An epigenetic circuit controls neurogenic programs during neocortex development

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MS TITLE: An epigenetic circuit controls neurogenic programs during neocortex development

AUTHORS: Andi Wang, Junbao Wang, Kuan Tian, Dawei Huo, Hanzhe Ye, Bo Zhang, Lichao Xu, Xiaojiao Hua, Kun Wang, Xudong Wu, Ying Liu, and Yan Zhou

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.

As you will see, the referees express considerable interest in your work, but have some very significant criticisms and recommend a substantial revision of your manuscript before we can consider publication. If you are able to revise the manuscript along the lines suggested, which will have to involve further experiments, I will be happy receive a revised version of the manuscript. Please pay particular attention to the comments of reviewer 1 and include explanations for the statistical methods and power that support the conclusions. 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

Manuscript by Wang et al investigates a novel mechanisms regulating the development of the cerebral cortex involving LncBAR that regulates the activity of the SWI/SNF chromatin remodeling complex BAF. The authors find that LncBAR loss of function leads to over-production of intermediate neural progenitor cells (IPCs) and increase in the relative proportion of deep cortical layer neurons. This mechanism seems to contradict the commonly held view that IPCs predominantly contribute to the production of upper layer neurons. The authors attribute this phenotype to the effects that LncBAR has on the cell cycle of the IPCs. The authors identify ZBTB20 as a transcriptional mediator of LncBAR function in cortical IPCs. The manuscript reports a very detailed and careful characterization of the phenotypes associated with LncBAR deletion, and a novel mechanism regulating IPC development.

Comments for the author

I find the study interesting, but have several reservations about the evidence presented that preclude me from recommending the publication of this manuscript in Development:
1. The only evidence provided to support the claim that LncBAR has been lost is fiugre S1 C, where the authors show one image of in situ hybridization from the knockout. However, the image is far from convincing, and it appears that most of the brain has retained the expression of this gene outside of the part of the image that the authors have chosen to highlight. I do not find this convincing.
2. The authors report changes in the proportions of upper and lower layer neurons in LncBAR knockout animals. They quantify a remarkably large sample size, four or five animals per cohort, but despite this, the observed effect sizes seem relatively small. The authors apply unpaired student t-test to ascertain the statistical significance, but this test is not appropriate for this experimental design. Correction for multiple hypotheses seems to be lacking. This criticism applies to most of the figures.
3. There are a number of quantifications presented in this manuscript, and in many cases it is not entirely clear what rostro-caudal position these are performed in. There is a substantial amount of variation in cell cycle parameters, cellular composition, and other features along the rostro-caudal and medio-lateral extent of the cerebral cortex. Given that many effects reported in this study are so subtle, it would be recommendable to include uncropped images of individual sections to enable the reader to ascertain that these quantifications were performed in comparable regions across the many comparisons presented in this paper.
4. I am confused about the role that authors attribute to LncBAR KO in regulating cell cycle progression. It is unclear if the authors argue that IPCs stall in cell cycle or are unable to differentiate. There seem to be arguments made in either case. First, their argument is that are more Tbr2+ cells with LncBARko (meaning less differentiation), but later say when they overexpress Zbtb20 there also are more Tbr2+ cells (which they now say is due to rescued cell cycle progression). Resolving these possibilities convincingly would significantly strengthen the paper.
5. Recent publication from the Crabtree lab has provided additional evidence for the role of the BAF complex in cell cycle stalling, and the authors provide only a very brief mention. A more comprehensive discussion of how findings from the two studies compare would strengthen the manuscript, and help to highlight points of novelty.

Minor:
Findings reporting epigenetic regulation (figures 5-6) are convincing, but could benefit from some clarifications. Further discussion of how IPC cell cycle is regulated by LncBAR would be ideal.
Titles of many subheadings could be adjusted to be more informative. Many titles sound vague.
Figure 5E: the WT replicates are quite different, at least for the first region examined, which puts less emphasis on their finding that BAF binds to Zbtb20 more without LncBAR
Figure 6F, G: why is there no difference between Zbtb20 and Zbtb20-Znf?

Figure S1F: It would be ideal if the authors included 100% loading to show overexpression but also similar gapdh expression
Figure S3D: this needs more replicates. There is one data point around 2 for WT that is bringing down the average and making it not significant, otherwise LncBARko would have significantly more cleaved caspase-3+ cells

Reviewer 2

Advance summary and potential significance to field

In this manuscript, Wang et al present a novel epigenetic pathway regulating cortical neurogenesis. They identified a novel long non-coding RNA, LncBAR, and investigated its role in cortical development using knockout mice. They show that depletion of LncBAR interferes with the regulation of cell-cycle and neurogenesis of intermediate progenitor cells, which is essential for proper neuron production. Applying several molecular methods, the authors found that LncBAR blocks the association of the BAF chromatin-remodeling complex with the genomic region of Zbtb20, a transcription factor known to be essential for neurogenesis and cortical layer formation. Thereby, LncBAR seems to control the fate of intermediate progenitors These findings are further supported by Zbtb20 overexpression via *in-utero* electroporation, which could reverse effects of the LncBAR knockout in neural progenitors.

The authors report a novel pathway showing how a long non-coding RNA modulates a specific activity of the BAF complex. Additionally, this work contributes to the understanding of regulatory RNAs in brain development, a field, which is largely unknown so far.

The experiments were conducted carefully and comprehensively. I recommend some improvements for the writing. At some points, the authors should give more information or explanations (specific comments) and also the style can be improved (minor comments). After correction of these, I strongly support the publication of the manuscript.

Comments for the author

Specific comments:

1. It should be mentioned in the introduction that lncBAR is not present in primates/humans because it appears in the results part like sth that is known
2. Pax6 and Sox2 are both marker for the same cells. But you observe unaltered levels of Pax6+ (line 161, Fig. 2D) and fewer Sox2+ cells (line 207, Fig. 4M-P) in E16.5. Please explain.
3. For the paragraph line 171-188: It should be explained in an additional sentence what Ki67 is and what BrdU is so that readers can understand what Ki67 and BrdU positive or negative actually means for the cells. Also putting Fig S4B into Fig 3 could help understanding. Additionally, line 179-180 is confusing: “but fewer being Ki67 positive and having exited cell-cycle”, shouldn’t it be “and **more** having exited cell-cycle”?
4. Line 196: 1. What are Calretinin+ and Calbindin+ OB cells and why do you look for them? 2. Regarding Fig. 4I, you cannot say fewer Calbindin+ cells so easily.
5. Line 200: What is DCX?
6. Line 253: 253 What is zinc-finger-deleted ZBTB20? Please mention why it is used and why do you expect it to be not incorporated into the nucleus
7. Lines 314-316: diminished expression of Zbtb20 in dcKO -> wouldn’t you expect the opposite according to your findings? Please explain or at least give suggestions
8. Fig. 5A: What does the relative intensity mean? Relative to what? Why is 0.001 considered as high intensity, what does it mean? Please explain in a few words
9. Fig. 5B: Please explain in the figure legend why we do not see a marker here
10. Fig. S5: add an illustration of an OB to show where the GCL, EPL and GLL areas are and explain the abbreviations GCL, EPL, GLL, DCX and RMS in figure legend
11. Fig. S6BC: Please mention in the figure legend why actin and H3 were used
12. Fig, S6G, legend: Where is a GO analysis here? Isn’t it just the Venn diagram of differentially-expressed genes and BRG1-bound genes in LncBAR^{KO} neurospheres?

13. Fig. S6E: why are there different numbers (not 223 up and 177 down)? It should be explained
14. Cite Encode as explained here https://www.encodeproject.org/help/citing-encode/

Minor comments:

1. Line 52: “Neocortical PNs […] gyrencephalic brains” Confusing sentence, reads like an enumeration even though it is a relative clause
2. Line 74: “showed that aNSC at V-SVZ **are** derived”
3. Line 80: what do you mean with “their”? Whose? “the” fits better
4. Line 160: “We thus look**ed**” stay consistent with the tense
5. Line 192: “Notably, the olfactory bulb**s** […] were”
6. Line 220: Here you write “E16.5” but in Fig.5A it is the E14.5 cortex -> is that correct?
7. Line 287: “play**s**”
8. Lines 290-294: please give a reference for this sentence
9. Line 371: “Numbers of and neurospheres were analyzed after 3 passages” There is either one word missing or one word too much in this sentence.
10. Line 581: “were add**ed**”

First revision

Author response to reviewers' comments

Responses

Reviewer 1 Advance Summary and Potential Significance to Field:

Manuscript by Wang et al investigates a novel mechanisms regulating the development of the cerebral cortex involving LncBAR that regulates the activity of the SWI/SNF chromatin remodeling complex BAF. The authors find that LncBAR loss of function leads to over- production of intermediate neural progenitor cells (IPCs) and increase in the relative proportion of deep cortical layer neurons. This mechanism seems to contradict the commonly held view that IPCs predominantly contribute to the production of upper layer neurons. The authors attribute this phenotype to the effects that LncBAR has on the cell cycle of the IPCs. The authors identify ZBTB20 as a transcriptional mediator of LncBAR function in cortical IPCs. The manuscript reports a very detailed and careful characterization of the phenotypes associated with LncBAR deletion, and a novel mechanism regulating IPC development.

Reviewer 1 Comments for the Author:

I find the study interesting, but have several reservations about the evidence presented that preclude me from recommending the publication of this manuscript in Development:
We are encouraged that the Reviewer found our study interesting and appreciate his/her very helpful comments and suggestions. We have extensively addressed these issues by performing experiments and modified the text. In *LncBAR*^{KO} neocortices, IPCs are overproduced but stall in cell cycle during mid-late neocortical neurogenesis, which accounts for the increase in the relative proportion of deep-layer neurons and decrease of upper-layer neurons. We’d like to point out that multiple studies indicated that IPCs contribute to both deep- and upper-layer projection neurons and defects of IPC behaviors result in disproportionate layering of neocortical projection neurons (PN) (PMID:19168665; PMID:18940588; PMID:27320921). Our point-by-point responses are listed below.

1. The only evidence provided to support the claim that LncBAR has been lost is fiugre S1 C, where the authors show one image of in situ hybridization from the knockout. However, the image is far from convincing, and it appears that most of the brain has retained the unpaired student t-test to ascertain the statistical significance, but this test is not appropriate for this experimental design. Correction for multiple hypotheses seems to be lacking. This criticism applies to most of the

figures.
This is a very important issue. In revision, we've applied corrections for multiple hypotheses for all applicable quantifications and comparisons (**Fig. 1B, 1K, 2H, 3C, 3F, 3I, 5C, 6C, S2A, S2I, S3C, S4C-S4D, S4H, S5H, S5O** and **S5R'**). Although in a few cases, the strength of difference was altered, most are not changed. Thus, all major conclusions still stand. We've included a supplementary table (**Table. S4**) to describe statistical processing for every comparison.

15. There are a number of quantifications presented in this manuscript, and in many cases it is not entirely clear what rostro-caudal position these are performed in. There is a substantial amount of variation in cell cycle parameters, cellular composition, and other features along the rostro-caudal and medio-lateral extent of the cerebral cortex. Given that many effects reported in this study are so subtle, it would be recommendable to include uncropped images of individual sections to enable the reader to ascertain that these quantifications were performed in comparable regions across the many comparisons presented in this paper.
We appreciate the reviewer's very important comment and suggestion, and agree that '*There is a substantial amount of variation in cell cycle parameters, cellular composition, and other features along the rostro-caudal and medio-lateral extent of the cerebral cortex*'. Thus, in revision, we've included all uncropped images of individual sections to show that all quantifications were performed in comparable regions (**Fig. 1A, 1F, 1 I, 2A, 2I, 3A, 3G, 4M, 4O, S3D-S3F, S4A, S4E, S5E-S5G** and **S5I**). Moreover, these uncropped images indicated that sections of WT and KO groups were identically processed, as they are bilaterally symmetrical and dorsal-ventrally not tilted.

16. I am confused about the role that authors attribute to LncBAR KO in regulating cell cycle progression. It is unclear if the authors argue that IPCs stall in cell cycle or are unable to differentiate. There seem to be arguments made in either case. First, their argument is that are more Tbr2+ cells with LncBARko (meaning less differentiation), but later say when they

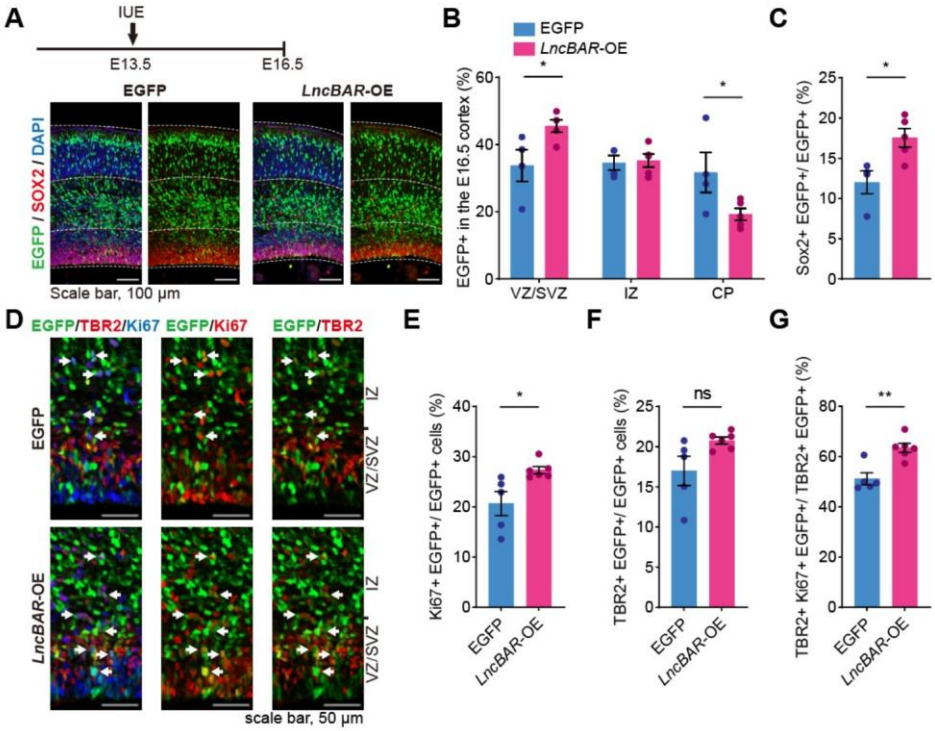


Fig. 7A-7G

17. Recent publication from the Crabtree lab has provided additional evidence for the role of the BAF complex in cell cycle stalling, and the authors provide only a very brief mention. A more comprehensive discussion of how findings from the two studies compare would strengthen the manuscript, and help to highlight points of novelty.
We thank the great suggestion. In revision, we've furthered discussion regarding BAF's role in regulating NPCs' cell cycle progression and compared it with our findings (line 353-360):
"*Notably, loss of BAF53a stalls the cell cycle of RGs and IPs at G2/M to disrupt neocortical*

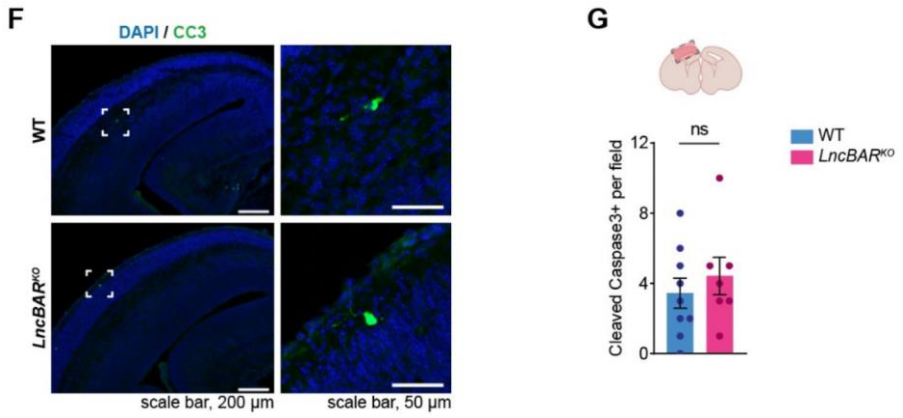
neurogenesis with an increase of PAX6+ and TBR2+ coexpression, which partially resembles defects of LncBAR^{KO} neocortices (Braun et al., 2021). Molecularly, BAF53a ablation leads to reduced chromatin accessibility at neurogenesis transcription factor binding sites perhaps due to Polycomb enrichment. Decreased expression of Zbtb20 in LncBAR^{KO} NPCs could be caused by altered chromatin accessibility and status of Polycomb-mediated repression, which deserves further exploration."

Figure 6F, G: why is there no difference between Zbtb20 and Zbtb20-Znf?

We re-examined the subcellular localization of overexpressed ZBTB20 and ZBTB20-ΔZnf. In line of previous report (PMID:30281617) by Jones *et al.*, although ZBTB20-ΔZnf is largely cytosolic, a fraction of it is also nucleus. Thus, the ZBTB20-ΔZnf might have residual transactivating effect or a transcription-dependent role of ZBTB20 in regulating IP divisions. We've stated the point in Line 287-289: "*Intriguingly, expression of ZBTB20-ΔZnf had minor effects on cell proliferation, suggesting a transcription-dependent role of ZBTB20 in maintaining IP divisions or residual transactivating effect of ZBTB20-ΔZnf*".

Figure S1F: It would be ideal if the authors included 100% loading to show overexpression but also similar gapdh expression.
The purpose of Northern blot is to validate the transcript size of *LncBAR* is a little less than 3000 nt, with the 10% overexpression (OE) lane being the positive control. If we load 100% of OE, the *LncBAR* band would be overwhelming. I hope the reviewer could understand our point.

Figure S3D: this needs more replicates. There is one data point around 2 for WT that is bringing down the average and making it not significant, otherwise LncBARko would have significantly more cleaved caspase-3+ cells
To clarify the point, we performed cleaved Caspase 3 staining on more E16.5 brains to show loss of *LncBAR* does not cause enhanced apoptosis (**Fig. S3F-3G**)



11. For the paragraph line 171-188: It should be explained in an additional sentence what Ki67 is and what BrdU is so that readers can understand what Ki67 and BrdU positive or negative actually means for the cells. Also putting Fig S4B into Fig 3 could help understanding. Additionally, line 179-180 is confusing: "but fewer being Ki67 positive and having exited cell- cycle", shouldn't it be "and more having exited cell-cycle"?
We thank these suggestions. 1) Meanings of BrdU and Ki67 were explained where they first appear in the revised manuscript. 2) **Fig. S4B** has been moved into **Fig. 3** in revision. 3) We've rephrased the sentence as followed in revision: "*…but fewer IPs being Ki67 positive (**Fig. 3A-3D**). Moreover, there were fewer TBR2+BrdU+Ki67+ cells, and a smaller portion of TBR2+BrdU+ IPs expressed Ki67 in LncBAR^{KO} neocortices (**Fig. 3E-3F**).*". (Line 191-193)

12. Line 196: 1. What are Calretinin+ and Calbindin+ OB cells and why do you look for them?
2. Regarding Fig. 4I, you cannot say fewer Calbindin+ cells so easily.

13. Line 200: What is DCX?

1) In revision, we added an introductory sentence in this part: "*Interneurons of OBs, including those expressing TBR2, Calretinin (CR) and Calbindin (CB), are continuously replenished by migrating Doublecortin- (DCX) expressing neuroblasts that are generated by aNSCs in the V-SVZ of the walls of the lateral brain ventricles*." (Line 206-209). 2) We apologize for not stating precisely. The description was changed into "*…numbers of TBR2+, CR+ but not CB+ OB cells were fewer in LncBAR^{KO} brains.*" (Line 211-212).

14. Line 253: 253 What is zinc-finger-deleted ZBTB20? Please mention why it is used and why do you expect it to be not incorporated into the nucleus.

The purpose of using the ZBTB20-ΔZnf is to set up a control with defective function. A previous report revealed that unlike full-length ZBTB20, ZBTB20-ΔZnf was not exclusively localized in nuclei and had defects in regulating dendritic arborization of neocortical neurons (PMID:30281617). Our study further showed that ZBTB20-ΔZnf is less potent in promoting IP divisions than the full-length ZBTB20 (**Fig. 6**). We thus inserted an introductory sentence in this part: "*ZBTB20-ΔZnf abolished the exclusively nuclear localization of ZBTB20 and showed defects in regulating dendritic arborization of neocortical neurons*" (Line 275-277).

Table S1. Significantly-enriched BAF-subunits in LncBAR-precipitated extracts

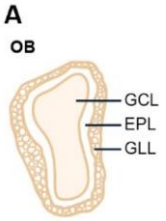
Protein symbol	Peptide counts		Normalized Abundance		Relative intensities	
	sense	antisense	sense	antisense	sense	antisense
Actl6a (BAF53A)	1	0	6100000	0	0.000257	0.000000
Arid1a (BAF250A)	5	0	6000000	0	0.000253	0.000000
Smarcc1 (BAF155)	11	0	5700000	0	0.000240	0.000000
Smarce1 (BAF57)	3	0	1700000	0	0.000072	0.000000
Pbrm1 (BAF180)	1	0	1300000	0	0.000055	0.000000
Dpf2 (BAF45D)	1	0	1000000	0	0.000042	0.000000
Smarcd1 (BAF60A)	2	0	900000	0	0.000038	0.000000
Smarcc2 (BAF170)	13	3	12000000	1500000	0.000506	0.000169
Smarca4 (BRG1)	23	6	26000000	5900000	0.001096	0.000664
Total precipitated proteome's abundance			23719610000	8883890000		

9. Fig. 5B: Please explain in the figure legend why we do not see a marker here

The marker lane was loaded with standard molecular weight marker and did NOT recognized by respective antibodies.

10. Fig. S5: add an illustration of an OB to show where the GCL, EPL and GLL areas are and explain the abbreviations GCL, EPL, GLL, DCX and RMS in figure legend

As per suggested, an illustration of an OB was added in revision to show different areas (**Fig. S5A**). Abbreviations for GCL, EPL, GLL, DCX and RMS were explained in revised figure legends.



11. Fig. S6BC: Please mention in the figure legend why actin and H3 were used

We've stated the reason why Actin and H3, two loading controls, were used in revised figure legend for **Fig. S6B-6C**. Since the BAF complex can bind to Actin, the anti-Brg1 antibody pulled down more Actin than antisense-*LncBAR* in **Fig. S6C**.

12. Fig, S6G, legend: Where is a GO analysis here? Isn't it just the Venn diagram of differentially-expressed genes and BRG1-bound genes in LncBARKO neurospheres?

Second decision letter

MS ID#: DEVELOP/2021/199772

MS TITLE: An epigenetic circuit controls neurogenic programs during neocortex development

AUTHORS: Andi Wang, Junbao Wang, Kuan Tian, Dawei Huo, Hanzhe Ye, Si Li, Chen Zhao, Bo Zhang, Yue Zheng, Lichao Xu, Xiaojiao Hua, Kun Wang, Qing-Feng Wu, Xudong Wu, Tao Zeng, Ying Liu, and Yan Zhou
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.

Reviewer 1

Advance summary and potential significance to field

The revised manuscript has been substantially improved and resolved my main concerns, I do not have any other issues that would preclude publication.

Comments for the author

The revised manuscript has been substantially improved and resolved my main concerns, I do not have any other issues that would preclude publication.

Reviewer 2

Advance summary and potential significance to field

See my comments in the previous submission.

Comments for the author

The revised manuscript has been significantly improved with clarification to the text. I have no further concerns.