Single-cell imaging of cell cycle reveals CDC25B-induced heterogeneity of G1 phase length in neural progenitor cells

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Original submission

First decision letter

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MS TITLE: Single-cell imaging of cell cycle reveals CDC25B-induced heterogeneity of G1 phase length in neural progenitor cells

AUTHORS: Angie Molina, Frederic Bonnet, Valerie Lobjois, Sophie Bel-Vialar, Jacques Gautrais, Fabienne Pituello, and Eric Agius

I have now received all the referees' reports on the above manuscript, and have reached a decision. I apologise that this has taken longer than usual, but one of the referees' reports was very delayed. The referees' comments are appended below, or you can access them online: please go to BenchPress and click on the 'Manuscripts with Decisions' queue in the Author Area.

As you will see, the referees express considerable interest in your work, but have some significant criticisms and recommend a substantial revision of your manuscript before we can consider publication. Both referees raise several questions and make a series of constructive comments. The reviewers raise concerns regarding the interpretation of data from the forced expression of CDC25B that should be addressed. Adding loss-of-function data to the single cell imaging analysis, or identifying CDC25B G1 substrates would greatly strengthen the study. In addition, the reviewers request further data and analysis to validate conclusions regrading some of the cell cycle length measurements.

If you are able to revise the manuscript along the lines suggested, which will involve further experiments, I will be happy receive a revised version of the manuscript. Your revised paper will be re-reviewed by one or more of the original referees, and acceptance of your manuscript will depend on your addressing satisfactorily the reviewers' major concerns. Please also note that Development will normally permit only one round of major revision.

We are aware that you may be experiencing disruption to the normal running of your lab that make experimental revisions challenging. If it would be helpful, we encourage you to contact us to

discuss your revision in greater detail. Please send us a point-by-point response indicating where you are able to address concerns raised (either experimentally or by changes to the text) and where you will not be able to do so within the normal timeframe of a revision. We will then provide further guidance. Please also note that we are happy to extend revision timeframes as necessary.

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

Reviewer 1

Advance summary and potential significance to field

The concept of the coupling of cell cycle dynamics with the acquisition of cell fate is a central one in developmental biology. The manuscript by Molina et al addresses this by using time lapse imaging of slices of embryonic chicken spinal cords to (1) carefully characterizing the cell cycle progression of individual neural progenitors cells (NPCs), (2) manipulate cell cycle by overexpressing the critical phosphatase CDC25B, which is thought to regulate G1-S and G2-M phase progression, and (3) use defined parameter stochastic modeling in an attempt to predict phase duration in a population of dividing progenitors.

The key argument of the study is that our understanding of cell cycle dynamics of a heterogeneous population of neural progenitors is understood only at a macro level, and could use a more careful lineage analysis of the single cell behaviors. The authors conclude that NPCs of the embryonic spinal cord display a large variance in cell cycle states, with each phase of each cell cycle being largely independent of each other, and the overall cell cycle length is mostly reflective of the G1 phase length. This is not entirely surprising as cell growth occurs in G1, and this phase is under heavy regulation by checkpoint controllers.

In order to characterize cell cycle dynamics, Molina et al used a FUCCI constructs in an optimized slice culture protocol coupled with spinning disk confocal imaging. They characterized their time lapse lineage analysis with PCNA, BrdU, and HuC/D staining to correlate cell cycle phase state with neurogenic determination. The manuscript is well written, and involves an impressive analysis of cell cycle dynamics. In addition to an established role in G2-M transition, the author now argue that CDC25B also plays are role in regulating G1 phase length, although the exact mechanism was not resolved. This work builds on previous studies (some from the same group) in the role of CDC25B in promoting cell cycle progression and neurogenesis. The difference here being the lineage resolution at the ‘single cell’ level. However, the overall conclusion that populations of NPCs in developing neural tissues are asynchronous and heterogeneous with respect to cell cycle status is not new. Nor is the fundamental role of CDC25B in this process. The authors now suggest the control G1 phase length by CDC25B is the main contributing factor to NPC cell cycle length heterogeneity. And this conclusion was principally drawn from overexpression studies via electroporation followed by time lapse imaging of embryonic spinal cords.

Comments for the author

Issues to address:
(1) While the single cell analysis was impressive and comprehension, the CDC25B gain-of-function experiments lead to some concerns regarding physiological roles for the protein. For example, CDKs are active in G1-S transition, so the overexpression of a phosphatase is bound to cause disruption in this process. This observations needs further exploration mechanistically. CDC25 is a phosphatase that promotes the destruction of cylins. So what is it targeting in the G1 phase? Another issue with this approach is that any manipulation by electroporation is necessarily mosaic: different cells receive different amounts of the phosphatase. This makes a population analysis of anything that affects cell cycle parameters challenging. The authors’ previous studies used CDC25B RNAi (Development, 2012, 139:1095), and they concluded CDC25B primarily affected G2 phase length,

which is more consistent with the established literature. It is unclear why they chose not to use this reagent in their time-lapse experiments.
Their analysis of the role of CDC25B contrasts with the findings from genetic loss-of-function studies (e.g. Ferguson et al., MCB, 2005, 25: 2853) which showed that mice lacking CDC25 are viable (but sterile) and their fibroblasts exhibit normal cell cycle and checkpoints. Thus any conclusion regarding CDC25B's role in the cell cycle from constitutive overexpression studies need to be tempered by the possibility that they are creating a non-physiological state.
(2) I appreciated the authors' efforts to limit photoxicity in long term time lapses experiments using the spinning disk confocal, however they did not evaluate DNA repair mechanisms as a result of prolonged exposure to high intensity light that may have contributed to heterogeneity in cell cycle length, most especially S phase, which showed the greatest variability. Specifically, S phase was the most variant out of all other phases (M: 55/20 = 2.75X; G1: 980/140 = 7X G2: 175/53 = 3.3X; S: 945/130 = 7.3X). Can the long variance in S phase is suggestive of DNA damage in a subset of cells? They should stain samples at the end of their imaging assay with markers for DNA fragmentation (e.g. Annexin V-FITC) and activation of DNA repair systems (e.g. gamma-H2AX, OGG1), to visualize DNA damage recruitment and see if it correlates with S-phase length.
(3) If cell cycle duration would be independent of one another, then you would expect drift within populations of cells once synchrony is achieved (e.g. by serum starvation). Is there not some way to test for this by incubating slice cultures with colchicine, following extensive washing? Would such an approach be too toxic for slice cultures, precluding its use?
(4) While it was impressive that slice cultures generated ventral spinal cord cell types somewhat normally, the timing of their differentiation was delayed relative to in ovo development, and the resulting neural tubes were smaller (Suppl. Fig. 1). This correlated with lengthened cell cycle duration in slice cultures, but possibly involved cell death as well (not evaluated). It highlights caution in conclusions regarding basic aspects of cell cycle dynamics from slice culture imaging and CDC25B overexpression. Correlating the in vitro data from the current study with published reports of cell cycle manipulations (KO of cyclins, Cdc25, p27, p57) in various NPCs using loss-of-function studies in vertebrates, (e.g. zebrafish, mice) is important.
(5) One counter-intuitive aspect of the modeling is the claim that S and M phase length are independent of one another. Wouldn't one assume a tightly coupling of at least the S/M phases, as cell divisions should not occur unless DNA is properly replicated? It deserves a bit more clarity in the text.

Reviewer 2

Advance summary and potential significance to field

In their manuscript, Molina et al. utilize a live imaging strategy of chick neural tube slices to study the cell cycle of neural progenitor cells. Their method allows them to image the tissue for 48h and thus can be potentially useful for understanding the changes in cell cycle dynamics that occur in the course of embryonic development. The authors show that the cell cycle of neural progenitors is heterogeneous, without much apparent correlation between the lengths of individual cell cycle phases. They further show that Cdc25 affects mainly the duration of G1 but also increases the heterogeneity in cell cycle lengths.
Altogether, the live imaging method presented in this study is valuable and has the potential to deliver interesting results. Furthermore, the measurements of cell cycle heterogeneity are novel and may be useful for understanding the regulation of cell cycle dynamics. However, beyond this, the paper does not deliver substantial new insight into the mechanisms that underlie cell cycle lengthening. It also suffers from major issues with the presentation and interpretation of results, as outlined below.

Comments for the author

Major issues:
1. The authors do not present sufficient validation that recordings from slices accurately reflect the cell cycle dynamics in the embryo. Crucially, the authors do not report the DV positions of the cells they used for their measurements, nor do they report whether differences between progenitors in different domains can be detected. As the authors mention in the introduction the kinetics of cell cycle exit depends on the DV position of neural progenitors

– the validity of the method can be greatly strengthened if the live imaging method can capture this difference. This information is also necessary to exclude the possibility that part of the observed cell cycle heterogeneity is derived from differences in cell identity, rather than being actual variability of cell cycle length amongst progenitors of the same subtype.
2. A key point of the manuscript is to demonstrate at the single-cell level that the cell cycle lengthens over time. The control condition in Fig. 5 where lineages were tracked contains only 11 data points, and most of them are close to the bisector, hence it is difficult to make a strong conclusion whether lengthening is indeed observed in the experiments. This result should be strengthened by adding more data points. Furthermore, they should show that the mean cell cycle length is only a function of the developmental stage, but not of time elapsed since dissection and imaging, to formally exclude the possibility that the cell cycle dynamics are artificially altered as a result of the culture and imaging.
3. A key value in demonstrating that the cell cycle of individual cells lengthens is that this can be distinguished from the possibility, left open by population studies, that one subpopulation of cells cycle at a near-constant rate, while a second subpopulation exit the cell cycle while remaining in the progenitor zone. To corroborate this point, the authors would need to demonstrate that the data from live imaging provides an accurate range of cell cycle heterogeneity. In their data, they report cell cycle lengths ranging from ~10h to ~25h – is this the upper limit for progenitors? Can they confirm this number with long EdU incorporation experiments in which they identify at what point all the progenitors have passed S phase?
A minor related point: the statement that the cell cycle is heterogeneous does not make much sense unless it is related to a reference value or concept (any measurement has variation). The authors should take care to rephrase their statements to take this into account and talk about the range of heterogeneity instead.
4. A key conclusion is that the lengths of the cell cycle phases are independent and uncorrelated (except for a small effect between M and S phase).
However, the authors make the point that the cell cycle changes over time and even have two distinct phases summarized in Fig. 6. It is unclear whether these distinct phases might have different mechanisms of cell cycle control. To address this, but also in general to make their data accessible and understandable to the readers, they should show the correlation analysis in the form of a plot, not just a table (Supplementary 2.3). The regression plots should be shown at least for some key pairwise comparisons (e.g. G1 vs G2). It should be reported whether early and late phase progenitors behave differently.
5. In their CDC25B overexpression experiments, the authors found that G1 phase and the overall Tc length are increased. According to a previous study (Peco et al., 2012), gain of CDC25B function leads to increased neurogenesis.
This raises the question as to whether the cells with increased G1 length in the CDC25B OE experiment actually go on to divide. The authors should report the results of lineages overexpressing CDC25B in Fig. 5, like they do for the control and delta cdk conditions, as well as report the analysis only for the G1 of those cells where a subsequent mitosis can be observed.
Minor points:
-The authors say that: “FUCCI G1 expressing cells localized on the basal side of the explants are most probably differentiating neurons. The G1 lengths measured for these differentiating cells are excluded from our analyses”. It is unclear what exactly is meant here. Are the authors saying that these are cells that will undergo a final division before becoming postmitotic, or are they saying that these cells will exit the cell cycle in G2? Their tool allows them to make this distinction clearly. Furthermore, the basal localization alone does not seem reliable for identifying cells that are becoming postmitotic (e.g. HuC positive cells are also observed in mid-apicobasal positions). In particular this could be relevant for interpreting the Cdc25B results. Can the authors give an estimate of how the HuC staining changes along the apico-basal axis of electroporated embryos? Or could they use the KO fluorescence intensity in G1 cells relative to PCNA (as electroporation control) to determine whether the reporter can be used to distinguish G1 vs G0 cells?
- The authors discuss changes in G1 length between mother and daughter cells.
But the S phase also seems to shorten. As the duration of S phase is considered an important factor in the maintenance of the proliferative capacity of neural progenitors, do the authors observe differences in S phase length preceding different modes of division?
-When describing the neural progenitor’s behaviour during live imaging, the authors mention that different division modes can be observed. However, it is not clarified how frequently different MoDs are registered.

- While the modelling of cell cycles phase distributions and relationships appear to be “well compatible with the observed one” some statistical analysis of the fit of the data would improve this analysis. The authors have also not fully explored other possibilities here, including, for example, weak coupling between phases. On page 15, the statement “This is confirmed using Monte Carlo permutation” is unclear and requires further clarity and justification. The line for the Monte Carlo permutations (Fig.3H) appears to fit somewhere between the actual data and the fully anti-correlated data, especially after Tc durations greater than 900 minutes; again, some analysis of the fit quality of these models would be good.

First revision

Author response to reviewers' comments

We are appreciative of reviewers’ 1 and 2 interest, and we would like to thank the referees for their constructive and helpful comments. The following text is our point-by-point answer. In blue are the referee comments in black our answers.

Reviewer 1 Advance Summary and Potential Significance to Field:
The concept of the coupling of cell cycle dynamics with the acquisition of cell fate is a central one in developmental biology. The manuscript by Molina et al addresses this by using time lapse imaging of slices of embryonic chicken spinal cords to (1) carefully characterizing the cell cycle progression of individual neural progenitors cells (NPCs), (2) manipulate cell cycle by overexpressing the critical phosphatase CDC25B, which is thought to regulate G1-S and G2-M phase progression, and (3) use defined parameter stochastic modeling in an attempt to predict phase duration in a population of dividing progenitors.

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Reviewer 1 Comments for the Author:

Issues to address:

While the single cell analysis was impressive and comprehension, the CDC25B gain-of- function experiments lead to some concerns regarding physiological roles for the protein. For example, **CDKs are active in G1-S transition, so the overexpression of a phosphatase is bound to cause**

disruption in this process. This observation needs further exploration mechanistically. CDC25 is a phosphatase that promotes the destruction of cylins. So what is it targeting in the G1 phase? Another issue with this approach is that **any manipulation by electroporation is necessarily mosaic: different cells receive different amounts of the phosphatase**. This makes a population analysis of anything that affects cell cycle parameters challenging. The authors' previous studies used **CDC25B RNAi** (Development, 2012, 139:1095), and they concluded CDC25B primarily affected G2 phase length, which is more consistent with the established literature. **It is unclear why they chose not to use this reagent in their time-lapse experiments.**

We want to thank the referee for his/her constructive comments. The first issue addressed by the reviewer is the question of the mechanism by which the CDC25B phosphatase could regulate progression in G1 phase. To misexpress CDC25B we used the mouse cell cycle dependent CDC25B cis regulatory element (ccRE) that reproduces the cell cycle regulated transcription of CDC25B (Korner et al., 2001). We anticipated that the periodic expression induced by the promoter and the intrinsic instability of CDC25B would result in CDC25B being actively degraded at the end of mitosis and would preclude its expression in G1. We have now verified that assumption using an active eGFP- CDC25B chimeric fusion protein (Lobjois et al., 2009) and showed that it is never co- expressed in cells with the G1 phase marker mKO2-zCdt1 after co-electroporation. These results are included in the new supplement figure 7 and described in the results section of the revised version of the manuscript. Therefore, we propose that CDC25B activity occurs during the G2/M phase.

Recently, S. Spencer and collaborators have interestingly showed that the information integrated during the G2 phase of mother cells (mitogens signaling, cyclin D protein synthesis) drive the timing of restriction point crossing during the G1 phase of daughter cells (Min et al., 2020; Moser et al., 2018; Spencer et al., 2013). We thus hypothesized that CDC25B, expressed in G2, could generate G1 phase length heterogeneity indirectly by delaying the timing of the restriction point passage in NPCs. As already described (Moser et al., 2018), we analysed the phospho-Rb (S807/811) to identify NPCs that have crossed the restriction point and are committed to the cell cycle (positive) or not (negative) after CDC25B electroporation. As shown in the new figure 5 of the revised version of the manuscript, CDC25B, but not CDC25B$^{\Delta CDK}$, induces an increase in the percentage of phospho-RB (S807/811) negative NPCs, suggesting that the expression of CDC25B in G2 induces a higher proportion of NPCs that are in G1 before the restriction point and not yet differentiated (Tuj1 negative). Therefore, CDC25B could induce lengthening and increase in the range of heterogeneity of the G1-phase by delaying the passage of restriction point during G1. These new and original results represent a first step in understanding the mechanism by which CDC25B expression during G2 can affect G1 length. We have added in the discussion of the revised version of the manuscript, a model of the mechanism that could be involved in this regulation of the restriction point by CDC25B. The investigation of this model will be challenging and cannot be done in a time frame compatible with the revision of this manuscript. We therefore really think that it will be the subject of a future article.

The second issue concerns the mosaic expression following electroporation. We agree with the referee that with the electroporation protocol, the cells receive different amounts of CDC25B. This means that cells expressing different amounts of plasmid will have different responses depending on the dose received. We were aware of that and already commented on it in the manuscript (cf. discussion: "*Electroporation in chick neural tube leads to mosaic expression...*"). We reinforced our conclusion that G1 phase heterogeneity is mainly due to CDC25B activity, by comparing the range of heterogeneity in all phases of the cell cycle in control versus CDC25B conditions using the exit time as a read out. This has been added to the discussion in the revised version of the manuscript.

Finally, as mentioned by the reviewer, we previously used CDC25B RNAi to test the role of this phosphatase in neurogenesis in relation with its expression pattern in the spinal cord. In the submitted manuscript, we wanted to analyze cell cycle kinetics with single cell resolution in proliferative and neurogenic progenitors. Because of its neurogenic activity, we used gain-of-function of CDC25B to be able to affect the proliferative vs neurogenic ratio in the dorsal neural tube where CDC25B is not expressed. Using RNAi will maintain the cells in the proliferative mode and will not give insight into the modification of the cell cycle kinetics associated with neurogenesis. We did not use RNAi for technical reasons as well: *CDC25B* transcripts are not present in all progenitors at the time we begin the movie (Fig. 1); RNAi experiments result in variable level

of knock down and are indeed even more mosaic than gain-of-function experiments. That is why we did not choose this strategy.

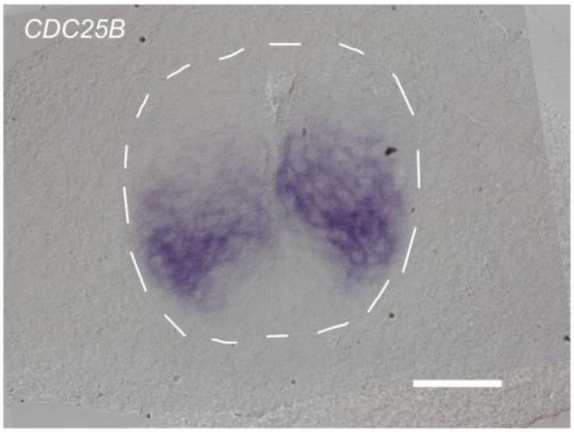

Figure 1: In situ hybridization against CDC25B on a 24 hours culture of E2 chick embryo explant. Dotted line around the neural tube. Scale bar represents 50 µm.

Their analysis of the role of CDC25B contrasts with the findings from genetic loss-of- function studies (e.g. Ferguson et al., MCB, 2005, 25: 2853) which showed that mice lacking CDC25 are viable (but sterile) and their fibroblasts exhibit normal cell cycle and checkpoints. Thus any conclusion regarding CDC25B's role in the cell cycle from constitutive overexpression studies need to be tempered by the possibility that they are creating a non-physiological state.

We agree with the referee that all gain-of-function experiments have to be analyzed carefully. However, we have several data in loss-of-function experiments that support our interpretation indicating that neural progenitor cells do not behave like mouse embryonic fibroblast (Ferguson et al., 2005). CDC25B downregulation performed using RNAi in chicken spinal NPCs results in a lengthening of the G2 phase without significantly modifying the S phase or total cell cycle duration, indicating that at the population level it might be associated with a shortening of the G1 phase (Peco et al., 2012). Such a mechanism of G1/G2 phase interplay in the developing spinal cord was also proposed by Kicheva (Kicheva et al., 2014). We have also generated a genetic loss-of-function in mouse (Bonnet et al., 2018). In spinal NPCs of mutant embryos, the proliferative index and mitotic index did not display a significant reduction compared to control embryos, but the G2-phase was clearly lengthened (from 1 hr 49 min in controls to 2 hr 19 min in mutants). More recently, we analysed the cell cycle of cortical NPCs in E14,5 dpc CDC25B mutant embryos (Roussat et al., bioRxiv doi: https://doi.org/10.1101/2021.12.14.472592). CDC25B loss-of-function severely increases G2-phase length in cortical NPCs (from 2h12 hours in controls to 4h05 in CKO) without affecting total cell cycle duration. In this condition, we were able to quantify the number of NPCs in G1 phase and we observed a significant reduction of the number of cells in G1, indicative of a shortening of the G1 phase, the total cell cycle length being unchanged.

(2) I appreciated the authors' efforts to limit photoxicity in long term time lapses experiments using the spinning disk confocal, however they did not evaluate DNA repair mechanisms as a result of prolonged exposure to high intensity light that may have contributed to heterogeneity in cell cycle length, most especially S phase, which showed the greatest variability. Specifically, S phase was the most variant out of all other phases (M: 55/20 = 2.75X; G1: 980/140 = 7X G2: 175/53 = 3.3X; S: 945/130 = 7.3X). Can the long variance in S phase is suggestive of DNA damage in a subset of cells? They should stain samples at the end of their imaging assay with markers for DNA fragmentation (e.g. Annexin V-FITC) and activation of DNA repair systems (e.g. gamma-H2AX, OGG1), to visualize DNA damage recruitment and see if it correlates with S-phase length.

We agree with the reviewer that our imaging strategy could induce DNA damage. During the course of our protocol we performed immunostaining against ▫H2AX. As shown in the new supplementary Figure 2, there is no increase in the expression of ▫H2AX after time lapse imaging. This suggests that our imaging protocol does not induce the DNA double strand break repair system recruitment that could induce S-phase lengthening.

(3) If cell cycle duration would be independent of one another, then you would expect drift within populations of cells once synchrony is achieved (e.g. by serum starvation). Is there not some way to test for this by incubating slice cultures with colchicine, following extensive washing? Would such an approach be too toxic for slice cultures, precluding its use?

From our experience, all attempts to apply synchronization and release protocols to study progenitors cell cycle progression, led to either cell differentiation or are too toxic and induce rapid cell death.

(4) While it was impressive that slice cultures generated ventral spinal cord cell types somewhat normally, the timing of their differentiation was delayed relative to in ovo development, and the resulting neural tubes were smaller (Suppl. Fig. 1). This correlated with lengthened cell cycle duration in slice cultures, but possibly involved cell death as well (not evaluated). It highlights caution in conclusions regarding basic aspects of cell cycle dynamics from slice culture imaging and CDC25B overexpression. Correlating the in vitro data from the current study with published reports of cell cycle manipulations (KO of cyclins, Cdc25, p27, p57) in various NPCs using loss-of-function studies in vertebrates, (e.g. zebrafish, mice) is important.
Cell death was evaluated and reported in the revised version (suppl. fig. 1C). We believe that a complete comparison with all the published reports on KO of cyclins, Cdc25, p27, p57 in various NPCs studies in vertebrates would fit better in a review.

(5) One counter-intuitive aspect of the modeling is the claim that S and M phase length are independent of one another. Wouldn't one assume a tightly coupling of at least the S/M phases, as cell divisions should not occur unless DNA is properly replicated? It deserves a bit more clarity in the text.

In our study, the model shows that duration of the S phase can be independent of the duration of mitosis. It does not mean that the phases are independent, but that their length is independent. We agree with the reviewer that a cell cannot enter mitosis if the DNA has not been correctly replicated. We have clarified this point in the revised version of the manuscript by adding in the discussion the sentence:" The experimental and theoretical Tc survival curves display a very similar pattern, suggesting that indeed cell cycle phase durations are stochastic and independent. *It does not mean that the phases are independent, as cell divisions should not occur unless DNA is properly replicated for example, but that their length is independent*."

Reviewer 2 Advance Summary and Potential Significance to Field: In their manuscript, Molina et al. utilize a live imaging strategy of chick neural tube slices to study the cell cycle of neural progenitor cells. Their method allows them to image the tissue for 48h and thus can be potentially useful for understanding the changes in cell cycle dynamics that occur in the course of embryonic development. The authors show that the cell cycle of neural progenitors is heterogeneous, without much apparent correlation between the lengths of individual cell cycle phases. They further show that Cdc25 affects mainly the duration of G1 but also increases the heterogeneity in cell cycle lengths. Altogether, the live imaging method presented in this study is valuable and has the potential to deliver interesting results. Furthermore, the measurements of cell cycle heterogeneity are novel and may be useful for understanding the regulation of cell cycle dynamics. However, beyond this, the paper does not deliver substantial new insight into the mechanisms that underlie cell cycle lengthening. It also suffers from major issues with the presentation and interpretation of results, s outlined below.

Reviewer 2 Comments for the Author:

Major issues:
1. The authors do not present sufficient validation that recordings from slices accurately reflect the cell cycle dynamics in the embryo. Crucially, the authors do not report the DV positions of the cells they used for their measurements, nor do they report whether differences between progenitors in different domains can be detected. As the authors mention in the introduction, the kinetics of cell cycle exit depends on the DV position of neural progenitors – the validity of the method can be greatly strengthened if the live imaging method can capture this difference. This information is also necessary to exclude the possibility that part of the observed cell cycle heterogeneity is derived from differences in cell identity, rather than being actual variability of cell cycle length amongst progenitors of the same subtype.

We agree with the reviewer that reporting the DV positions of the cells is an important issue. We analyzed the position of our clones in our time lapses, in control conditions, by measuring the distance to the roof plate of the progenitors for which cell cycle kinetics was analyzed. Most of our clones are localized in the dorsal most 50 micrometers of the neural tube, which corresponds to the dorsal half of the spinal cord in our experimental design. We reported the DV positions of the cells we used for our measurements in the new suppl. Fig. 4A. The latter shows that heterogeneity of the G1 phase length is not linked to different localizations along the D/V axis.

2.A key point of the manuscript is to demonstrate at the single-cell level that the cell cycle lengthens over time. The control condition in Fig. 5 where lineages were tracked contains only 11 data points, and most of them are close to the bisector, hence it is difficult to make a strong conclusion whether lengthening is indeed observed in the experiments. This result should be strengthened by adding more data points. Furthermore, they should show that the mean cell cycle length is only a function of the developmental stage, but not of time elapsed since dissection and imaging, to formally exclude the possibility that the cell cycle dynamics are artificially altered as a result of the culture and imaging.

Lengthening of the cell cycle, and of the G1-phase, during development of the spinal cord has been precisely demonstrated in mouse and chick embryo at the progenitors population scale (Kicheva et al., 2014; Molina and Pituello, 2017). Our reason to study cell cycle dynamics at the single-cell level, was to decipher heterogeneity among progenitors. As mentioned in the manuscript, we found that the average G1 phase length is 257 +/- 28 min and 249 +/- 28 min in mother cells in the control and CDC25B$^{\Delta CDK}$ conditions, respectively, versus 309 +/- 38 min and 315 +/- 29 min in daughter cells. These durations (between 240 and 300 min) are consistent with those published by Kicheva et al. at similar developmental stages.

To nevertheless exclude the possibility that imaging could cause a progressive increase of G1-phase length that could lead to artefactual G1-phase length heterogeneity, we analyzed the duration of G1-phase of progenitors, depending on the time-point since the beginning of the time-lapse experiment. The graph sup fig 4B shows that the duration of G1 phase does not depend on the time elapsed since imaging. These data are added in the revised manuscript.

However, we agree with the referee that we have a lonely few points in the control condition, which clearly show a cell cycle and a G1-phase lengthening over time, and this is the reason why we added the data from the CDC25B$^{\Delta CDK}$ condition, considering the fact that there is no modification of cell cycle phases compared to control.

The strategy for single-cell analysis requires long-term live imaging and careful cell lineage analyses to be able to track mother and daughter cells which is time consuming. Given the current health crisis and difficulties in accessing equipment, we will not be able to add a significant number of cells to the control condition as requested by the reviewer. We propose therefore to tone down our words in the conclusion of the Figure 6 in the revised version of the manuscript.

3.A key value in demonstrating that the cell cycle of individual cells lengthens is that this can be distinguished from the possibility, left open by population studies, that one subpopulation of cells cycle at a near-constant rate, while a second subpopulation exit the cell cycle while remaining in the progenitor zone. To corroborate this point, the authors would need to demonstrate that the data from live imaging provides an accurate range of cell cycle heterogeneity. In their data, they report cell cycle lengths ranging from ~10h to ~25h – is this the upper limit for progenitors? Can they confirm this number with long EdU incorporation experiments in which they identify at what point all the progenitors have passed S phase?

We performed a cumulative EdU experiments with EdU incorporation every 3 hours for 27 hours to answer this point. At 27 hours of EdU incorporation, we could see progenitor nuclei that did not yet incorporate EdU (arrows in Suppl. Fig. 3) indicating that they display cell cycle longer than 27 hours. We have added this data in Suppl. Fig. 3.

A minor related point: the statement that the cell cycle is heterogeneous does not make much sense unless it is related to a reference value or concept (any measurement has variation). The authors should take care to rephrase their statements to take this into account and talk about the range of heterogeneity instead.

We have rephrased our text in the revised version of the manuscript according to the reviewer's recommendation.

4.A key conclusion is that the lengths of the cell cycle phases are independent and uncorrelated (except for a small effect between M and S phase). However, the authors make the point that the cell cycle changes over time and even have two distinct phases summarized in Fig. 6. It is unclear whether these distinct phases might have different mechanisms of cell cycle control. To address this, but also in general to make their data accessible and understandable to the readers, they should show the correlation analysis in the form of a plot, not just a table (Supplementary 2.3). The regression plots should be shown at least for some key pairwise comparisons (e.g. G1 vs G2). It should be reported whether early and late phase progenitors behave differently.

The regression plots are provided in the revised version of the SI manuscript (Figure SI 2.3.2).

5. In their CDC25B overexpression experiments, the authors found that G1 phase and the overall Tc length are increased. According to a previous study (Peco et al., 2012), gain of CDC25B function leads to increased neurogenesis. This raises the question as to whether the cells with increased G1 length in the CDC25B OE experiment actually go on to divide. The authors should report the results of lineages overexpressing CDC25B in Fig. 5, like they do for the control and delta cdk conditions, as well as report the analysis only for the G1 of those cells where a subsequent mitosis can be observed.

The analysis for CDC25B is added to the figure, see the new version of the figure In the manuscript, all the G1 data analyzed correspond to proliferating cells. Specifically, for the G1 phase, we only took into account the G1 phase length of cells re- entering the cell cycle, i.e. cells that re-enter S phase after G1. The cells for which we could not see the beginning of S phase (cells with a G1 phase superior to 1000 min or lost during the tracking) were excluded from the analysis.

Minor points:

-The authors say that: "FUCCI G1 expressing cells localized on the basal side of the explants are most probably differentiating neurons. The G1 lengths measured for these differentiating cells are excluded from our analyses". It is unclear what exactly is meant here. Are the authors saying that these are cells that will undergo a final division before becoming postmitotic, or are they saying that these cells will exit the cell cycle in G2? Their tool allows them to make this distinction clearly. Furthermore, the basal localization alone does not seem reliable for identifying cells that are becoming postmitotic (e.g. HuC positive cells are also observed in mid-apicobasal positions). In particular, this could be relevant for interpreting the Cdc25B results. Can the authors give an estimate of how the HuC staining changes along the apico-basal axis of electroporated embryos? Or could they use the KO fluorescence intensity in G1 cells relative to PCNA (as electroporation control) to determine whether the reporter can be used to distinguish G1 vs G0 cells?

We are sorry that our text is not clear. These "FUCCI G1 expressing cells" are differentiating neurons obtained from electroporated proliferating progenitors. The FUCCI G1 reporter remains expressed in G0 cells (Sakaue-Sawano et al., 2008). In our hands, the HuC/D marker is used as a neuronal differentiation marker. It is expressed in young neurons around 12 hours after their last mitosis and is observed sometimes in young neurons migrating apico-basally. The cells that we observe in the basal side are never seen re-entering mitosis during our experiments. All these observations suggest that they are young neurons.

- The authors discuss changes in G1 length between mother and daughter cells. But the S phase also seems to shorten. As the duration of S phase is considered an important factor in the maintenance of the proliferative capacity of neural progenitors, do the authors observe differences in S phase length preceding different modes of division?

In our experiments, when we analyzed the duration of the S phase of the mother cell and the outcome of its division, we did not see a clear relationship (see Supplement Figure 8B).

-When describing the neural progenitor's behaviour during live imaging, the authors mention that different division modes can be observed. However, it is not clarified how frequently different MoDs are registered.

In our experiments, we followed 32, 27 and 48 divisions of progenitors in the control, CDC25B and CDC25B$^{\Delta CDK}$ conditions, respectively. The results are summarized in the table 2 of the revised manuscript.

- While the modelling of cell cycles phase distributions and relationships appear to be "well compatible with the observed one" some statistical analysis of the fit of the data would improve this analysis. The authors have also not fully explored other possibilities here, including, for example, weak coupling between phases. On page 15, the statement "This is confirmed using Monte Carlo permutation" is unclear and requires further clarity and justification. The line for the Monte Carlo permutations (Fig.3H) appears to fit somewhere between the actual data and the fully anti-correlated data, especially after Tc durations greater than 900 minutes; again, some analysis of the fit quality of these models would be good.

The two-samples KS goodness of fit test are now added in the new version of the manuscript.

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Second decision letter

MS ID#: DEVELOP/2021/199660

MS TITLE: Single-cell imaging of cell cycle reveals CDC25B-induced heterogeneity of G1 phase length in neural progenitor cells

AUTHORS: Angie Molina, Frederic Bonnet, Julie Pignolet, Valerie Lobjois, Sophie Bel-Vialar, Jacques Gautrais, Fabienne Pituello, and Eric Agius

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

The overall evaluation is positive and we would like to publish a revised manuscript in Development, provided that the referees' comments can be satisfactorily addressed. The referees recognise the advance provided by the single cell resolution live-imaging of neural progenitors, however, both referees raise similar concerns about the analysis and interpretation of the phospho-Rb data (Fig 5). These issues could be addressed by using HuC/D or NeuN to mark neurons and being more explicit about the caveats associated with these experiments. Please attend to all of the reviewers' comments in your revised manuscript and detail them in your point-by-point response. If you do not agree with any of their criticisms or suggestions explain clearly why this is so.

We are aware that you may currently be unable to access the lab to undertake experimental revisions. If it would be helpful, we encourage you to contact us to discuss your revision in greater detail. Please send us a point-by-point response indicating where you are able to address concerns raised (either experimentally or by changes to the text) and where you will not be able to do so within the normal timeframe of a revision. We will then provide further guidance. Please also note that we are happy to extend revision timeframes as necessary.

Reviewer 1

Advance summary and potential significance to field

The revised manuscript by Molina et al explores cycle dynamics in individual cells within the developing chicken spinal cord and argues for a role for the well-studied phosphatase, CDC25B, in lengthening G1 in progenitors. The mechanism of action is unclear and not fully addressed in the manuscript. But the study involves significant advances in single cell imaging analysis of a mixed population of neural progenitors and provides evidence linking G1 phase length heterogeneity with neurogenesis (an idea proposed by a number of other studies). The broad applicability of the study is the argument that G1 phase lengthening in progenitors is a driver of differentiation within growing tissues.

Comments for the author

There were several issues raised with the previous manuscript version. These included phototoxicity, possible involvement of DNA repair mechanisms accounting for G1 lengthening, shortcomings on the use of a gain of function approach with CDC25B, the lack of identification of downstream targets of CDC25B in G1 phase regulation, and clarifications on the modeling aspects of the study. For the most part, these issues were addressed with additional control experiments, clarifications in the text, and the proposal that the Retinoblastoma protein make be a possible target of CDC25B in G1 phase lengthening (see below).
The manuscript is generally well written, and nicely evaluated cell heterogeneity in cell cycle dynamics in a growing tissue—which is a challenging phenomenon to capture dynamically. The authors should be commended for this technical tour-de-force. While it is unfortunate the authors could not more fully explore potential targets of CDC25B in cell cycle lengthening, nor fully account for the discrepancies of their findings with that of other studies, they did address most concerns thoughtfully and have improved their manuscript.

Concerns to address:
The pRB staining in Fig. 5 was widespread—perhaps all the DAPI stained nuclei. If this is the case, how useful of a marker it is? Focusing on the pRB/Tuj1 staining in challenging, especially given the TuJ1 is cytoplasmic. A better marker would have been NeuN or HuC/D to pick out differentiated nuclei.
Assuming the pRB/Tuj1 fraction quantification was accurate, however, the difference in pRB/Tuj1 positivity went from 12% in controls to nearly 20% in CDC25B electroporated cells. This is a relatively small change that alone is unlikely to account for all the G1 phase lengthening heterogeneity observed in the single cell analysis. I would be careful in concluding that pRB levels/activity is sufficient to account of CDC25B-mediated G1 lengthening. This conclusion was emphasized in the abstract and discussion and should be toned down with a generous helping of caveats. As indicated in my earlier critique, the overexpression of CDC25B is probably having a much broader effect on a number of cell cycle regulatory factors to effect changes in cell cycle length.

Reviewer 2

Advance summary and potential significance to field

The authors have improved the manuscript and addressed the raised points.

Comments for the author

In the revised version, the authors propose that CDC25B activity in G2 delays Rb phosphorylation in G1, thereby causing G1 lengthening. The evidence they present to support this is that OE of CDC25B leads to an increase in the number of cells negative for phospho-Rb. This part could be strengthened. On one hand, it is important to demonstrate that the cells that are negative for Rb are in G1. It is possible that CDC25B overexpression pushes cells out of the cell cycle instead of increasing G1. So far the authors look at Tuj staining to exclude postmitotic cells, however, this is not nuclear and it would be easy to miss cells that are exiting the cell cycle. HuC/D staining could make this point clearer. Furthermore to strengthen the link between CDC25B activity and Rb phosphorylation, it would be reassuring to see that Rb phosphorylation changes across time and in particular test whether it is decreased when cells typically start producing CDC25B at the onset of neurogenesis.

Second revision

Author response to reviewers' comments

We appreciate the opportunity to re-submit a revised version of our manuscript and we hope that the additional experiments we made will overcome the limitations that the reviewers saw and we hope you will find it now suitable for publication in Development.

Reviewer 1

Concerns to address:

The pRB staining in Fig. 5 was widespread—perhaps all the DAPI stained nuclei. If this isthe case, how useful of a marker it is? Focusing on the pRB/Tuj1 staining in challenging,especially given the TuJ1 is cytoplasmic. A better marker would have been NeuN orHuC/D to pick out differentiated nuclei.

We added more information regarding our strategy in the result section to make things clearer for the reader and to strengthen our data as recommended by reviewer 2. “Immunostaining for phospho-Rb (S807/811)is classically used to analyze crossing of therestriction point (Moser et al., 2018; Spencer etal., 2013). Our goal was then to identify NPCsdisplaying Rb dephosphorylated

in G1 (phospho-Rb negative cells) as a readout ofthe nuclei that are in the G1 pre restriction point phase. To restrict quantification to NPCsand avoid counting young neurons expected tobe also phospho-Rb negative, we combinedphospho-Rb staining and markers of youngneurons (Tuj1/Tubb3 or HuC/D). We clearlyidentified phospho-Rb and Tuj1 or HuC/D negative cells in the ventricular zone (Fig.5 andnot shown). We also verified that phospho- Rb/HuC/D negative cells were in G1 by using our FUCCI G1 and PCNA reporter (data not shown). We then quantified the percentage ofphospho-Rb (S807/811) negative NPCs afterCDC25B electroporation (Fig.5)."

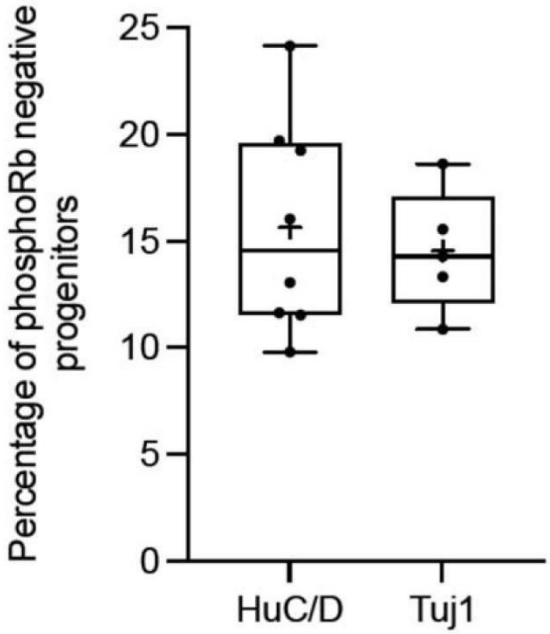

Figure 1: Comparison of the percentage ofphospho-Rb negative NPCs, negative for HuC/D or Tuj1 immunostaining. Counting performed on 5 adjascent sections from 3 embryos stained with either HuC/D or Tuj1.

In the manuscript figure 5, we therefore quantified the percentage of nuclei that are NOTstained by phospho Rb. As mentioned in the text 12.2%+/-0.8 of control electroporated cells where phospho-Rb/Tuj1 negative. This percentage raised to 19.9%+/-1.3% for CDC25B electroporated cells but was not affected by CDC25BΔCDK electroporation(10.9%+/-0.7%).

We did not use NeuN because it is expressed late during neuronal differentiation (Kim et al., 2013). Indeed, in our hands Tuj1 is often detected earlier than HuC/D. Nevertheless, as recommended, we compared Tuj1 and HuC/D expression to pick out differentiated cells. As shown, in the Fig. 1, there is no significant difference in the percentage of phospho Rb negative NPCs, the use of either marker gives comparable results.

Assuming the pRB/Tuj1 fraction quantification was accurate, however, the difference in pRB/Tuj1 positivity went from 12% in controls to nearly 20% in CDC25B electroporatedcells. This is a relatively small change that alone is unlikely to account for all the G1 phase lengthening heterogeneity observed in the single cell analysis. I would be careful inconcluding that pRB levels/activity is sufficient to account of CDC25B-mediated G1 lengthening. This conclusion was emphasized in the abstract and discussion and shouldbe toned down with a generous helping of caveats. As indicated in my earlier critique, the overexpression of CDC25B is probably having a much broader effect on a number of cell cycle regulatory factors to effect changes in cell cycle length.

We agree with the reviewer comments on "careful in concluding that pRB levels/activity is sufficient to account of CDC25B-mediated G1 lengthening". We thought it was already thecase as we had written it. We now modify the sentences in the abstract as follows: "Weidentify that the core cell cycle machinery CDC25B phosphatase, known to regulate G2/M transition, indirectly increases the duration of the G1 phase. Part of the mechanism possibly relies on delaying restriction point crossing" In the discussion we also tone downthe conclusion " CDC25B control of

restriction point crossing could therefore contribute, at least partly, to G1 phase length heterogeneity. Deciphering this hypothesis in our model system is challenging and will require further investigations, including single cellmeasurement of the time spent in G1 prior restriction point crossing.".

Reviewer 2

Comments for the Author:

In the revised version, the authors propose that CDC25B activity in G2 delays Rb phosphorylation in G1, thereby causing G1 lengthening. The evidence they presentto support this is that OE of CDC25B leads to an increase in the number of cells negative for phospho-Rb. This part could be strengthened. On one hand, it is important to demonstrate that the cells that are negative for Rb are in G1. It is possible that CDC25B overexpression pushes cells out of the cell cycle instead of increasing G1. So far the authors look at Tuj staining to exclude postmitotic cells, however, this is not nuclear and it would be easy to miss cells that are exiting the cell cycle. HuC/D staining could make this point clearer. Furthermore, to strengthen the link between CDC25B activity and Rb phosphorylation, it would bereassuring to see that Rb phosphorylation changes across time and in particular test whether it is decreased when cells typically start producing CDC25B at the onset of neurogenesis.

We have now compared the HuC/D and Tuj1 staining and show that they are equivalent in our hands (Fig.1). To verify that the phospho-Rb negative cells are in G1, we haveelectroporated the Fucci G1 PCNA markers and we performed phospho-Rb and HuC/D staining 24h hours later (Fig 2).

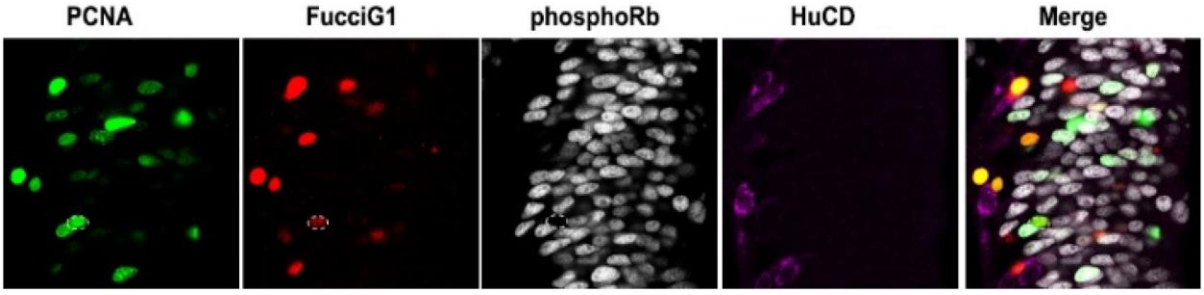

Figure 2: Cross-sections of chick neural tube expressing mKO-zCdt1-pIRES-NLS-EGFP-L2-PCNA reporter and stained with anti phosphoRb and HuCD antibodies. The dashed line shows a electroporated cell (PCNA positive), which is negative for phosphoRb and expresses the FucciG1 reporter.

We counted 11 sections on 3 different embryos and out of the 48 electroporated progenitors that were phospho-Rb negative, we observed 8 cells in mitosis and 40 cells Fucci G1 positive and HuC/D negative. Thus, NPCs located in the ventricular zone, FucciG1 positive and HuC/D negative are most likely cells in G1.

The data are described in the result section as data not shown.

As mentioned in the introduction, neurogenesis progresses from ventral to dorsal regions in the chicken neural tube (Kicheva et al., 2014). To test whether Rb phosphorylationcorrelates with neurogenesis, we have compared the percentage of neural progenitor cellsnegative for phospho-Rb protein in the ventral and dorsal regions of the neural tube atstage (E 2.5, HH18). This stage corresponds to a peak period of motor neuron production in the ventral neural tube whereas very few HuC/D positive cells are detected in the dorsal region (Fig. 3). CDC25Bis highly expressed in the ventral spinal cord at this stage (Bonnet et al., 2018; Peco et al., 2012). We clearly detected a significant higher percentage of NPCs negative for both HuC/D and phospho-Rb in the ventral neural tube than in the dorsal region. We also checked at 2 more stages (HH12, HH 20) and also observed a correlation between the percentage of phospho-Rb and HuC/D negative NPCs and neurogenesis (not shown). We do not plan to addthese data to the present manuscript.

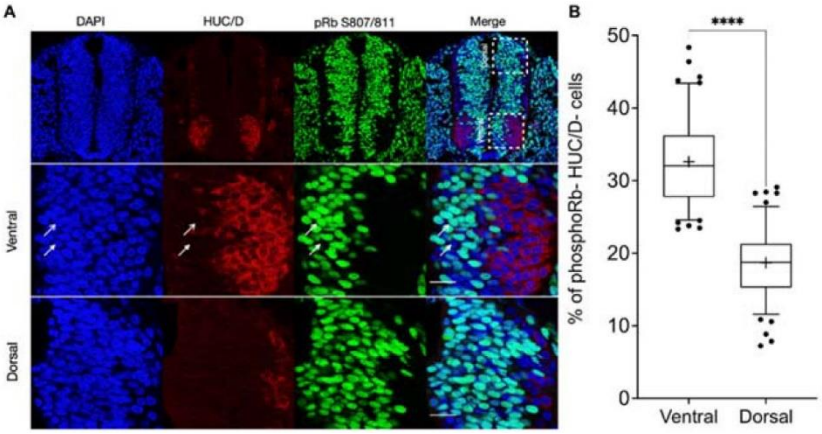

Figure 3 : The ventral region of the chicken neural tube shows a higher percentage of NPCs negative for phosphorylated retinoblastoma protein than in the dorsal region. A- Representative cross-sections of chick neural tube with immunofluorescence against HuC/D and phospho-Rb (S807/811). Zoom in pictures in the ventral and dorsal region. The full arrow shows a negative cell for phospho-Rb staining and negative for HuC/D; the empty arrow shows a positive cell for phospho-Rb staining and negative for HUC/D. B- Box and whiskers plots (5-95 percentile) of the proportion of phospho-Rb negative progenitors (HuC/D negative cells) in the ventral and the dorsal region. The results correspond to 108 sections from 8 embryos from 3 independent experiments. The cross represents the mean.
*****p<0,0001.*

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Third decision letter

MS ID#: DEVELOP/2021/199660

MS TITLE: Single-cell imaging of cell cycle reveals CDC25B-induced heterogeneity of G1 phase length in neural progenitor cells

AUTHORS: Angie Molina, Frederic Bonnet, Julie Pignolet, Valerie Lobjois, Sophie Bel-Vialar, Jacques Gautrais, Fabienne Pituello, and Eric Agius
ARTICLE TYPE: Research Article

I am happy to tell you that your manuscript has been accepted for publication in Development, pending our standard ethics checks.