell possessing the basal process and adopting renewing radial glial progenitor cell fate. While this might be the case when two daughter cells are just born at the ventricular zone surface (Konno et al., 2008), the relative basal/apical position of two daughter cells could change as development proceeds, as the cell bodies of both cells migrate radially with different speed (Tsai J.-W. et al., Nat Neurosci 2010). For example, based on the images it is impossible to figure out which cell possesses the basal and apical process in Fig. 4 K-N'. Similarly, in Fig. 4 G-J', one keeps wondering how it could be possible that the authors know with which cell the broken pieces of basal process associate.

Response: We thank the Referee for this very pertinent criticism, but also agree with the Referee that morphological association of the basal process with a given cell is not trivial. We also agree that position of the daughter cell *per se* is not an indicator for future self-renewal. These points accepted, we wish to emphasize that we consistently observe the inheritance of the basal process by the basally-positioned daughter cell. This point is further demonstrated by a 3-D reconstruction movie showing the basally-localized daughter cell inheriting its basal process (Supplementary movie1) and add description about 3D construction (p.10, line 1).

b) The real key experiment is to examine the inheritance of cD2/the basal process in dividing progenitor cells and then correlate the inheritance pattern with fate specification. However, the authors just glossed over the M-phase analysis, and simply used phospho-Vimentin staining (Fig. 4 C-F'). Even in these images, it is unclear where the high-magnification images (Fig. E-F') were from.
Given that the authors were able to achieve single cell fluorescence labeling using lentivirus, to fundamentally address the issue the authors should perform time-lapse imaging to trace the mitosis of individual progenitor cells and then carry out cD2 staining and/or cell fate marker staining to directly link cD2 inheritance and asymmetric cell fate specification. Furthermore, given that the authors had the cD2-/- mice, they should perform the same analysis in the mutant mice to address the functional significance of asymmetric inheritance of cD2.

Response: Regarding Fig. 4, since C and D are Z-stuck images (total 20 mm thickness) and Figs. E-F' are more thinner ones (2 mm thickness), their appearance may be slightly different. However, as same as other figures in Fig. 4, Figs. 4E-F' are high-magnification images of a centerly positioned cell body and a basal endfoot shown in Fig. 4C. We have clarified this relationship in the Legends (p. 34, line 5).

We agree with this Referee that one way to capture the 'smoking gun' would be to conduct time-lapse experiments to correlate basal process inheritance with daughter cell fate. We will certainly pursue this as a separate study but for the purpose of this manuscript, we provide supporting data in the form of a 3D-reconstruction movie (Supp Movie 1). However, the suggested experiments are fraught with difficulties, and potentially difficult to interpret: e.g. correctly allocating ownership of a given basal process, in stained sections, with a dividing cell body identified in time-lapse movies. We also feel that this demand by the Referee raises the bar unfairly somewhat, given the knowledge of the field gained from techniques similar to ours (Kosodo et al., 2008).

Minor concerns:
1. Fig. 6: It is unclear why the authors combined SVZ and IZ in quantification. Given that these two regions harbor different types of cells, this is rather uncommon in the field.

Response: To address this concern, we have reclassified the cells in the VZ, SVZ, IMZ/OSVZ, and CP according to the criteria of Lange & Calegari (2010), and revised Fig. 6 and related text in the Results (p. 15, line 7 to p.16 line 6) and Legends (p. 35, line 12).

2. Fig. 7: It is not convincing that cD2 is expressed in the basal processes of the oSVZ cells. Overall the human cortex part is a bit over-stretched.

Response: Given the scarcity of human tissue, we feel that inclusion of Cyclin D2 staining in human tissue is complementary to the overall study. It was never intended to parallel our analysis in mouse tissue, and hence descriptive in nature. We note other studies describing staining of different antigens in human embryonic cortical tissue are couched in similar terms (e.g. Fietz et al., 2010; Hansen et al. 2010).

3. P. 22: line 15 "site" instead of "sight"?

The typo was revised in the text (p. 22, line 19).

Referee #3:

To be precise, showing i) transport of cD2 mRNA, ii) a correlation between cD2 inheritance and neurogenesis, and ii) perturbation of neurogenesis upon manipulation cD2 is not formally proving the authors' hypothesis because G1-cyclins may still influence neurogenesis independently from endfoot localization of their mRNAs. Perhaps the authors should discuss this and moderate some of their perhaps too strong conclusions. Nevertheless, this is a very novel hypothesis and interesting piece of work which elegantly integrates asymmetric distribution of a cell cycle regulator with asymmetric cell division and fate.

Response: We thank the Referee for this insight, and have accordingly discussed alternative interpretations of our findings under the Discussion, i.e. the possibility that perturbation of cyclin D2 may affect neurogenesis via non-endfeet mediated mechanisms. This will be addressed in our future time-lapse experiments suggested by Referee 2, which we feel lies outside the scope of the present work. As noted by this Referee, our work sets a new benchmark for the field by integrating the basal inheritance of a cell-cycle regulator with asymmetric cell division.

While similar mechanisms were described in invertebrates, most notably drosophila, to the best of my knowledge, this is the first work clearly showing the specific subcellular distribution of a mRNA that is important for cell fate change and its asymmetric inheritance in mammalian neural stem cells (perhaps Schwamborn et al., 2009 being an exception that the authors may wish to discuss). This work is also very timely and of broad interest because an increasing literature is being produced indicating that cell cycle length and, specifically G1 (which is regulated by cD1 and 2), controls differentiation of neural and also other somatic stem cells. Here Tsunekawa et al. can provide a first insight into this process at the cellular and mechanistic level. With a few exceptions, the manuscript is also well written and the experiments well performed.

Response: Again, we concur with the Referee regarding the novelty of our work showing asymmetric subcellular mRNA localication as a mechanism for determining cell fate. We have included Schwamborn et al (2009) in our Discussion (p. 18, line 4).

Altogether, while formal prove of this hypothesis may still require several years of work, I believe that this manuscript contains enough intellectual novelty and robust evidence to deserve prompt attention and diffusion and I am very confident that many scientists in various fields will find the information provided valuable.

Response: We are encouraged by this Referee's remark concerning the intellectual niche occupied by this manuscript.

Yet, I also think that this manuscript requires a few major improvements before publication in a prestigious journal is being considered.

According to the constructive suggestions made by this reviewer, we revised the manuscript as follows:

1) The key novelty of this work is that basal-cD2 mRNA may be critical for cell fate change. RNAi of cD2 shows increased neurogenesis. This is consistent with the authors hypothesis but has already been shown by others. The missing link is that the authors have not shown that RNAi can deplete the basally localized mRNA of endogenous cD2. Panels in Fig S7 are cut at the level of the IZ and in situ hybridizations are not shown. It is in principle possible that shRNAs, which are efficient in depleting cD2 in the VZ, could sill not reach the cell's endfoot several microns away and that basal-cD2 mRNA is unperturbed. This can disprove the authors' hypothesis and should be addressed.

Response: To address this concern, we now include new *in situ* hybridization data to demonstrate changes in *Cyclin D2* mRNA following gain/loss of function experiments. Thanks to this reviewer, we now clearly show effects of the gain/loss experiments in Fig. S7B-E and insets of S7F-I. Notably, knockdown of Cyclin D2 almost vanished localization of mRNA and protein of Cyclin D2 from the basal endfoot, which had a greater impact on the fate of neural progenitor compared with overexpression (Fig. S7O). We believe this revision indeed supports our hypothesis (p. 19, line 8).

2) Interpreting overexpression data becomes even more difficult. The authors state that cD2 is present in G2-M (depicted in Fig 4P) but this cannot be the case because G1-cyclins (D/E) are known to be degraded prior to S. Frankly, while controls for this antibody are very good (Fig S1), I have problems to define nuclei/soma of cells in Fig 4B and F as positive. I tend to believe instead that the threshold used by the authors is simply too low and these may be negative cells. importantly, if this should be true, it may even strengthen the authors' hypothesis because it would emphasize the role of the basal-cD2 that is "protected away" from ubiquitination and S degradation.

Response: Cyclin D2 is known to be degraded by the SCF (Skp1, cullin, F-box) complex that degrades G1-cyclin in a cell-cycle independent manner (reviewed in Murray, Cell, 2004) and not by

an anaphase promoting complex that degrades Cyclin B and A in a cell-cycle specific manner. Previous studies suggested Cyclin D1 is expressed in S-G2 phase of proliferating cells (Guo et al., 2002), and we believed that the jury is still out regarding the expression of Cyclin D2 in the cycling neuroepithelium. The pertinent point for us is that we have carried out our experiments *in vivo*, by gene electroporation into the cortical primordium, and we observed nuclear expression of Cyclin D2 in cortical progenitors not only in G1 phase but also in G2-phase and M-phase. We concede that Cyclin D2 expression during the latter two phases was weaker but still discernible above background. As noted by this Referee, this may also alert the reader to the novelty of our hypothesis.

A number of questions about overexpression also arise. Is the overexpressed cD2 mRNA also localized to the endfoot? Is cD2 protein in the cell body/nucleus also degraded in S phase as the endogenous cD2 is? These two points are very important because a given combination of answers may invalidate the authors' hypothesis and addressing them only requires a few stainings. Also, it is unclear to me whether the electroporated cD2 construct contains its 3'UTR. Even with 3'UTR, ISH should be shown to demonstrate proper localization.

Response: The overexpression experiments were performed with plasmid containing Cyclin D2 ORF without 3' UTR, which therefore resulted in generalized Cyclin D2 overexpression in the VZ. We heed the referee's point about ISH and now present new data to display the spatial localization of mRNA (Fig. S7B-E). In addition, since the expression vector we had a robust promoter activity in the cortical progenitor cells, approximately 90% of VZ cells strongly expressed Cyclin D2 (Fig. S7C). It is thus very likely that this overexpressed Cyclin D2 stays in all phases of cell cycle. By doing this, we successfully disrupted natural asymmetric localization patterns of Cyclin D2 among daughter cells, and cause hyperproliferation of cortical progenitor cells.

In revision of Fig. S7, we also show *Cyclin D2* mRNA and protein at the basal side (Fig. S7B-E, insets of F-I). These data further demonstrate diminished localization of *Cyclin D2* mRNA and protein in the loss-of-function condition (Fig. S7E, inset of I), which resulted in precocious neuronal differentiation (Fig. S7O). According to the revised figure, the corresponding text was rewritten (p. 14, line 5).

2) It is important that the authors make some point clear. For examples, cD2 is said to be actively transported to the endfoot. I believe that this is convincingly demonstrated but a superficial reader may ask why is passive diffusion excluded. Differences between GFP and RFP in Fig 2 and cD1/3 and cD2 in Fig S2 may still be due to differences in expression levels. This is clearly not the case because deletion experiments show a clear specificity of the 3'UTR domain (Fig S3). However, these data are relegated to the supplement and diffusion is not even mentioned in the manuscript.

Response: We now discuss the point about diffusion in the revised text (p. 8, line 7).

Moreover, cD2 mRNA may be accumulated to the endfoot because of a miRNA titrating down cD2 mRNA in the soma. This can be very interesting and the authors may comment on that. Did they check the cD2 3'UTR for mouse miRNAs?

Response: This is a very interesting idea that had not occurred to us. Certainly we are prepared to entertain the possibility that differential miRNA activity may titrate down the cyclin D2 mRNA closer to the soma. There are a few potential miRNA that are differentially expressed during cortical neurogenesis (Shibata et al., 2011, Gaughwin et al., 2011). We searched the database and found several potential target sites against mir-1192, mir-15b, mir-195 etc. However, these miRNA are not long enough to carry a localization signal, and because of this, we believe that miRNAs (if indeed they are capable of degrading cyclin D2 mRNA) may be localized mostly in the soma. This implies that some *Cyclin D2* mRNA might be trapped by these miRNAs and degraded closer to the soma. Eventually, *Cyclin D2* mRNA that escapes from the soma by a transporting machinery, including a 50 bp element that we have identified, might accumulate within the basal endfoot. The confirmation of whether *Cyclin D2* mRNA is degraded by these miRNA is outside the scope of this study, but this could work in parallel with the basal transportation system that we have identified. This is a very interesting discussion and we have included it in the text (p. 19, line 21).

More importantly, a substantial set of data are based on the identification of cell in G1/S/G2/M. The authors write "A transfection protocol was employed to express EGFP in the basal process and immunostaining for Cyclin D2 was conducted to detect protein distribution at different stages of the cell cycle (Fig. 4)". What is this protocol about? How could the authors be sure that a given cell is in G2 rather than G1? Did they do BrdU/EdU experiments to corroborate their criteria? Is the proportion of cells in G1/S/G2/M representing the relative length of these phases known from the literature? Without convincing evidence about the validity of the authors' criteria, data in this figure cannot be interpreted. Moreover, it seems form Fig 4 that the authors find many single cells after 24 hours but all infected cells should have divided at least once so they should not find any single cell...

Response: We admit that this is clearly a case of the meaning being lost in translation! We do not have a protocol that can differentially express GFP in the basal process; or detect GFP in G2 rather than G1. To avoid confusion, we now refrain from claiming detection at G2-phase from Fig. 4.

3) I cannot understand why have the authors merged SVZ and IZ in their analysis of cell fate (Fig 6). These two layers contain very different cell types, dividing intermediate progenitors and migrating postmitotic neurons. By mixing them the authors may lose significance. Splitting may show, for example, a more substantial increase in intermediate progenitors and even stronger inhibition of neurogenesis. Very similar experiments making this distinction were described in Lange et al., 2009. I feel that the authors may be missing an important occasion to detect an even stronger phenotype.

Response: We thank the Referee for this suggestion, and accordingly in the revised manuscript, re-submit our data following Cyclin D2 gain/loss of function into different zone categories (Figure 6) (p. 15, line 7 to p.16 line 6 for Results and p. 35, line 12 for Legends).

Minor comments.

4) Introduction: since this is a work in mouse, it would be nice to correlate the steps in corticogenesis with gestation days i.e. onset of neurogenesis (E11) and so forth.

We have added gestation days in the text as suggested by this reviewer (p. 3, lines 4 and 10).

5) Throughout the manuscript we often find "endfoot of neuroepithelial cells" while at the stages being analyzed one should refer to them more as radial glia.

The terminology has been changed into “endfoot of radial glia” throughout the text.

6) The authors point out an inconsistency about cD2 being in intermediate progenitors or apical progenitors (Glickstein versus current work). While this is not a major point, quantifications of cD2 and Tbr2/Sox2 may corroborate the explanation they provide.

Inconsistency about Cyclin D2 in intermediate progenitors and apical progenitors is not a major claim in our study, and thus we omitted the issue from the Discussion.

7) The authors state that BrdU data upon overexpression of cD2 (Fig S8) suggest that "neurons may have been forced to divide". First, BrdU immunolabeling is no evidence for mitosis and, second, despite all the manipulations performed in the history of science neurons were never seen to complete a mitosis. Thus, this ectopic BrdU is more likely due to delamination of progenitors and/or increase in the abundance of the so-called outer-radial glia (oRG) which, while very modest, has also been described in mouse (Wang et al 2011). These cells preserve a basal endfoot... perhaps containing cD2 mRNA.

We apologize for the misleading phrasing. Although neurons may divide in a genetically engineered condition (Ajioka, Cell, 2007), this is not the case for our study in normal corticogenesis. Considering the importance of oRGs, we have mentioned oRGs according to this reviewer’s suggestion, referred a paper by Wang et al. (2011) (p. 15, line 11), added the term “outer sub-ventricular zone (OSVZ)” to Fig. 6 and SFig. 8, and revised the related text in Results (p. 15, line 7 to p.16 line 6) and Legends (p. 35, line 19 and p. 45, line 16).

8) References are not always optimal: division of intermediate progenitors in the SVZ was described by Noctor, Haubensak, and Miyata in 2004, and certainly not by the subsequent Kosodo, Attardo or Kowalczyk works (see intro). Some references are also out-dated. Taking asymmetric cell division and cell cycle as examples one may think that the relatively old reviews by Fishell & Kriegstein and Ohnuma & Harris 2003 should be complemented (or replaced) by more recent reviews such as Kosodo & Huttner 2009 and Salomoni and Calegari 2010 (and/or Lange & Calegari 2010 concerning cyclins in stem cell differentiation). Later in discussion, Fietz et al. and Hansen et al. 2010 should be accompanied by the independent and equally nice report on ferrets by Reillo at al. 2011 (but published online in 2010). Again, these three works seems very incomplete without the following up paper in mouse by Wang et al. 2011. Perhaps, Schwambron et al. 2009 may be discussed in the frame of asymmetric inheritance and m(i)RNAs.

We carefully revised the text by referring to various relevant papers suggested by this reviewer (p. 3, lines 9 and15, p. 4, line 21, p. 5, line 8, p. 21, line 1).

9) Figures: the nuclei in the top part of Fig 1I are confusing, the brain should finish with the basal lamina like the other panels. Fig 2 should include data from S3 because these are too important to burry in the supplement (discussed above). This figure may then become too crowded. Perhaps the diagram of electroporation (which is a copy-and-paste from the previous Inoue & Osumi 2001 without proper reference!) could go to the supplement. I believe that the readers from this field are fully aware of this technique, which the Osumi lab has pioneered. A little inset showing basal lamina and endfeet should be shown in all figures including Fig 6 and S7.

The previous Fig. 1I was obtained from a sample in which the cranial mesenchyme was still attached. This resulted in many Cyclin D2 positive cells appearing above the basal lamina. Fig. 2 has been restructured by including important data from previous Fig. S3 and by moving the diagram of electroporation to new Fig. S3. Accordingly, the relevant text was rewritten in the Results (p. 7, line 16 and p.8 line5) and Legends (p. 32, line 21 and p. 42, line 17). Insets were added to all figures of Fig. 5 (we suppose the reviewer did not mean Fig. 6 from the context) and Fig. S7, with the revised text in Results (p. 13 line 20 to p.14 line 11), and Legends (p. 34, line 18 and p. 44, line 7).

Additional Correspondence 20 January 2012

Thank you for submitting your revised manuscript to the EMBO Journal. Your study has now been seen by referee #2 and 3 and their comments are provided below. As you can see, both referees appreciate the introduced changes and support publication in the EMBO Journal. I am therefore pleased to proceed with the acceptance of the paper for publication here.

Thank you for submitting your interesting study to the EMBO Journal.

Yours sincerely,

Editor
The EMBO Journal

REFEREE REPORTS

Referee #2

The authors have addressed most of my previous concerns. I support the publication.

Referee #3

The authors have significantly improved their manuscript and addressed most of my comments and

concerns. I think that the novelty of this work fully justifies certain technical limitations in providing formal proof of the authors hypothesis and since the authors have now tuned down some of the strong claims present in their previous submission, I believe that the revised manuscript is entirely appropriate for the standards of EMBO

