Heterogeneous nuclear ribonucleoprotein A3a controls mitotic progression of neural progenitors via interaction with cohesin

Min-Yi Ou, Xiang-Chun Ju, Yi-Jun Cai, Xin-Yao Sun, Jun-Feng Wang, Xiu-Qing Fu, Qiang Sun and Zhen-Ge Luo
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Original submission

First decision letter

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MS TITLE: Heterogeneous nuclear ribonucleoprotein A3 controls neural progenitor division and temporal patterning of cortical neurons

AUTHORS: Min-Yi Ou, Xiang-Chun Ju, Yi-Jun Cai, Xin-Yao Sun, Jun-Feng Wang, Qiang Sun, and Zhen-Ge Luo

I have now received the reports of three referees on your manuscript and I have reached a decision. The reports are appended below and 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, all the referees express great interest in your work, but they also have significant criticisms and recommend a substantial revision of your manuscript before we can consider publication. In particular, they request further analysis of the mechanism by which HNRNPA3 promotes cell cycle progression, and referee 2 suggests to examine activation of the spindle assembly checkpoint. If you are able to revise the manuscript along the lines suggested by the referees, which may involve further experiments, I will be happy to receive a revised version of the manuscript. Your revised paper will be re-reviewed by the original referees, and its acceptance will depend on your addressing satisfactorily all their major concerns. Please also note that Development will normally permit only one round of major revision.

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

Reviewer 1

Advance summary and potential significance to field

This paper documents the expression pattern of the RNA binding protein HNRNPA3, showing that it is associated with chromosomes in cortical neural progenitors and assesses its loss of function phenotype in HNRNPA-KO mice, the human neural stem cell line ReN and human cerebral organoids. The study reveals a consistent phenotype of delayed mitosis and fewer deep layer neurons and the authors conclude that HNRNPA3 is required to maintain chromosome integrity and to promote neuronal competency. The study is largely descriptive and phenomenological and does not provide mechanistic insight into how this RNA binding protein regulates chromosome organization during mitosis or how it influences neuronal differentiation.

Comments for the author

Detailed comments

1) It is not clear whether HNRNPA is neural specific or simply enriched in certain neural progenitor cell populations – do other cells express it in the embryo and are these other tissues /cell types affected in the KO condition?
2) The authors show that γH2AX, the DNA damage driven histone modification, is upregulated in ReN neural stem cells – is this also observed in the KO mouse and human cerebral organoids?
3) Can the authors provide details on how regions of the cerebral organoids were selected for analysis.
4) Have the authors performed any gain of function approaches in these three cellular contexts?
5) This study would be more informative if regulation of HNRNPA3 was also addressed and in particular if its relationship with other proteins (noted in the Introduction) that regulate similar cell behaviours, mitotic progression and spindle orientation in cortical neurons was addressed.
6) The major weakness of the paper is that it does not provide mechanistic insight into how this RNA binding protein regulates chromosome organization during mitosis or how it influences neuronal differentiation.

Reviewer 2

Advance summary and potential significance to field

Ou et al show that HNRNPA3 is expressed in progenitors of the developing cortex in both, mice and humans. During the cell cycle, HNRNPA3 seems to be required for M-phase progression. Genetic deletion of Hnrnpa3 in mice as well as knock-down in human organoids induced a reduction of deep layer neurons, underlining a conserved function of Hnrnpa3 in regulating neuronal output of early neurogenic progenitors. The authors provide evidence that loss of HNRNPA3 leads to mitotic delay by reducing the amount of early progenitors entering anaphase. Thus, they hypothesize that reduced proliferative capacity results in decreased production of lower layer neurons.

Comments for the author

1. The authors show that loss of Hnrnpa3 results in a reduction of lower layer neurons in the developing cortex. To fully appreciate the severity of the phenotype analysis at a later time point should be done, e.g. at P21.

2. The origin of the mispositioned PH3+ cells (shown in Fig 3E) is not clear. It seems an attractive hypothesis that these cells are derived from mitotically delayed progenitors. Why not testing this hypothesis by injecting EdU at E12.5, collect brains at E13.5 and perform EdU/PH3 staining on these samples?

3. It appears striking that despite the formation of anagen bridges and resulting chromosome fragmentation, mitotically delayed progenitors don't undergo apoptosis. What is happening to these progenitors? Do they activate p53 and/or upregulate DNA repair machinery to continue the cell cycle at one point? Do they never complete the cell cycle (upper layer neuron production does not

seem to be affected, despite E17.5 cortex still presenting a significantly higher number of PH3+ cells)? The authors should perform gH2AX, p53 and DNA repair machinery stainings in mouse cortices at E13.5 or E14.5 to follow up on this question.

4. The data presented in the manuscript strongly suggest activation of the spindle assembly checkpoint. Is this the case? And if yes, it should be analyzed in some more detail, e.g. by staining for Bub1 or by characterizing Cohesin positioning in prometaphase cells. The prometaphase arrest and the time lapse analysis shown in Fig S2 (which even suggests repeated condensation-decondensation of the chromosomes) might be indicative of a failure of Cohesin degradation upon Hnrnpa3-deficiency. Unless checkpoint activation is clearly confirmed, the statement in paragraph 1 page 7 "… abnormal distribution of mitotic cells might be due to arrest of cell division" should be rephrased.

5. The authors have a good HNRNPA3 antibody in their hands. Please also show protein expression or HNRNPA3 in mouse cortex of E12.5, E14.5 and E15.5 to complement the RNA expression data presented in Fig S1 and demonstrate that the protein is specifically expressed in early progenitors producing lower layer neurons, but not later-stage progenitors. Along this line, show HNRNPA3 staining on human tissue and organoids together with HOPX staining for a more precise characterization of the cell type expressing HNRNPA3.

Textual/Presentation Issues:
1. The title of the manuscript should be changed. The role of Hnrnpa3 was not investigated in neurons, but in progenitors. Thus, "temporal patterning of cortical neurons" should be removed.
2. The introduction is lacking any information about Hnrnpa3. Some information provided in the discussion should be moved to introduction to formulate a clear scientific question why this gene might play a role in neurogenesis.
3. The first paragraph on page 4 should be rephrased. It does not appear to have a clear message. The authors should again be careful with wordings, e.g. they should be cautious to not mix up cell cycle length with cell cycle exit.
4. The discussion should elaborate on the possibility that Hnrnpa3 itself does not necessarily need to be involved in correct M-phase progression, but that its loss can result in alterations of alternative splicing. Thus, gene products of impaired alternative splicing might slow down M-phase progression.
5. The abbreviation "AP" found in the introduction was not explained anywhere in the text.
6. Please use a different crop for either Fig. 3B or Fig. 3E. Both crops are derived from the same image.
7. I suggest to show Figure 5 as last figure of the manuscript. The current arrangement is slightly confusing, since current Figure 6 is jumping back to mouse cortex.
8. Fig. S6: is it a representative image for the Hnrnpa3 KO cortex? If yes, the Ki67 staining in the VZ is strikingly different between control and KO mice. What is the explanation for this phenomenon.
9. The manuscript requires rigorous proofreading and correction of many grammatical and typographical errors.

Reviewer 3

Advance summary and potential significance to field

DEVELOP/2019/185132
Heterogeneous nuclear ribonucleoprotein A3 controls neural progenitor division and temporal patterning of cortical neurons
Min-Yi Ou, Xiang-Chun Ju, Yi-Jun Cai, Xin-Yao Sun, Jun-Feng Wang, Qiang Sun, and Zhen-Ge Luo

In this manuscript, Min-Yi et al report the expression and role of heterogeneous nuclear ribonucleoprotein A3 (HNRNPA3) in regulating the division of early cortical neuronal precursors. They generate a novel knock out mice line and demonstrate that HNRNPA3 is essential for the correct generation of deep-layer neurons. Interestingly, the authors interpret that upper layers are not majorly affected. This would have implications for our understanding cell fate acquisition and segregation of precursor cells.

Comments for the author

This is an interesting manuscript that will be of interest to readers in the field. The experiments are well performed, figures are clear and the manuscript is clearly written. Nevertheless, I consider that there are some issues that should be addressed before publication as suggested in the r.

Major.
1-The major concern I have is the interpretation that the upper layers are not affected. I am not convinced that the data shows this and this is a forced interpretation given the current data. In fact, I do consider that the manuscript would be also interesting if it turned out that the authors can not strongly support the claim that upper layers precursors are not affected.
My concern arises because most of the experiments shown do not analyze upper layer precursors and upper layer generation directly, but extract conclusions from more indirect evidence.
In order to support this claim the authors need to:
a- Show whole sections of the cortex of postnatal animals of at least 1 week old with stainings for Cux1 and Ctp2 or equivalent in order to illustrate the extension of the loss of Ctip3 compare to upper layers.
b- In Figure 6 the authors show experiments of BrdU incorporation at E12.5 and (A-B) and BrdU at E14.5 S6A-B and Ki67 staining. They also analyze the generation of Ctip2+ and Cux1. They claim that upper layers are not affected because the fraction of E14.5 BrdU cells exiting the cell cycle is not affected and because the fraction (10%) of Cux1/E12.5 BrdU positive cells is not altered.
I find some concerns in these figures and hence the interpretations. In Figure 6H-J the authors analyze the fraction of Cux1 positive cells generated at E12.5, which in accordance with their late origin is very low, 10%. from the literature and also when looking at the panels I would expect even less. I hardly see a few positive BrdU cells in the upper layers and they do not seem to represent 10% of brdu+ total cells. It is also of note that the arrow points to a layer I neuron. This BrdU cells could be interneurons generated in ventral telencephalon. Please re-check/ re-analyze the data.
In Figure S6A, the panel showing Hnrnpa3 KO is disturbing. The VZ is devoid of Ki67 signal and there are many Brdu+ Ki67- cells in the VZ, which confounds the data and interpretations. Is this an artifact of staining or confocal analysis or true data? Please clarify. If this is not artefactual, then the authors have to separate the analysis of BrdU/Ki67 cells in the VZ and SVZ. If artefactual, then the authors have to quantify again with new stainings In these experiments of BrdU at E12.5 and at E14.5 the authors could also count the fraction of Ki67+ cells that are brdU+ in order to estimate differences in the cell cycle re-entry. This will help support or reinterpret their data.
2-The description of the knock out is very poor. The diagram in Figure 2A should be improved. The interpretation of the blots shown as short exposure and long exposure should be explained at length in the result section. Are the authors implying that there is some protein? Is it the product of alternative splicing (the band in ko seems a bit high).
Also, the viability and general development of the KOs should be described. Do these animals survive to adulthood? Do they present any gross malformations, defects, etc…This is important to understand the importance of HNRNPA3 and also because for the analysis of upper layers it would be best to analyze animals older than P0.

Minor
1-In Figure 1D-in y-axis indicate expression of HNRNPA3
2-In pg 6 ln 13-14 “layer II-III marker Cux1” should be corrected. Cux1 is a marker for layers II-IV.

First revision

Author response to reviewers' comments

Point-by-point responses to Reviewers comments:

Reviewer 1
This paper documents the expression pattern of the RNA binding protein HNRNPA3, showing that it is associated with chromosomes in cortical neural progenitors and assesses its loss of function phenotype in HNRNPA-KO mice, the human neural stem cell line ReN and human cerebral organoids. The study reveals a consistent phenotype of delayed mitosis and fewer deep layer neurons and the authors conclude that HNRNPA3 is required to maintain chromosome integrity and to promote neuronal competency. The study is largely descriptive and phenomenological and does not provide mechanistic insight into how this RNA binding protein regulates chromosome organization during mitosis or how it influences neuronal differentiation.

Response: We are grateful to this Reviewer for the constructive comments that have helped us to improve the quality of this study. We have performed a number of new experiments to further analyze the mechanism by which HNRNPA3 promotes cell cycle progression, and found that HNRNPA3 acts via modulating the cohesin-association with chromosomes to regulate chromosome organization during mitosis of neural progenitors (see points 4 and 5, and revised Fig. 5).

Detailed comments
1) It is not clear whether HNRNPA is neural specific or simply enriched in certain neural progenitor cell populations – do other cells express it in the embryo and are these other tissues /cell types affected in the KO condition?

Response: This is a reasonable concern. We analyzed the expression of Hnrnpa3 in various tissues. As shown in revised Fig. S1E, Hnrnpa3a was widely expressed in various tissues, including cerebrum, cerebellum, lung, pancreas, and spinal cord, whereas Hnrnpa3b was expressed in limited tissues such as cerebrum, kidney and skeletal muscle. The KO mice died soon after birth (within 1-2 days), probably due to neonatal hypoxia caused by lung developmental defects (see revised Fig. S2). Because of the neonatal lethality, we also examined neuromuscular synapses in the diaphragm, which controls breathing, and found no defect in neonatal KO mice (Fig. S2C). Given that Hnrnpa3 is enriched in neural progenitors at early stages of cortex development (Fig. 1 and Fig. S1A-D), this study mainly focuses on its role in cortex development.

2) The authors show that γH2AX, the DNA damage driven histone modification, is upregulated in ReN neural stem cells – is this also observed in the KO mouse and human cerebral organoids?

Response: Yes! This phenomenon is also observed in the KO mice and human cerebral organoids with HNRNPA3 down-regulation (see Revised Fig. S4C-F and Fig. S7G-I).

3) Can the authors provide details on how regions of the cerebral organoids were selected for analysis.

Response: As shown in previous studies (Lancaster MA et al 2013 Nature; Lancaster MA & Knoblish J 2014 Nature Protocols), the neuroepithelial buds emanated from outer surface of cerebral organoids were selected for imaging analysis. This information has been added in revised manuscript (see Page 19, lines 539-541 and legends of Fig. S7B, D).

4) Have the authors performed any gain of function approaches in these three cellular contexts?

Response: As shown in the revised manuscript, we performed gain-of-function studies by over-expressing HNRNPA3 in human neural progenitor ReN cells and in mice. Indeed, forced expression of HNRNPA3 caused an increase in cell proliferation of ReN cells (see revised Fig. S5G, H), suggesting that HNRNPA3 might facilitate mitotic progression. In line with positive association between the level of Hnrnpa3 and NP stemness, over-expression of Hnrnpa3 in mice at E14.5 via in utero electroporation (IUE) caused an increase in Pax6-labeled RG cells in mice at E16.5 (see revised Fig. S6I, J).

5) This study would be more informative if regulation of HNRNPA3 was also addressed and in particular if its relationship with other proteins (noted in the Introduction) that regulate similar cell behaviours, mitotic progression and spindle orientation in cortical neurons was addressed.

Response: We performed new sets of experiments to understand the relationship between HNRNPA3 and other proteins that regulate mitotic progression and/or spindle orientation. We found that HNRNPA3 associates with the core cohesin complex protein SMC1 and regulates its release from sister chromosomes during cell division (see revised Fig. 5). It is known that cohesin proteins, including SMC1, play important roles in sister chromatids separation during cell division. The regulation of cohesin by HNRNPA3 can explain why its deficiency caused formation of chromosomes bridges and mitotic delay. Notably, a recent study has implicated a relationship between cohesion complex and human brain developmental disorder (Kruszka P et al Brain 2019).

6) The major weakness of the paper is that it does not provide mechanistic insight into how this RNA binding protein regulates chromosome organization during mitosis or how it influences neuronal differentiation.

Response: As mentioned above, the regulation of cohesin association with chromosomes provides a mechanistic insight into how HNRNPA3 regulates chromosome organization during mitosis of NPs. We believe that the hindered cell division caused by the loss of HNRNPA3 also impaired neuronal competency.

Reviewer 2 Advance summary and potential significance to field
Ou et al show that HNRNPA3 is expressed in progenitors of the developing cortex in both, mice and humans. During the cell cycle, HNRNPA3 seems to be required for M-phase progression. Genetic deletion of Hnrnpa3 in mice as well as knock-down in human organoids induced a reduction of deep layer neurons, underlining a conserved function of Hnrnpa3 in regulating neuronal output of early neurogenic progenitors. The authors provide evidence that loss of HNRNPA3 leads to mitotic delay by reducing the amount of early progenitors entering anaphase. Thus, they hypothesize that reduced proliferative capacity results in decreased production of lower layer neurons.

Reviewer 2 Comments for the author
1. The authors show that loss of Hnrnpa3 results in a reduction of lower layer neurons in the developing cortex. To fully appreciate the severity of the phenotype analysis at a later time point should be done, e.g. at P21.

Response: This is an interesting point! Unfortunately, the Hnrnpa3 KO mice died soon after birth (within 1-2 days), probably due to neonatal hypoxia caused by lung developmental defects (see revised Fig. S2A, D, E). Considering that cortical layer lamination in mice already complete before birth, we are confident that our analysis in late embryonic stages (E18.5) and P0 is sufficient to conclude Hnrnpa3's role in the generation of early-born deep layer neurons (Fig. 2). This conclusion was further strengthened by the analysis for layer-specific cell types in BrdU pulse labeling (Fig. 6H-K) and in utero electroporation (IUE) experiments (Fig. S6).

2. The origin of the mispositioned PH3+ cells (shown in Fig 3E) is not clear. It seems an attractive hypothesis that these cells are derived from mitotically delayed progenitors. Why not testing this hypothesis by injecting EdU at E12.5, collect brains at E13.5 and perform EdU/PH3 staining on these samples?

Response: This is a good point! As suggested by the Reviewer, we pulse labelled cycling NPs by BrdU injection at E12.5 and examined 6 hr later to avoid the intervention of cells that may re-enter another round of cell cycle, according to the protocol introduced recently (Ye et al., 2017 Neuron 96, 1041-1054). We found that the Hnrnpa3 KO mice exhibited an increase in the percentage of PH3+BrdU+ cells (see revised Fig. S3C, D), which represents cells arrested in mitosis (Ye et al., 2017 Neuron 96, 1041-1054). This result also indicates that the mispositioned PH3+ cells were originated from NPs with mitotic delay.

3. It appears striking that despite the formation of anagen bridges and resulting chromosome fragmentation, mitotically delayed progenitors don't undergo apoptosis. What is happening to these progenitors? Do they activate p53 and/or upregulate DNA repair machinery to continue the cell cycle at one point? Do they never complete the cell cycle (upper layer neuron production does not seem to be affected, despite E17.5 cortex still presenting a significantly higher number of PH3+

cells)? The authors should perform gH2AX, p53 and DNA repair machinery stainings in mouse cortices at E13.5 or E14.5 to follow up on this question.

Response: This is a reasonable concern. As suggested by the Reviewer, we examined levels of DNA damage response marker γH2AX and DNA repair activation marker phosphor-Atm (pS1981) in mice at E13.5, and found that both of them were elevated in KO mice (see revised Fig. S4C-F). The activation of DNA repair pathway may help to maintain cells at steady state and prevent cell death. Indeed, the NPs with chromatin bridges still finished mitotic process, and finally gave rise to two daughter cells, although this process was abnormally prolonged (Fig. 4D). This also explains why the production of upper layer precursors (revised Fig. S3A, B) and neurons (Fig. 2C, F) was not affected.

4. The data presented in the manuscript strongly suggest activation of the spindle assembly checkpoint. Is this the case? And if yes, it should be analyzed in some more detail, e.g. by staining for Bub1 or by characterizing Cohesin positioning in prometaphase cells. The prometaphase arrest and the time lapse analysis shown in Fig S2 (which even suggests repeated condensation-decondensation of the chromosomes) might be indicative of a failure of Cohesin degradation upon Hnrnpa3-deficiency. Unless checkpoint activation is clearly confirmed, the statement in paragraph 1 page 7 "… abnormal distribution of mitotic cells might be due to arrest of cell division" should be rephrased.

Response: Good point! As suggested by the Reviewer, we first examined localization of the spindle check point protein BubR1 and found that it remained associated with chromosomes in HNRNPA3 knockdown NPs at prometaphase (revised Fig. 5A), suggesting that HNRNPA3 may not be involved in the activation of the spindle assembly checkpoint directly. Interestingly, we found that HNRNPA3 associated with the key component of cohesion complex protein SMC1 and down-regulation of HNRNPA3 influenced the release of SMC1 from chromosomes in NPs at metaphase (revised Fig. 5B-F). These results support the possibility that HNRNPA3 regulates NP division through modulating dynamic association of cohesin proteins with chromosomes during mitotic progression. The mentioned statement has been rephrased.

5. The authors have a good HNRNPA3 antibody in their hands. Please also show protein expression or HNRNPA3 in mouse cortex of E12.5, E14.5 and E15.5 to complement the RNA expression data presented in Fig S1 and demonstrate that the protein is specifically expressed in early progenitors producing lower layer neurons, but not later-stage progenitors. Along this line, show HNRNPA3 staining on human tissue and organoids together with HOPX staining for a more precise characterization of the cell type expressing HNRNPA3.

Response: As suggested by the Reviewer, we performed several sets of staining to determine the expression of HNRNPA3 protein in mouse cortex, human tissue and organoids. As shown in revised Fig. S1D, Hnrnpa3 was expressed highly in VZ/SVZ region of mice at E12.5, and gradually decreased in E14.5 and E15.5. This result complements the RNA expression data. In human tissue and cerebral organoids, many RGs labeled by HOPX also expressed HNRNPA3 (Fig. 1D and Fig. S1A), in line with the conclusion that HNRNPA3 is enriched in NPs with high stemness.

Textual/Presentation Issues:
1. The title of the manuscript should be changed. The role of Hnrnpa3 was not investigated in neurons, but in progenitors. Thus, "temporal patterning of cortical neurons" should be removed.

Response: As suggested by the Reviewer, the title of revised manuscript is changed to "Heterogeneous nuclear ribonucleoprotein A3 controls mitotic progression of neural progenitors".

2. The introduction is lacking any information about Hnrnpa3. Some information provided in the discussion should be moved to introduction to formulate a clear scientific question why this gene might play a role in neurogenesis.

Response: As shown in the revised manuscript (page 4, lines 90-99), more background about Hnrnpa3 has been added to the introduction section.

3. The first paragraph on page 4 should be rephrased. It does not appear to have a clear message. The authors should again be careful with wordings, e.g. they should be cautious to not mix up cell cycle length with cell cycle exit.

Response: Sorry for the confusion. These sentences have been rephrased.

4. The discussion should elaborate on the possibility that Hnrnpa3 itself does not necessarily need to be involved in correct M-phase progression, but that its loss can result in alterations of alternative splicing. Thus, gene products of impaired alternative splicing might slow down M-phase progression.

Response: This point has been added to the discussion section of revised manuscript (see page 15, lines 419-423).

5. The abbreviation “AP” found in the introduction was not explained anywhere in the text.

Response: “AP” represents “apical progenitors” (see revised manuscript line 68, page 3).

6. Please use a different crop for either Fig. 3B or Fig. 3E. Both crops are derived from the same image.

Response:Corrected (see revised Fig. 3B and 3E).

7. I suggest to show Figure 5 as last figure of the manuscript. The current arrangement is slightly confusing, since current Figure 6 is jumping back to mouse cortex.

Response:As suggested by the Reviewer, the order of these two figures has been changed (see revised Fig. 6 and 7).

8. Fig. S6: is it a representative image for the Hnrnpa3 KO cortex? If yes, the Ki67 staining in the VZ is strikingly different between control and KO mice. What is the explanation for this phenomenon.

Response: The original image was an artifact produced during image acquisition. It has been replaced by a representative image (see revised Fig. S6A) and data was re-analyzed accordingly.

9. The manuscript requires rigorous proofreading and correction of many grammatical and typographical errors.
Response: Thanks. We have done rigorous proofreading and corrected all identified errors.

Reviewer 3 Advance summary and potential significance to field
In this manuscript, Min-Yi et al report the expression and role of heterogeneous nuclear ribonucleoprotein A3 (HNRNPA3) in regulating the division of early cortical neuronal precursors. They generate a novel knock out mice line and demonstrate that HNRNPA3 is essential for the correct generation of deep-layer neurons. Interestingly, the authors interpret that upper layers are not majorly affected. This would have implications for our understanding cell fate acquisition and segregation of precursor cells.

Reviewer 3 Comments for the author
This is an interesting manuscript that will be of interest to readers in the field. The experiments are well performed, figures are clear and the manuscript is clearly written. Nevertheless, I consider that there are some issues that should be addressed before publication.

Response: We are grateful to the Reviewer for the positive comments. The constructive suggestions have helped to improve the quality of the revised manuscript greatly.

Major.
1-The major concern I have is the interpretation that the upper layers are not affected. I am not convinced that the data shows this and this is a forced interpretation given the current data. In fact, I do consider that the manuscript would be also interesting if it turned out that the authors can not strongly support the claim that upper layers precursors are not affected.

My concern arises because most of the experiments shown do not analyze upper layer precursors and upper layer generation directly, but extract conclusions from more indirect evidence.

Response: In the revised manuscript, we analyzed intermediate progenitors (IPs) labeled by Tbr2, which give rise to late-born upper layer neurons, and found no difference between WT and KO mice (revised Fig. S3A, B). The conclusion that Hnrnpa3 mainly affects early-born deep layer neurons, but not late-born upper layer neurons, was also supported by the results of BrdU pulse labeling (Fig. 6H-K) and in utero electroporation (IUE) experiments (Fig. S6D-H). We believe Hnrnpa3 may not play a direct role in determining the specification of neuronal fates. The temporal dynamic expression pattern of Hnrnpa3 may underlie its specific role, or alternatively, highly proliferative NPs might be more vulnerable to the loss of Hnrnpa3. This point has been discussed in revised manuscript.

In order to support this claim the authors need to: a-Show whole sections of the cortex of postnatal animals of at least 1 week old with stainings for Cux1 and Ctp2 or equivalent in order to illustrate the extension of the loss of Ctip3 compare to upper layers.

Response:This is an interesting point! Unfortunately, the Hnrnpa3 KO mice died soon after birth (within 1-2 days), probably due to neonatal hypoxia caused by lung developmental defects (see revised Fig. S2A, D, E). Considering that cortical layer lamination in mice has already completed before birth, we are confident to conclude that Hnrnpa3 mainly affects the generation of early-born deep layer neurons by analyzing cortical layers in late embryonic stages (E18.5) and P0 (Fig. 2). This conclusion was further strengthened by the analysis for layer-specific cell types in BrdU pulse labeling (Fig. 6H-K) and in utero electroporation (IUE) experiments (Fig. S6).

b-In Figure 6 the authors show experiments of BrdU incorporation at E12.5 and (A-B) and BrdU at E14.5 S6A-B and Ki67 staining. They also analyze the generation of Ctip2+ and Cux1. They claim that upper layers are not affected because the fraction of E14.5 BrdU cells exiting the cell cycle is not affected and because the fraction (10%) of Cux1/E12.5 BrdU positive cells is not altered.
I find some concerns in these figures and hence the interpretations. In Figure 6H-J the authors analyze the fraction of Cux1 positive cells generated at E12.5, which in accordance with their late origin is very low, 10%. from the literature and also when looking at the panels I would expect even less. I hardly see a few positive BrdU cells in the upper layers and they do not seem to represent 10% of brdu+ total cells. It is also of note that the arrow points to a layer I neuron. This BrdU cells could be interneurons generated in ventral telencephalon. Please re-check/ re-analyze the data.

Response: Although the fraction of Cux1 positive cells in E12.5 mice is very low due to their late origin, they were still recognizable. We only analyzed the percentage of Cux1+BrdU (full retained) cells among cells with full retained BrdU. As suggested by the Reviewer, we have re-analyzed the data without including layer I neurons (see revised Fig. 6H, K).

In Figure S6A, the panel showing Hnrnpa3 KO is disturbing. The VZ is devoid of Ki67 signal and there are many Brdu+ Ki67- cells in the VZ, which confounds the data and interpretations. Is this an artifact of staining or confocal analysis or true data? Please clarify. If this is not artefactual, then the authors have to separate the analysis of BrdU/Ki67 cells in the VZ and SVZ. If artefactual, then the authors have to quantify again with new stainings.

Response: Sorry for the confusion. The original image was an artifact produced during image acquisition. It has been replaced with a representative image from the same mouse (see revised Fig. S6A). We also re-analyzed the data without including the deleted image. The new analysis did not change the previous conclusion (see revised Fig. S6B and legend).

In these experiments of BrdU at E12.5 and at E14.5 the authors could also count the fraction of Ki67+ cells that are brdU+ in order to estimate differences in the cell cycle re-entry. This will help support or reinterpret their data.

Response: As suggested by the Reviewer, we also analyzed the percentage of BrdU+Ki67+ cells among BrdU+ cells, which showed reduction in E12.5 mice (revised Fig. 6C) but not E14.5 mice (revised Fig. S6C).

2-The description of the knock out is very poor. The diagram in Figure 2A should be improved. The interpretation of the blots shown as short exposure and long exposure should be explained at length in the result section. Are the authors implying that there is some protein? Is it the product of alternative splicing (the band in ko seems a bit high).

Response: Thanks to the Reviewer for pointing out this. The diagram in Fig. 2A has been changed. To more clearly demonstrate the knock out effects, the brain samples of mice were blotted with antibodies against Hnrnpa3a or pan-Hnrnpa3. As shown in revised Fig. 2B, the homozygous Hnrnpa3a KO mice showed marked reduction in the level of Hnrnpa3a, but not Hnrnpa3b.

Also, the viability and general development of the KOs should be described. Do these animals survive to adulthood? Do they present any gross malformations, defects, etc…This is important to understand the importance of HNRNPA3 and also because for the analysis of upper layers it would be best to analyze animals older than P0.

Response: As mentioned above, although there was no apparent abnormality in the morphology of whole body and the brain (Fig. S2A, B), the Hnrnpa3a KO mice died shortly after birth (within 1-2 days after birth) and could not survive to adulthood, probably due to neonatal hypoxia caused by lung developmental defects (Fig. S2A, D, E). The neuromuscular synapses in the diaphragm were roughly normal (Fig. S2C), excluding the effect on the development of motor system which controls breathing. These information has been provided in revised manuscript.

Minor
1-In Figure 1D-in y-axis indicate expression of HNRNPA3
Response: Changed.

2-In pg 6 ln 13-14 “layer II-III marker Cux1” should be corrected. Cux1 is a marker for layers II-IV.
Response: Corrected (see revised manuscript, page 6, line 166).

Second decision letter

MS ID#: DEVELOP/2019/185132

MS TITLE: Heterogeneous nuclear ribonucleoprotein A3 controls mitotic progression of neural progenitors

AUTHORS: Min-Yi Ou, Xiang-Chun Ju, Yi-Jun Cai, Xin-Yao Sun, Jun-Feng Wang, Xiu-Qing Fu, Qiang Sun, and Zhen-Ge Luo

I have now received the reports of the three referees who had reviewed the earlier version of your manuscript and I 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 reviewers’ evaluation is positive and we would like to publish a revised manuscript in Development, provided that you satisfactorily address the remaining suggestions and comments of referees 1 and 3. In particular, referee 3 requests that you integrate better the data added to the revised version into the main figures and the text, that you clarify throughout the text which Hnrnpa isoform you analyse, and that you report the temporal expression pattern of the Hnrnpa3a isoform. Please attend to all the referees’ 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.

Reviewer 1

Advance summary and potential significance to field

This is a revision - summary is included in initial review

Comments for the author

Ou et al have carried out extensive revisions which have addressed most of the reviewers' comments. These include new/retrieved on-line data showing HNRNPA3 expression in non-neural tissues, further data on the γH2AX up regulation in the KO mouse and human cerebral organoids, HNRNPA3 mis-expression data showing induction of mitotic progression. The additional data providing evidence for an association of HNRNPA3 with a protein in the cohesin complex (new Figure 5) and hence a link to regulation of chromosome segregation should also provide a more mechanistic explanation for the HNRNPA3 KO phenotype that they describe, however, some further clarifications need to be addressed.

1) Figure 5 and legend – should include an indication of the reproducibility of the protein localisation studies and co-IP (3 independent experiments?), especially B and E. I am not sure what (B) shows – there is a size ladder missing to show that SMC1A ab is co-IP ing HNRNPA3.

2) If this data linking to the cohesin complex holds up the authors may wish to include this potential mechanism in the title.

3) Text editors should read through the MS and adjust English grammar/use.

Reviewer 2

Advance summary and potential significance to field

The authors show that HNRNPA3 is expressed in progenitors of the developing cortex in both, mice and humans. During the cell cycle, HNRNPA3 seems to be required for M-phase progression. Genetic deletion of Hnrnpa3 in mice as well as knock-down in human organoids induced a reduction of deep layer neurons, underlining a conserved function of Hnrnpa3 in regulating neuronal output of early neurogenic progenitors. Overall the findings of the study contribute to our understanding of neurogenesis during cortex development.

Comments for the author

The authors have carefully addressed all points that were raised in the initial review of this paper.

Reviewer 3

Advance summary and potential significance to field

This manuscript provides insights into the regulation of cortical specific precursors.

Comments for the author

I thank the authors for taking the time and effort of performing the suggested experiments and for incorporating the criticisms. However, I still feel that there is a need for a much stronger revision to be of sufficient quality for publication. Within the actual revision, the authors have incorporated most of the suggested experiments in the supplementary files and added some extra lines in their manuscript. However, the majority of the experiments suggested by the three reviewers actually improve and refine substantially the manuscript and deserve to be presented in the main figures. These new results should be carefully integrated, elaborated and reflected in the main text. It is important that the authors work on a profound revision and rearrange all figures to include the new data in the best logical sequence and in a way that everything is properly displayed. I consider that

most data shown in supplementary belongs to the main figures, including time-lapse images. The authors do not have to restrict themselves to their first version.
In regard to the data, I still have some major concerns that have not been sufficiently addressed by the authors

1-In regard to the concern about the loss of upper and lower layer neurons, the new data and images make it very clear that the most affected neurons are Ctip2 positive neurons but not Tbr1 or Cux1 positive neurons. This is quite surprising and interesting. However, the claim that deep neurons are affected is an over-interpretation. The authors should aim for rigorousness and more precision in their descriptions and conclusions. Layer 6 Tbr1 early-born neurons are minimally affected but Ctip2+ are affected. No other L5 markers have been tested and therefore the authors should be more precise in their conclusions and conclude that the Ctip2+ deep layer neurons are reduced. The authors should also count the number of cells per column, not only the thickness of layers. If possible, it would be interesting if they can test some other layer 5 makers.
This is not to undermine the interest of a reduction of Ctip2+ neurons. Together with the BrdU experiments, it indicates that Hnrnpa3a regulates intermediate rounds of the proliferation of radial glial cells as the authors conclude, but data also suggests that there is selective regulation of layer 5 precursors. The authors should discuss this in the context of the expression of Hnrnpa3a expression in OSVZ in humans.
2-In regard to the two hnrnp3 isoforms a and b, the authors have to revise the manuscript and make sure that in the description of the results and conclusions they refer only to the functions of hnrnp3a for the mutant and that they clarify if the antibodies they use are against a or b isoforms. This includes the title.
3- The expression pattern and temporal dynamics of the hrnnp3a isoform should be reported carefully. The stainings with the specific hrnnp3a antibody, this should be shown both in WT and KO at E12.5, E13.5 and E16.5 at low and high magnification. Low magnifications such as the one shown in Figure 1 for Hnrnpa3 total antibody will allow distinguishing the location and proportion of precursors expressing the protein. The temporal dynamics of the expression will allow determining if the gene is regulated in a temporal manner.
4-In figure 6, panels J and K, the proportion of Brdu+ (full retaining neurons) that are Ctip2+ and Cux1+ do not add up. Are these neurons that change their fate in the absence of Hnrnpa3a? Which is the molecular identity or at least layer location, of the Brdu+ cells that are negative for Citp2 and Cux1?

Second revision

Author response to reviewers' comments

Point-by-point responses to Reviewers comments (in Italic)

Reviewer 1
Ou et al have carried out extensive revisions which have addressed most of the reviewers' comments. These include new/retrieved on-line data showing HNRNPA3 expression in non-neural tissues, further data on the γH2AX up regulation in the KO mouse and human cerebral organoids, HNRNPA3 mis-expression data showing induction of mitotic progression. The additional data providing evidence for an association of HNRNPA3 with a protein in the cohesin complex (new Figure 5) and hence a link to regulation of chromosome segregation should also provide a more mechanistic explanation for the HNRNPA3 KO phenotype that they describe, however, some further clarifications need to be addressed.

1) Figure 5 and legend – should include an indication of the reproducibility of the protein localisation studies and co-IP (3 independent experiments?), especially B and E. I am not sure what (B) shows – there is a size ladder missing to show that SMC1A ab is co-IP ing HNRNPA3.

Response: Thanks for pointing out this. The presented data (previous Fig. 5B and E) were representative results of three independent experiments and this information has been added to

revised manuscript (legends for Fig. 6B, E). The size ladder was also added to revised Figure (see new Fig. 6B and E). NOTE: We have removed unpublished data that had been provided for the referees in confidence.

2) If this data linking to the cohesin complex holds up the authors may wish to include this potential mechanism in the title.

Response: As suggested by the Reviewer, the title has been changed to “Heterogeneous nuclear ribonucleoprotein A3a controls mitotic progression of neural progenitors via interaction with cohesion”

3) Text editors should read through the MS and adjust English grammar/use.

Response: The revised manuscript has been thoroughly checked for grammar or word usage.

Reviewer 2 Advance summary and potential significance to field
The authors show that HNRNPA3 is expressed in progenitors of the developing cortex in both, mice and humans. During the cell cycle, HNRNPA3 seems to be required for M-phase progression. Genetic deletion of Hnrnpa3 in mice as well as knock-down in human organoids induced a reduction of deep layer neurons, underlining a conserved function of Hnrnpa3 in regulating neuronal output of early neurogenic progenitors. Overall the findings of the study contribute to our understanding of neurogenesis during cortex development.

Reviewer 2 Comments for the author
The authors have carefully addressed all points that were raised in the initial review of this paper.

Response: Thanks again to this Reviewer for the comments.

Reviewer 3 Advance summary and potential significance to field
This manuscript provides insights into the regulation of cortical specific precursors.

Reviewer 3 Comments for the author
I thank the authors for taking the time and effort of performing the suggested experiments and for incorporating the criticisms. However, I still feel that there is a need for a much stronger revision to be of sufficient quality for publication. Within the actual revision, the authors have incorporated most of the suggested experiments in the supplementary files and added some extra lines in their manuscript. However, the majority of the experiments suggested by the three reviewers actually improve and refine substantially the manuscript and deserve to be presented in the main figures. These new results should be carefully integrated, elaborated and reflected in the main text. It is important that the authors work on a profound revision and rearrange all figures to include the new data in the best logical sequence and in a way that everything is properly displayed. I consider that most data shown in supplementary belongs to the main figures, including time-lapse images. The authors do not have to restrict themselves to their first version.

Response: We thank the Reviewer for the great suggestion. As suggested, we have incorporated many important results that were previously presented in supplementary files into main figures, which were also displayed and elaborated in better logical sequence. The major figures have been expanded from 7 to 9.

In regard to the data, I still have some major concerns that have not been sufficiently addressed by the authors

1-In regard to the concern about the loss of upper and lower layer neurons, the new data and images make it very clear that the most affected neurons are Ctip2 positive neurons but not Tbr1 or Cux1 positive neurons. This is quite surprising and interesting. However, the claim that deep neurons are affected is an over-interpretation. The authors should aim for rigorousness and more precision in their descriptions and conclusions. Layer 6 Tbr1 early-born neurons are minimally affected but Ctip2+ are affected. No other L5 markers have been tested and therefore the authors should be more precise in their conclusions and conclude that the Ctip2+ deep layer neurons are

reduced. The authors should also count the number of cells per column, not only the thickness of layers. If possible, it would be interesting if they can test some other layer 5 makers.
This is not to undermine the interest of a reduction of Ctip2+ neurons. Together with the BrdU experiments, it indicates that Hnrnpa3a regulates intermediate rounds of the proliferation of radial glial cells as the authors conclude, but data also suggests that there is selective regulation of layer 5 precursors. The authors should discuss this in the context of the expression of Hnrnpa3a expression in OSVZ in humans.

Response: We thank the Reviewer for this point. Although Ctip2 is most widely used putative marker for layer 5 neurons, it is possible that there are other subtypes that may not express Ctip2. From current references and in our hands, we failed to find other reliable markers that could be used to specifically label layer 5 neurons, and thus this study used Ctip2 as a layer 5 marker and Tbr1 as a layer 6 marker and the description has been modified accordingly. As suggested by the Reviewer, we also counted the number of Ctip2+, Cux1+, Tbr1+, and Tbr2+ cells (see revised Fig. 2G, I, L, N). Both the number of Ctip2+ or Tbr1+ cells and the thickness of these cell layers were significantly reduced in KO mice, whereas that of Cux1+ or Tbr2+ were not affected. The selective regulation might reveal the different feature between early and late neurogenesis in mouse. The temporal dynamic expression pattern of Hnrnpa3 may underlie its specific role, or alternatively, highly proliferative NPs might be more vulnerable to the loss of Hnrnpa3a. It has been shown that early neurogenesis from aRGs is more vulnerable to chromosome defects (Lee et al., 2012a, Lee et al., 2012b, Vargas-Hurtado et al., 2019). Progenitors in human oSVZ still remain high proliferative, which contributes to expansion of superficial layer in folding cortex, whereas basal progenitors in mouse tend to be neurogenic with limited rounds of division. Indeed, the continued high expression of HNRNPA3 in oSVZ reveals that basal progenitors and apical progenitors may share similar gene expression pattern in humans but own distinct transcriptome in mouse (Florio, Albert et al. 2015). In line with this notion, down-regulation of HNRNPA3 in cultured human cerebral organoids caused a more severe defects in NP proliferation. One probable reason is that human corticogenesis requires more rounds of NP divisions to meet the needs for lateral and vertical expansion, and the mitotic delay caused by down-regulation of HNRNPA3 impaired the NP pool size. This point has been discussed in revised manuscript.

2-In regard to the two hnrnp3 isoforms a and b, the authors have to revise the manuscript and make sure that in the description of the results and conclusions they refer only to the functions of hnrnp3a for the mutant and that they clarify if the antibodies they use are against a or b isoforms. This includes the title.

Response: This is a good suggestion! The mutant mice were specific for Hnrnpa3a. Considering that human tissues mainly express a-isoform (Papadopoulou et al., 2012), the description of the results and conclusions have been modified to refer to Hnrnp3a, including the title. Antibodies against pan-Hnrnpa3, which recognized both a- and b-isoforms, as well as that specific for a-isoform were used in immunoblotting analysis for the verification of knockdown effect (see revised Fig. 2B). The results clearly indicate the reduction of a-isoform but not the b-isoform, and a-isoform is the major isoform expressed in the mouse cortex (revised see revised Fig. 2B). The antibody specific for a-isoform did not work very well in immunostaining and thus we chose pan-Hnrnpa3 antibody for immunostaining analysis. As shown in revised Fig. 2C and 5A, the Hnrnpa3a KO mice exhibited barely detectable signal of Hnrnpa3, suggesting that the signals recognized by the antibody for pan-Hnrnpa3 in immunostaining mainly represented a-isoform.

3- The expression pattern and temporal dynamics of the hrnnp3a isoform should be reported carefully. The stainings with the specific hrnnp3a antibody, this should be shown both in WT and KO at E12.5, E13.5 and E16.5 at low and high magnification. Low magnifications such as the one shown in Figure 1 for Hnrnpa3 total antibody will allow distinguishing the location and proportion of precursors expressing the protein. The temporal dynamics of the expression will allow determining if the gene is regulated in a temporal manner.

Response: As mentioned above, Hnrnpa3a is the major isoform expressed in cortical neural progenitors. As suggested by the Reviewer, we determined temporal dynamic expression pattern of Hnrnpa3 in mouse cortex in various embryonic stages by co-staining with RG marker Pax6 and IP marker Tbr2, in WT (revised Fig. 1E, F) and Hnrnpa3a KO mice (revised Fig. 2C). The results clearly showed that Hnrnpa3, here mainly a-isoform, was highly expressed in RGs, but not IPs, at early

stages (E12.5) and then declined at later stages after E14.5. Thus, the expression of Hnrnpa3a is regulated in a temporal and cell-type specific manner.

4-In figure 6, panels J and K, the proportion of Brdu+ (full retaining neurons) that are Ctip2+ and Cux1+ do not add up. Are these neurons that change their fate in the absence of Hnrnpa3a? Which is the molecular identity or at least layer location, of the Brdu+ cells that are negative for Citp2 and Cux1?

Response: To address the question of whether the absence of Hnrnpa3a changed neuronal fates, we analyzed percentage of neurons expressing pan-neuronal marker NeuN among BrdU fully retained cells (see revised Fig. S5D, E). We found that NeuN+BrdUfull cells occupied almost all BrdUfull population and there was no difference between WT and Hnrnpa3a KO mice (see revised Fig. S5E). This result together with the observation of reduction in Ctips+BrdUfull cells support the conclusion that mitotic delay caused by the loss of Hnrnpa3 impairs early neurogenesis rather than neuronal fate specification. The BrdU+ cells that were negative for Ctip2 and Cux1 were distributed in all layers and might be other types of mitotic neurons or that with delayed maturation.

References

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Lee Y., et al (2012a). Neurogenesis requires TopBP1 to prevent catastrophic replicative DNA damage in early progenitors. Nat Neurosci, 15, 819-26.
Lee Y et al (2012b). ATR maintains select progenitors during nervous system development. EMBO J, 31, 1177-89.
Papadopoulou C., et al. (2012). Expression profile and interactions of hnRNP A3 within hnRNP/mRNP complexes in mammals. Arch Biochem Biophys 523, 151-160.
Vargas-Hurtado D., et al (2019). Differences in mitotic spindle architecture in mammalian neural stem cells influence mitotic accuracy during brain development. Curr Biol, 29, 2993-3005 e9.

Third decision letter

MS ID#: DEVELOP/2019/185132

MS TITLE: Heterogeneous nuclear ribonucleoprotein A3a controls mitotic progression of neural progenitors via interaction with cohesin

AUTHORS: Min-Yi Ou, Xiang-Chun Ju, Yi-Jun Cai, Xin-Yao Sun, Jun-Feng Wang, Xiu-Qing Fu, Qiang Sun, and Zhen-Ge Luo
ARTICLE TYPE: Research Article

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