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Mitotic spindle orientation directs cell fate and bias notch activity in chick neural tube

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The manuscript was previously reviewed by the EMBO Journal. A revised version was directly transferred to EMBO reports, including the original referee reports with the authors response:

Correspondence – Original Referee Reports 10 January 2012

Referee #1:

This manuscript addresses the important issue of asymmetric divisions in the vertebrate neural tube. Such divisions are essential to ensure growth and differentiation in the embryonic CNS. The most useful data in this manuscript confirms the recently published work in zebrafish neural tube, that the more apical daughter in asymmetrically fated divisions is destined to be the neuron and the more basal daughter is destined to become the progenitor. As in the zebrafish work the more basal daughter is found to reattach to the apical surface in order to regain apical progenitor status. This is important data as it shows the unexpected mechanism revealed in the fish is conserved in other vertebrates. Also this manuscript confirms another fish observation that asymmetric inheritance of the apical Par3 domain can result from cleavage planes that are very close to perpendicular. This data supports the proposal derived from studies in mouse neural tube that very small alterations in cleavage plane can have significant consequences for asymmetric inheritance.

The most novel aspect of the current manuscript is the attempt to monitor Notch activation during asymmetrically fated divisions using live imaging. Authors claim this data shows that the more basal daughter develops a higher level of Notch activation than the apical daughter. Although the authors do not show this directly, it is assumed that they are monitoring asymmetrically fated divisions and

the more basal daughter will become the progenitor. In the illustrated example (Movie S7) it is not convincing that the orientation of cleavage can be accurately monitored in these experiments, as the image shown is too fuzzy for the cleavage furrow to be sufficiently clear. I think it is possible to interpret this movie as showing telophase separating the daughters along the plane of the apical surface, and by the time the authors have labelled the daughters as apical and basal one nucleus has moved away from the apical surface. It just isn't very clear, and I assume this is the best of the 6 examples they have. The elevated Notch activity is not apparent until 9 hours after mitosis. So I'm not sure this really tells us anything new, but if we assume the authors can strengthen their apical/basal designation, then this data confirms what we would expect from our previous knowledge of Notch activation during neurogenesis. So it could be nice confirmation of a firm prediction at the level of individual cells (it would be stronger if the data directly showed the high Notch daughter became a progenitor and the low Notch daughter became a neuron). The authors also conclude that this shows that Notch signalling is influenced by mitotic spindle orientation, but given the difficulty of deciding spindle orientation in the example they show in Movie S7 I do not think this is yet very compelling. Also it is far from clear how directly spindle orientation might be influencing Notch activation; I think it more likely that Notch activation will be the result of integrating many influences. It would also of course be interesting (indeed critical to understand mechanism) to test whether the asymmetric Notch activation is somehow autonomous to the dividing progenitor and its daughters or whether signals from neighbouring cells are important. I think this last point would be too much to ask for in this work however.

I do not think the authors should use the term stem cell in their title, as they are not studying stem cells. They are studying asymmetric divisions in neural progenitors. And at the moment I do not think the data supports their claim that spindle orientation directs Notch activation, at best it correlates with this (if they can strengthen the relevant data)

Referee #2:

In this manuscript, Das & Storey described their study on the role of mitotic spindle orientation during neurogenesis in chick neural tube. They show that Insc-induced horizontal (parallel to the apical surface) cleavage of the neuroepithelial cells leads to typical asymmetric stem cell mode divisions-one daughter cell remains to be a progenitor while the other differentiates into a neuron. Interestingly, in contrast to previous assumption, the basal daughter cell derived from such asymmetric cell division turns to be a progenitor while the apical daughter cell becomes a neuron. Using a new real-time reporter of Notch activity, the authors show that differential Notch activation follows asymmetric cell division.

In vertebrate, whether spindle orientation-mediated asymmetric partitioning of apical and basal components of neural stem cells results in defined and differential cell fates of the daughter cells is still controversial. Although many of the observations described in this manuscript are similar to a recent study in zebrafish (Alexandre et al, 2010), confirming such intriguing stem cell mode division in a higher vertebrate central nervous system is still of significance. The real-time analysis of Notch activities through asymmetric cell division is certainly a plus for the manuscript.

One major concern of the manuscript is that most of the results and conclusions were derived from cells over-expressing Insc which could raise the possibility of artificial effects. It is possible that over-expression of Insc might not only affect spindle orientation, but also interfere with normal cell fate specification, for example, by affecting the localization and/or function of Par3. The authors need to demonstrate: (1) How often is the basal progenitor/apical neuron mode of asymmetric cell division observed during normal neurogenesis? (2) How often does spindle orientation-mediated asymmetric apical and basal inheritance result in basal progenitor/apical neuron mode of asymmetric cell divisions during normal neurogenesis? (3) Does such basal progenitor/apical neuron mode of asymmetric cell divisions rely on endogenous Insc?

Importantly, if the asymmetric inheritance of the apical and basal cells poles leads to basal progenitor/apical neuron mode of asymmetric cell division, as proposed by the authors in Fig. 8, it will be in contrast to previous clonal analysis in chick neural tube by others (Morin, 2007) and by the authors' own group (Wilcock, 2007) which demonstrated that many of the parallel (0-30o) and

intermediate (30-60o) cleavages actually led to the production of both progenitor daughter cells. The authors need to explain such obvious discrepancies.

In Fig. 6, the authors showed that asymmetric inheritance of apical-basal components takes place during normal neurogenesis, but did not address the questions raised above. In page 11, the authors stated that "Furthermore, in divisions in which cellular processes and the cell fates of both daughter cells were clearly apparent neuronal cell fate correlated with retention of a process extending to the apical surface, while its basally located sibling re-grew a new apical process and subsequently divided again." I do not understand such statement. If "the cell fates of both daughter cells were clearly apparent neuronal cell fate", how could "its basally located sibling re-grew a new apical process and subsequently divided again"?

In Fig. 6D and Supplementary Movie S6, the red dotted cell looks like a basal cell to me, based on the angle of the cleavage plane.

In Fig. 7B, the authors intended to demonstrate that rapid loss of apical polarity complex is part of the neuronal differentiation mechanism. First of all, it is difficult to observe the localization of aPKC and Par3 in the merged images. Separated single staining of aPKC or Par3 should be presented. Second, in fixed tissue, I do not know how the authors can be sure that these Tuj1 positive cells are derived from the apical daughter cells. The authors have performed live imaging and showed asymmetric partitioning of GFP-Par3 upon cell division (Fig. 6A and 6B). In principal, it should be possible to follow the changes of GFP-Par3 inherited by the apical daughter cells when they differentiate.

Transfer date – authors response 08 February 2012

Das and Storey, response to referees:

Referee #1:

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We note the reviewer's recognition of the importance of this work and the role it will play in reconciling controversial observations in other species.

The most novel aspect of the current manuscript is the attempt to monitor Notch activation during asymmetrically fated divisions using live imaging. Authors claim this data shows that the more basal daughter develops a higher level of Notch activation than the apical daughter. Although the authors do not show this directly, it is assumed that they are monitoring asymmetrically fated divisions and the more basal daughter will become the progenitor. In the illustrated example (Movie S7) it is not convincing that the orientation of cleavage can be accurately monitored in these experiments, as the image shown is too fuzzy for the cleavage furrow to be sufficiently clear. I think it is possible to interpret this movie as showing telophase separating the daughters along the plane of the apical surface, and by the time the authors have labelled the daughters as apical and basal one nucleus has moved away from the apical surface. It just isn't very clear, and I assume this

is the best of the 6 examples they have. The elevated Notch activity is not apparent until 9 hours after mitosis. So I'm not sure this really tells us anything new, but if we assume the authors can strengthen their apical/basal designation, then this data confirms what we would expect from our previous knowledge of Notch activation during neurogenesis. So it could be nice confirmation of a firm prediction at the level of individual cells (it would be stronger if the data directly showed the high Notch daughter became a progenitor and the low Notch daughter became a neuron).

We are concerned that the reviewer has missed important details of this Notch reporter experiment and in this movie in particular. The Movie S7 is of a cell expressing Insc-IRES-H2B RFP as well as the Hes5-1 reporter construct. The expression of Insc reproducibly increases the apico-basal orientation of divisions and H2B labels the chromatin and hence orientation of the mitotic spindle. At time 1h 59mins, the cell is in late anaphase and about to enter telophase, and the chromatin is marked by the H2B RFP in the red channel. The cleavage plane is already determined at this stage as being perpendicular to the spindle pole (marked by H2B RFP) (we wonder whether the reviewer may have mistaken the H2B-RFP localization to chromatin as a "fuzzy" appearance)? The apical and basal daughter cells are marked with a yellow and pink dot at this point (i.e. from the division) and then for the duration of the movie. To additionally directly visualise the cleavage plane, we would have to electroporate a membrane marker, in addition to the Insc-ires-H2B and Hes5-1 constructs. This is technically very challenging and is likely to be detrimental to cell viability. We do think that H2B-RFP is a good marker for spindle orientation. It also serves to label the nucleus throughout movies, so that changes in Hes5-1 activity can be attributed to alteration of Notch activity and not to the nucleus coming in and out of focus. To address the reviewer's request for further clarity, we have revisited this data and are able to provide several further stills frames leading up to the division at the anaphase image in which we indicate basal and apical daughter cells in the figure and movie. These new images show the classic back and forth rotation of the mitotic spindle at metaphase (first described by Richard Adams in the cortex and also observed by us in the chick neural tube, Wilcock et al 2007) and the fixing of mitotic spindle orientation at anaphase. We have re-made the figure to show this and have slowed down the movie so that this cell behaviour is more apparent.

The reviewer comments on our finding that Notch activity increase is detected at 9h post-mitosis (in the cell shown). We have now up dated the paper to provide the range of observations we have made (between 7 and 10h to reach 3 fold increase over the apical daughter cell). This reporter is transfected and we follow activation in sibling cells following mitosis, but the precise time at which we detect increased Notch activity to some extent reflects the amount of plasmid transfected into the mother cell. This assay therefore does not allow statements about the precise timing of activation – the striking finding here is that the basal daughter consistently shows this increase - the reviewer's comment has made us realize that presenting the activation timing data in the main paper might mislead and for this reason we have now placed this graph in the supplementary figures and note this point in the legend.

The authors also conclude that this shows that Notch signalling is influenced by mitotic spindle orientation, but given the difficulty of deciding spindle orientation in the example they show in Movie S7 I do not think this is yet very compelling. Also it is far from clear how directly spindle orientation might be influencing Notch activation; I think it more likely that Notch activation will be the result of integrating many influences. It would also of course be interesting (indeed critical to understand mechanism) to test whether the asymmetric Notch activation is somehow autonomous to the dividing progenitor and its daughters or whether signals from neighbouring cells are important. I think this last point would be too much to ask for in this work however.

We have published recently a baseline study characterizing normal Notch signalling dynamics revealed by this reporter in the chick neural tube and this shows that the vast majority of divisions at early stages generate daughter cells that both activate Notch, consistent with most divisions at these stages giving rise to two progenitors (Vilas-Boas et al, 2011 *BMC Biology* **9:** 58). Here, when we mis-express Insc almost all divisions are now apico-basally orientated and we see a consistent switch to asymmetric activation of Notch

in daughter cells and strikingly that it is the basal daughter cell that fires up Notch signalling. This does indicate a correlation between spindle orientation and Notch activity in daughter cells. Real-time imaging of the signalling activity of single cells within a developing neuroepithelium is a significant new achievement - although of course it would be nice if we could track individual cells for even longer (i.e to demonstrate a further division) we do note that the basal daughter, which is Notch active, moves back towards the apical surface consistent with entry into G2 and pending mitosis (movie 7). This technique and these observations are still a major advance in the field; previous work has either been in fixed tissue or used a luciferase reporter which does not provide cellular resolution and does not allow analysis of cell behaviour (Williams et al, 2011 *Nature* **470:** 353-358, El-Hashash et al, 2011 *Development* **138:** 1395-1407, Shimojo et al, 2008 *Neuron* **58:** 52-64) (and others reviewed in Vilas-Boas et al. 2011). Our further data in the paper demonstrates that Insc mis-expression generates apico-basal divisions that systematically give rise to a basal daughter, which divides again (i.e. a progenitor) and an apical daughter that differentiates. The Notch reporter data here further confirms the progenitor status of the basal daughter and together these data demonstrate a remarkable correlation between division orientation, cell behaviour and signalling activity. Our conclusion is that mitotic spindle orientation biases Notch signalling – this does not exclude the influence of signalling from neighboring cells. We have clarified this in the text and changed the title accordingly. We agree with the reviewer that it is beyond the scope of this paper to establish the underlying mechanism(s) that direct asymmetric Notch activation.

I do not think the authors should use the term stem cell in their title, as they are not studying stem cells. They are studying asymmetric divisions in neural progenitors. And at the moment I do not think the data supports their claim that spindle orientation directs Notch activation, at best it correlates with this (if they can strengthen the relevant data)

We agree that these cells are neural progenitors undergoing asymmetric divisions. This is a stem cell mode division, but we acknowledge that it is important to avoid any confusion with embryonic stem cells. We have revised the text accordingly. We also agree that directs may be too strong a word, we have replaced this with “biases” Notch activity.

Referee #2:
In this manuscript, Das & Storey described their study on the role of mitotic spindle orientation during neurogenesis in chick neural tube. They show that Insc-induced horizontal (parallel to the apical surface) cleavage of the neuroepithelial cells leads to typical asymmetric stem cell mode divisions-one daughter cell remains to be a progenitor while the other differentiates into a neuron. Interestingly, in contrast to previous assumption, the basal daughter cell derived from such asymmetric cell division turns to be a progenitor while the apical daughter cell becomes a neuron. Using a new real-time reporter of Notch activity, the authors show that differential Notch activation follows asymmetric cell division.

In vertebrate, whether spindle orientation-mediated asymmetric partitioning of apical and basal components of neural stem cells results in defined and differential cell fates of the daughter cells is still controversial. Although many of the observations described in this manuscript are similar to a recent study in zebrafish (Alexandre et al, 2010), confirming such intriguing stem cell mode division in a higher vertebrate central nervous system is still of significance. The real-time analysis of Notch activities through asymmetric cell division is certainly a plus for the manuscript.
We note this reviewer’s analysis of our work as a significant contribution to the field and their acknowledgement of the novelty of real time imaging of Notch activity during asymmetric cell division in the neuroepithelium.

One major concern of the manuscript is that most of the results and conclusions were derived from cells over-expressing Insc which could raise the possibility of artificial effects. It is possible that over-expression of Insc might not only affect spindle orientation, but also interfere with normal cell fate specification, for example, by affecting the localization and/or function of Par3.

Insc mis-expression is an established tool for manipulating the mitotic spindle and has been used as such in numerous papers published in high profile journals **(**Postiglione et al, 2011 *Neuron* **72:** 269-284, Konno et al, 2008 *Nat Cell Biol* **10:** 93-101, Zigman et al, 2005 *Neuron* **48:** 539-545). In particular, we note recent mis-expression of Insc in the mouse cortex, (Postiglione et al, 2011), where it is argued that Insc manipulation is the most specific way of altering mitotic spindle orientation because unlike *Drosophila* Pins, Par3, Par6 and aPKC, Insc has a single vertebrate homolog (Zigman et al, 2005 *Neuron* **48:** 539-545, Lechler and Fuchs, 2005 *Nature* **437:** 275-280, Katoh M, 2003 *Int J Mol Med* **11:** 111-116), whose only reported function is in asymmetric cell division. In addition, we have carefully titrated the levels of Insc mis-expression in our experiments and determined the lowest level at which we are able to consistently alter spindle orientation. We also demonstrate that in these conditions Insc mis-expression does not interfere with apical localization of Par3-GFP (Figure 2D and movieS4) and endogenous Par3 protein (Fig S4B) suggesting that this increase in Insc levels does not disrupt protein localization. Importantly, our paper presents the first work that shows real time cell behaviour following Insc mis-expression and our findings are not inconsistent with conclusions drawn from Insc mis-expression analyzed at a cell population level in fixed cortical tissue (Postiglione et al, 2011 *Neuron* **72:** 269-284)**.** On a more general point of mis-expression studies, we also note manipulations carried out by Alexandre et al in the Zebrafish also involved mis-expression by transfection. Clearly, if this is carefully controlled it can be a valuable approach to understanding gene function.

The authors need to demonstrate: (1) How often is the basal progenitor/apical neuron mode of asymmetric cell division observed during normal neurogenesis?

We interpret this to mean how often does the apical daughter give rise to a neuron during normal neurogenesis? In all divisions in which we observe neuron birth following mitosis the cell that differentiates into a neuron inherited the apical process (6/6 cases, page 11). This suggests that asymmetric inheritance of the apical process correlates very well with neuronal cell fate during normal neurogenesis.

(2) How often does spindle orientation-mediated asymmetric apical and basal inheritance result in basal progenitor/apical neuron mode of asymmetric cell divisions during normal neurogenesis?

We interpret this question to mean how many cells with apico-basally oriented divisions taking place during normal neurogenesis generate a basal daughter that divides again and an apical daughter that becomes a neuron.

The frequency of apico-basal divisions at early stages of development of the spinal cord is low, and the majority of divisions have symmetric fate outcomes, (Wilcock et al 2007). Our measurement of division orientations with low level Insc confirm this and indicate 4/20 divisions between 0-30 degrees (Fig.1B"). To resolve the apparent discrepancy between the low number of apico-basal divisions and the rate of neurogenesis, we have focused on: i) asymmetric inheritance of apical complex components. We find that Par3 is asymmetrically inherited in cells undergoing divisions with near vertical cleavage planes (Figure 3A and movie S5) suggesting that small shifts in mitotic spindle orientation are sufficient for assignment of asymmetric cells fates; ii) longer, more technically challenging live imaging of normal neurogenesis captured 6 lineages where we could measure cleavage plane at division and follow the fate of both daughter cells, here the original cells divide with within 60-90 degree cleavage plane, but still only one of the daughter cells inherited the apical process while the other cell (the basal daughter) extends a new apical process. In every case the apical daughter differentiated into a neuron while the basal daughter remains as a progenitor and divides again (Figure 3B and movie S6). These data demonstrate that asymmetric inheritance of apical components in neural progenitors of the spinal cord correlates with differentiation of the cell that inherits the original apical membrane, and that this does not require overtly apico-basal divisions.

(3) Does such basal progenitor/apical neuron mode of asymmetric cell divisions rely on endogenous Insc?

We find that the levels of *Insc* expression in the spinal cord increase during development and coincide well with the increase in neurogenesis (Supplementary figure 1). This is consistent with a role for endogenous Insc in the regulation of mitotic spindle orientation. However, the predicted phenotype for reducing Insc expression is to generate more divisions with a vertical cleavage plane (as observed in Postiglione et al, 2011 *Neuron* **72:** 269-284, Zigman et al, 2005 *Neuron* **48:** 539-545). This may have little effect at early stages when Insc is expressed at low levels and so may not help us to address the central question of this paper, which is the role of asymmetric inheritance of apical and basal components in cell fate choice. We use Insc mis-expression in this paper as a tool to manipulate mitotic spindle orientation, rather than to study Insc function.

Importantly, if the asymmetric inheritance of the apical and basal cells poles leads to basal progenitor/apical neuron mode of asymmetric cell division, as proposed by the authors in Fig. 8, it will be in contrast to previous clonal analysis in chick neural tube by others (Morin, 2007) and by the authors' own group (Wilcock, 2007) which demonstrated that many of the parallel (0-30o) and intermediate (30-60o) cleavages actually led to the production of both progenitor daughter cells. The authors need to explain such obvious discrepancies.

We have addressed apparent contradictions with our findings here (and these two studies in the Introduction and the Discussion), but will clarify these further here. In particular, our previous work was at stages when proneural gene expression is not widely present in the spinal cord and we proposed in Wilcock et al, that mitotic spindle orientation might not be significant until these neuronal differentiation promoting genes are expressed. There are differences in the technical approaches used by Morin and ourselves to determine changes in cell fate; the Morin work uses a triple transfection assay to create/follow clones which is not as reliable as our approach of simply observing individual cell behaviour in real time. In addition, Morin et al randomize the position of the mitotic spindle, while Insc mis-expression systematically generates apico-basally orientated divisions. We have re-written to clarify these points in the Introduction and the beginning of the Discussion.

In Fig. 6, the authors showed that asymmetric inheritance of apical-basal components takes place during normal neurogenesis, but did not address the questions raised above. In page 11, the authors stated that "Furthermore, in divisions in which cellular processes and the cell fates of both daughter cells were clearly apparent neuronal cell fate correlated with retention of a process extending to the apical surface, while its basally located sibling re-grew a new apical process and subsequently divided again." I do not understand such statement. If "the cell fates of both daughter cells were clearly apparent neuronal cell fate", how could "its basally located sibling re-grew a new apical process and subsequently divided again"?

We apologize for confusing the reviewer, there is a comma missing after “apparent” in this sentence, which has lead to this misreading. We have rewritten this sentence.

“Furthermore, in divisions in which cellular processes were visible and subsequent cell fates of both daughter cells **could be established,** neuronal cell fate correlated with retention of an apical process, while the basal sibling grew a new apical process and divided again” – end of page 7

In Fig. 6D and Supplementary Movie S6, the red dotted cell looks like a basal cell to me, based on the angle of the cleavage plane.

This division takes place with a cleavage close to vertical but its precise orientation with respect to the apical surface, which is not straight in this particular example, is difficult to present in a two dimensional image - which we agree here in 2D appears to show the cell with the red dot slightly higher (more basal) than its sibling. We therefore focused on determining which cell inherits the apical process, a structure that is clearly apparent in such GFP-tubulin labeled cells. This revealed that the cell that inherited the apical process (cell with the red dot) is the cell that becomes the neuron – while the cell that generates a new process (which must be by definition the basal cell) goes on to divide again. To convince further the referee of this pattern of inheritance we have made a rotating movie of

the division, so that the inheritance and emergence of a new apical process can be more clearly observed. The regrowth of the apical process is very clear here and is consistent with observations made when we induce apico-basal division by mis-expressing Insc.

In Fig. 7B, the authors intended to demonstrate that rapid loss of apical polarity complex is part of the neuronal differentiation mechanism. First of all, it is difficult to observe the localization of aPKC and Par3 in the merged images. Separated single staining of aPKC or Par3 should be presented. Second, in fixed tissue, I do not know how the authors can be sure that these Tuj1 positive cells are derived from the apical daughter cells. The authors have performed live imaging and showed asymmetric partitioning of GFP-Par3 upon cell division (Fig. 6A and 6B). In principal, it should be possible to follow the changes of GFP-Par3 inherited by the apical daughter cells when they differentiate.

We have improved Figure 7B (now Figure 3D) as requested to show single labelling of endogenous aPKC and Par3 proteins. While we cannot determine whether these TuJ1 +ve cells were apical or basal daughters this is not relevant to the study of the subsequent process of differentiation. We conclude from this data and our real-time analyses of cell behaviour that although the cell that inherits apical components differentiates, a critical next step is that the cell must now detach from the apical surface, withdrawing its apical process. This must involve a change in cell polarity as apical process withdrawal is followed by the projection of a new process (the axon) from close to the cell body located at the basal surface. We test here whether cells that are withdrawing their apical process retain apical complex proteins at the tip and find that these are indeed quickly lost as the process withdraws. Importantly, although the basal daughters from an apico-basal division do not inherit the original apical complex, they are able to re-polarise and thus re-establish their apico basal polarity. We conclude that maintenance of apico-basal polarity is important for progenitor cell status. Following the dynamics of Par3 in living cells is a difficult proposition, as, like previous studies (Alexandre et al, 2010), we use a Par3-GFP fusion that is driven by a constitutive promoter. So while this allows us to monitor the localization of Par3 in real time, it is not possible to image the loss of apical complex using this technique.

1st Editorial Decision 22 February 2012

I am glad to tell you that we have finally received the enclosed report from referee 1. The referee still has a few suggestions concerning the discussion of the data, that I would like you to incorporate before we can proceed with the official acceptance of your manuscript. I also would like to ask you to change the last sentence of the abstract "These findings .... identify a new step in the regulation of apical polarity." It is unclear to me what the new step in the regulation of apical polarity is.

I further noticed that the microscopic images are missing scale bars, which need to be added. Also, Figure 1D', D" and 1E' contain statistical analyses, but the figure legend does not define the error bars, nor the statistical test used to calculate the p-value. Please also add the total number of cells when percentages are given.

I apologize again for my late reply, and I look forward to seeing a new revised version of your manuscript as soon as possible.

Yours sincerely,

Editor
EMBO Reports

REFEREE REPORT:

Referee #1:

This manuscript reports some nice data that will definitely be of high interest to the field. Some of

the data on asymmetric segregation and daughter fate confirms what has previously been shown in other systems notably the fish embryo, but it is helpful to see this confirmation in other systems. I think the authors could usefully discuss more precisely what their results show and what they don't show.

Data shows that following artificial induction of apicobasal divisions, the basal daughters have elevated Notch activity. Other experiments show these daughters will become progenitors. The observation of elevated Notch in the progenitor daughter in the context of an asymmetric division is novel, and although not entirely unexpected it is nice to see it confirmed experimentally. The question is, is Notch activity regulated by spindle orientation? Yes in this experimental situation. I'm not however convinced that we can yet say whether normal fluctuations in spindle orientation determine differential Notch activation in the daughters. Also this experimental approach does not determine whether the differential Notch activation is intrinsic to the asymmetric division or whether alterations to spindle orientation alter Notch activation non-cell autonomously from neighboring cells. I think the authors should make this clear in the discussion.

Authors should discuss in the ms how elevated Hes5 activity approximately 10 hours after mitosis could be reporting events that are initiated nearly 10 hours earlier.

The analysis of apical components during normal neurogenesis (Figs 3A and B) shows that apical components can be asymmetrically inherited even though cleavage planes are close to perpendicular. This is a nice observation and similar to that already reported in zebrafish. The authors suggest this is due to slight deviation of the cleavage plane from perpendicular, but its not very convincing that they can accurately measure small deviations in a 3-dimension slice preparation. Therefore whether small deviations normally regulate apical inheritance remains unclear. The authors statement that their work "demonstrate(s) small changes in mitotic spindle orientation can lead to asymmetric segregation....." is too strong, the data at best shows a correlation because they have not systematically induced small deviations. They do not test whether there has to be a deviation from perpendicular cleavage to obtain asymmetric segregation. Also is it not possible that asymmetric distribution of apical components leads to changes in spindle orientation, rather than the other way round?

I think the authors could be more generous in alluding to results previously shown in the fish. For example asymmetric segregation of apical protein in divisions with near perpendicular cleavage and the rapid re-establishment of Par3 polarization in the basal daughter where both shown in the earlier zebrafish study.

Authors claim "de novo synthesis of apical complex proteins" in the more basal daughter. Its probably more accurate to say "ne novo polarisation" of apical proteins, as we don't know they synthesised rather than just redistributed.

I would prefer to see the section heading of Results section2 to be "Induced apico-basal divisions....." because basal progenitors in the normal neural tube do not retain apicobasal polarity.

I think the title should be modified to say: "Mitotic spindle orientation can direct cell fate and bias notch activity in chick neural tube" because I do not think we can yet conclude that spindle orientation is a normal mechanism of notch regulation and daughter cell fate. It might be but I don't think we know this for sure.

The descriptor of birds and mammals as "higher" vertebrates is no longer accepted usage; authors should just say other vertebrate classes.

1st Revision - authors' response 28 February 2012

We have addressed all requested changes below and in the revised paper. I have emailed you a tracked changes version for ease.

We have clarified the last sentence of the abstract and provided the scale bars, cell counts and error bar definitions requested (Stats tests were provided in the Methods).

We hope that the paper is now in a form that can be formally accepted for publication.

Referee #1:

This manuscript reports some nice data that will definitely be of high interest to the field. Some of the data on asymmetric segregation and daughter fate confirms what has previously been shown in other systems notably the fish embryo, but it is helpful to see this confirmation in other systems. I think the authors could usefully discuss more precisely what their results show and what they don't show.

Data shows that following artificial induction of apicobasal divisions, the basal daughters have elevated Notch activity. Other experiments show these daughters will become progenitors. The observation of elevated Notch in the progenitor daughter in the context of an asymmetric division is novel, and although not entirely unexpected it is nice to see it confirmed experimentally. The question is, is Notch activity regulated by spindle orientation? Yes in this experimental situation. I'm not however convinced that we can yet say whether normal fluctuations in spindle orientation determine differential Notch activation in the daughters.

We have made it clear in the Discussion that our conclusions are drawn from an experimental situation in which apico-basal divisions are induced. (page 11, line 2)
"This study shows, for the first time, that Notch signalling is differentially activated following ***induction*** *of an apico-basal division in the live neuroepithelium, strongly suggesting that mitotic spindle orientation influences Notch activity during normal neurogenesis".*

Also this experimental approach does not determine whether the differential Notch activation is intrinsic to the asymmetric division or whether alterations to spindle orientation alter Notch activation non-cell autonomously from neighboring cells. I think the authors should make this clear in the discussion.

We have now made a direct statement on this in the Discussion (Page11, 1ine 6)
" The systematic detection of elevated Notch activity in the basal daughter also confirmed the progenitor status of this cell. This strongly suggests that asymmetric division results in a cell intrinsic difference in the ability of sibling cells to respond to Notch signalling, although it is formally also possible that asymmetric partition of cellular components leads to changes that affect the ability of neighbouring cells to deliver Notch signalling."

Authors should discuss in the ms how elevated Hes5 activity approximately 10 hours after mitosis could be reporting events that are initiated nearly 10 hours earlier.

We chose to report elevated levels based on the time to 3 fold increase as this is clearest when presented as a series of still images – this is apparent at 7-10h. We had also explained in our letter and figure legend (Figure S5) that cells will be transfected with different levels of reporter plasmid and so the precise time when Notch activity is detected is also influenced by this – we have noted this now in the Results text (page 8, line 18) – we therefore do not place strong functional significance on the timing of Notch detection following mitosis.

The analysis of apical components during normal neurogenesis (Figs 3A and B) shows that apical components can be asymmetrically inherited even though cleavage planes are close to perpendicular. This is a nice observation and similar to that already reported in zebrafish. The authors suggest this is due to slight deviation of the cleavage plane from perpendicular, but its not very convincing that they can accurately measure small deviations in a 3-dimension slice preparation. Therefore whether small deviations normally regulate apical inheritance remains unclear. The authors statement that their work "demonstrate(s) small changes in mitotic spindle

orientation can lead to asymmetric segregation....." is too strong, the data at best shows a correlation because they have not systematically induced small deviations. They do not test whether there has to be a deviation from perpendicular cleavage to obtain asymmetric segregation. Also is it not possible that asymmetric distribution of apical components leads to changes in spindle orientation, rather than the other way round?

The word "demonstrates" has been replaced by "suggests" – page 8 line 2

I think the authors could be more generous in alluding to results previously shown in the fish. For example asymmetric segregation of apical protein in divisions with near perpendicular cleavage and the rapid re-establishment of Par3 polarization in the basal daughter where both shown in the earlier zebrafish study.

This is already directly addressed and referenced in the text (page 10 line 5).
"This strongly suggests that re-establishment of apical polarity is a normal neurogenesis step (Figure 4), in keeping with observations in the zebrafish [10]."

We have now added a further reference to this work (page 10, line16)(this was removed when the length the MS had to be reduced by 50%).
"However, given the rapid regulation of apico-basal polarity in basal daughter cells observed by live imaging, ***here and in the zebrafish [10]****, the incidence of this event may have been under-estimated."*

Authors claim "de novo synthesis of apical complex proteins" in the more basal daughter. Its probably more accurate to say "ne novo polarisation" of apical proteins, as we don't know they synthesised rather than just redistributed.

We do not claim, but conclude that the data "suggest that re-polarisation involves de novo synthesis" – this seems reasonable as we look and do not detect basal endogenous Par3 in cells induced to undergo AB divisions. To suggest that de novo synthesis might be involved does not exclude re-localisation of protein that is beneath detection levels. During normal neurogenesis most of the Par3 is asymmetrically inherited even in near perpendicular divisions. We have amended the Discussion as follows (page 10 line 1)

"The finding that on division Par3 largely localises in the apical daughter further ***suggests that re-polarisation of the basal cell may involve synthesis of new apical complex proteins as well as re-localisation of any inherited proteins."***

I would prefer to see the section heading of Results section2 to be "Induced apico-basal divisions....." because basal progenitors in the normal neural tube do not retain apicobasal polarity.
This has been changed

I think the title should be modified to say: "Mitotic spindle orientation can direct cell fate and bias notch activity in chick neural tube" because I do not think we can yet conclude that spindle orientation is a normal mechanism of notch regulation and daughter cell fate. It might be but I don't think we know this for sure.
This has been changed

The descriptor of birds and mammals as "higher" vertebrates is no longer accepted usage; authors should just say other vertebrate classes.
This has been changed

2nd Editorial Decision 28 February 2012

I am very pleased to accept your manuscript for publication in the next available issue of EMBO reports.

Thank you for your contribution to EMBO reports and congratulations on a successful publication. Please consider us again in the future for your most exciting work.

Yours sincerely,
Editor
EMBO Reports

