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Peer Review File

Dominant Clones Leverage Developmental Epigenomic States to Drive Ependymoma

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Any redactions in this file are there to maintain patient confidentiality, the confidentiality of unpublished data, or to remove third-party material.

This file contains all reviewer reports in order by version, followed by all author rebuttals in order by version.

Version 0:

Reviewer comments:

Referee #1

(Remarks to the Author)
In this manuscript, Kardian, Sun and colleagues examine pediatric supratentorial ependymomas using murine models and patient samples to show that ZFTA-RELA gene fusions may maintain chromatin accessibility of PLAGL motifs that are otherwise repressed during typical neurogenic differentiation. The techniques used are cutting-edge (scMultiome, lineage tracing), the manuscript is well-written, and the figures are beautifully presented. However, functional/mechanistic studies into the role of the PLAGL gene family during normal development and tumorigenesis in EPNs are missing. Furthermore, some of the in vitro and in vivo murine models have not been fully characterized, control groups are missing, and some of the correlational data in Fig. 2 and Fig. 3 may be overstated. Overall, this group already implicated several years ago (PMID: 33741710) PLAGL motif binding in ZFTA-RELA tumors. The novelty in this new work relates specifically to the findings of lineage specific commitment, and this component of the work requires additional substance. Concerns are highlighted below:

Major concerns:

1. Although these concerns are largely chronologically ordered, one of my main issues is with the lineage tracing component of the work. TrackerSeq is a powerfully applied tool, but this component of the work seems quite underdeveloped. The methods section is scant on details, and little information is provided in terms of how clones were identified (for example, minimum UMI counts required per barcode). In perusing the raw data in table 7, it appears that many “clones” are questionably called, where within a single putative LB clone multiple cells may have only one overlapping barcode with several others differing. For this to be an effect of under sequencing would need to be proven. Along these lines, the clones aren’t really shown on UMAPs with the exception of the dominant LB1. Finally, these experiments are performed on a small number of animals (n = 3 I believe) and it appears one of those (P4) has low tumor purity. Essentially, my concern is that this part of the data analysis is critical to the main conclusions, but lacks rigor currently. The authors could take many additional steps to validate their clone calling pipeline, convince the readers of the integrity of the system, add additional animals, and present the data in a convincing manner. I think it’s a beautiful experiment, no matter the findings, but as presented it is the major weakness of the manuscript in my opinion.

2. The authors should characterize the RGCs isolated from Fabp7-eGFP mice. Were the Fabp7+ RGCs biased toward specific subtypes of RGCs? Do those previous studies that the authors cite suggest whether specific subtypes of RGCs are more likely to be involved in EPNs? Fabp7+ cells can mark some subsets of immature OPCs and astrocyte-like cells. This is particularly apparent in the dotplot in Ext. Fig. 1E where OPC expression of FABP7 is quite high. The lack of changes in chromatin accessibility between ZR-expressing vs. control RGCs could be attributed to some of these cell type-specific selection biases of the model.

3. Please show the appropriate Western blots confirming ZFTA-RELA expression following infections.

4. From Fig. 1G, it is evident that the PLAGL1 motifs are also accessible in the OPC lineage, although this cluster is relatively small at E14.5 in the mouse forebrain. What roles do OPCs play in EPNs? Is there also accessibility of ZR-specific

DNA binding sites in OPCs?

5. Can the authors explain why the IUE experiments were performed slightly later (at E16.5) than the experiments in Fig. 1, where they characterized chromatin accessibility in the developing mouse forebrain (at E14.5)? Could some of the differences related to cell type-specific PLAGL1 motif activity between normal forebrain development and ZR-EPN mouse tumors be attributed to a difference in developmental time of the experimental paradigms?

6. The authors used the GLAST promoter to target RGCs; however, like most early promoters this is not completely unbiased and leans towards gliogenic progenitors. Have the authors confirmed cell type-specific integration of ZR?

7. For the experiments in Fig. 2, a control (non ZR-expressing) group is missing, but may be critical to understand how ZR-induced tumorigenesis impacts cell type distribution, PLAGL1 motif activity, etc. in this model.

8. The inferCNV data in Ext. Fig. 2A-B suggest high variability in CNV patterns between the three ZR-EPN mouse tumors. In addition, the CNV data for the single GBM mouse tumor do not display the classical chr 7 amplification. How do the authors explain these findings?

9. From Fig. 2E, it seems as though a subset of the RGC-like cluster has very high PLAGL1 activity, compared to other RGC-like cells within the same cluster, which may drive the differences between normal brain development and the ZR-EPN tumors. The authors could consider subclustering the RGC-like cluster to resolve these differences – perhaps some subtypes of RGCs are more likely to harbor high PLAGL1 motif activity in ZR-EPN tumors? Or perhaps this is related to earlier critiques on using GLAST as a promoter, performing IUE experiments later in developmental time, compared to experiments in Fig. 1, and missing a non ZR-expressing control group assayed at the same developmental time as the ZR-EPN tumor-expressing mice?

10. Fig. 2G-H: The authors state that the correlation between ZR and PLAGL1 target activity is observed preferentially in neuronal-like EPN cells, however, from 2G-H this is not necessarily true: correlation with neuron-like cells may display a lower p-value (greater statistical significance), but the r-values – which denote the strength of the correlation – are slightly greater in RGC-like cells (0.6) vs. neuron-like cells (0.5). Given this and the previous critique, it is unclear if the differences in correlation between RGC and neuron-like cells are biologically significant.

11. I have a similar comment for Fig. 3H-I, where, in fact, RGC-like and C1-neuron-like clusters show a largely similar correlational “r” value for PLAGL1, although the “r” value for nIPC clusters is slightly higher. Thus, it may be incorrect to state that the “positive correlation between ZR signature and PLAGL family motif activity were detected largely in neuronal-like cells”. On examining Fig. 3H-I in more detail, it is also apparent that other neuron-like clusters (C2 & C0), in fact, show a significantly smaller “r” value, suggesting that neuron-like clusters differ in their ZR signature / PLAGL1 motif activity correlations. The authors could assess how these three neuronal subclusters differ at the transcriptomic and functional levels.

12. The functional studies in this manuscript are rather limited. The authors accurately state and nicely show that their data suggests that “ZR expression maintains chromatin accessibility of PLAGL family motifs that are normally repressed during neuronal differentiation”. However, the consequences of keeping PLAGL family genes active during normal development, within RGCs or within cells along the neuronal lineage would also be important to examine as a part of this manuscript. The authors could also study the mechanistic role of PLAGL following transduction with ZR-expressing lentiviral vectors, as was done in the earlier part of the manuscript.

Minor concerns:

1. In addition to the CNV heatmaps shown by sample (on the Y-axes) in Ext. Fig. 2, can the authors also display heatmaps of the EPN and GBM tumors by cluster identity on the Y-axes, so the readers can fully visualize which clusters were annotated as malignant vs. non-malignant?

2. The authors should discuss in detail their observation that RGCs are not the dominant cell types within murine ZR-EPN tumors (as depicted in the barplots of Ext. Fig. 2E) in the context of their statements in the text citing prior work suggesting that RGCs may be “putative EPN cells of origin”.

Referee #2

(Remarks to the Author)
In this study, Kardian and colleagues investigate the susceptibly of developmental neural cells to transformation and elucidate the mechanism employed by fusion oncoproteins to drive tumorigenesis in pediatric supratentorial (ST) ependymoma (EPN). Using combined single cell ATAC and RNA sequencing in mouse and human EPN they build upon their previous study and find that accessible motifs in progenitor cells are leveraged by the oncogenic transcription factor ZFTA-RELA. They demonstrate that the accessibility program in radial glial cells is found to be enriched in PLAGL family DNA motifs which are open at oncogenic loci known to play a role in ependymoma progression. The study then confirms the EPN tumor cell hierarchy and demonstrates the clonal evolution of EPN.

This is a beautiful and important paper that contributes key insights to mechanistic understanding of ST-EPN. I have the following recommendations to improve an already excellent paper:

In Figures 1h-j, the motif activity score, DNA and chromatin accessibility are compared between RGC and neurons. As the RGC progenitor population is compared to mature neurons that harbor condensed chromatin, it would be interesting to look at this analysis comparing to a different progenitor cell type to further understand intrinsic vulnerabilities. OPCs appear to have accessible Plagl family TF motifs, how do these differ to the RGCs?

Similar to the data shown in Fig.1i and Ext Data Fig.1i for the mouse embryo forebrain analysis, how does the cell type specific accessibility alter for known EPN oncogenes (e.g. EphB2, Notch1) in the presence of ZR in the IUE mouse model?

Given that ependymal-like cells are a known cell state in EPN and have been identified in previous transcriptomic analyses of ST-EPN (Gojo et al., Cell 2020), it is striking that they aren't present in any of the ST-EPN samples analyzed. The authors should discuss/clarify why that may be the case. Are ependymal cell markers present in the cell-type reference gene list used to characterize these samples?

Given the ependymal-like cell state is not present for the ST-EPN samples analyzed here, the authors should perform complimentary staining of the mouse model (and, if possible, human ST-EPN tissue) to validate the cell states identified by sequencing.

The reference gene lists used for cell-type annotations is not clear. For example, Line 150, the developmental mouse brain atlas reference [23] is La Manno Nature 2021, yet the corresponding figure Ext Data Fig 1b is labeled as Bella Nature 2021 (should be Di Bella?). This is not included as a referenced paper. Similarly, line 239 reference [33] is Braun Science 2023, yet the Ext data Fig 4k states Trevino Nature 2021. Again, this is not included as a referenced paper.

On Line 177, the cell states that are specified include "cycling neural intermediate progenitor cells (IPC)", is this referring to the CycProg-Like group displayed in the figure? The IPC cell state is later discussed for Figure 4 (Line 243), where it is termed as nIPC-like. As this is the first time using nIPC, the authors should write the full term, or adjust the abbreviation in the previous line. Are these CycProg-Like and nIPC-like cell states identical as suggested on Line 258?

For the three neuronal-like cell states identified in ZFTA-RELA EPN (Figure 4f), are the markers for these cell-states suggestive of any specific neuronal types?

Minor :

All figures must be displayed in the order that they are discussed in the results.

Ext data Fig.1f is not referenced in results.

Referee #3

(Remarks to the Author)
Kardian and colleagues present a manuscript on ZFTA-RELA-driven ependymoma. Ependymoma are among the more common malignant childhood brain tumors, and the ZFTA-RELA-driven subtype makes up for a relatively small fraction. Patient outcomes are poor. The authors apply single-cell multiome profiling to study human and mouse tumors, as well as the developing mouse forebrain (E14.5). In the second part of the study, the authors combine their previously established in utero electroporation mouse model with a molecular barcoding strategy to distinguish malignant clones. Using advanced computational analyses on resulting genomic datasets, the authors conclude that specific chromatin states during brain development are vulnerable to transformation by ZFTA-RELA, and that differentiation hierarchies of malignant cells are conserved between tumors collected from humans and the mouse model. Using molecular barcoding coupled to single cell sequencing, the authors show that the entire hierarchy of malignant cells is present within a single clone.

The presented work is a continuation of a previous study by the same senior authors that investigated the effect of ZFTA-RELA on ependymoma formation (Arabzade, Cancer Discovery, 2021). The mechanisms by which ZFTA-RELA transforms a specific cell type during normal development is of interest to better understand the biology of these tumors and to motivate novel therapeutic approaches. I believe this kind of research can be very impactful and is much needed in the field of childhood cancer research. The authors apply a number of state-of-the-art experimental approaches (mouse modeling, single-cell genomics, molecular barcoding). Similar analyses have recently been performed in other types of cancer and the conceptual/methodological novelty is rather small, making the study less relevant for the broader research community. The manuscript is well written with some room for clarification. A number of main figure panels are not required for the narrative and could be made more concise. Overall, I find the study to be interesting and of decent quality, but I struggle to see novelty that would mandate publication in Nature.

These are my main comments:

1) Figure 1b shows that there are only minimal changes in global accessibility profiles between ZFTA-RELA (ZR) expressing radial glial cells and empty vector controls, suggesting that “patterns of chromatin accessibility were already established in specific lineage programs and subsequently commissioned by ZR”, a central claim of the manuscript. This claim is not supported by the violin plot showing the total number ATAC-seq peaks (without any statistical tests). An analysis like the one performed on the corresponding RNA-seq dataset shown in EDF1a, which shows major differences, needs to be performed.

2) I am unclear about the EWSR1-PLAGL1 (EWP) tumors that function as a contrast for the ZR tumors in the single cell analysis of patient samples in Figure 3. Important background/references are missing on these tumors. Do they reflect histologically diagnosed ependymoma that form their own DNA methylation class of PLAGL1-fused neuroepithelial tumors (e.g., Sievers et al, 2021)? The comparison to ZR tumors is not sufficiently motivated considering presumed differences in cellular origins. Also, methylation array profiling can only suggest the presence of these fusion events.

3) A main concern I have about the mouse model is the choice of the GLAST promoter for expression of the fusion oncogene. While this promoter is active in radial glia and other neural cell types, it might show different activity than the endogenous promoter driving ZR expression in patient tumors. This is important when studying the effect of the oncogene on transformation or in regulating malignant cellular hierarchies. For example, expression of ectopic ZR might occur in cell types in which the fusion would not be expressed in the natural context. At a minimum, this shortcoming needs to be clearly stated and potential implications discussed in more detail. Expression of wild-type ZFTA should be investigated in the mouse developmental dataset. A more ambitious approach would be to model the ZR fusion by CRISPR-guided deletion of the genomic region (1.5Mb) between ZFTA and RELA leading to the fusion oncogene.

4) Several other datasets of the developing mouse brain have been published in recent years. Relying on a single time-point seems like a missed opportunity to map the developmental correlates of ZR-driven ependymoma with greater accuracy.

5) The molecular barcoding approach is interesting, but the number of samples are too small and the analyses are too premature to yield conclusive results. Most findings are rather obvious, and it is unclear what could have been an alternative finding. For example, it can be expected that the clonal diversity is higher in early neoplastic lesions. It needs to be clarified if the non-tumor cells in Figures 5f and 5i carry the dominant lineage barcode (I assume not?). The concluding paragraph in the discussion seems to be a bit of an overstatement (“lineage tracing tracing experiments demonstrated the emergence of dominant, often single clones”, and “conventional therapies may miss single aggressive tumor clones capable of driving tumor recurrence” – how can it be both?).

6) I am a bit confused by the proposed cellular hierarchy of ZR-driven ependymoma. Pseudotime inference analysis resulted in a model in which cycling nIPC cells give rise to gliogenic and neurogenic differentiation programs. How does this relate to the UMAP presented in Figure 4b? Where do RGC-like cells fall? Ideally such analyses should be shown in individual samples as cell types and their abundance may vary between patient tumors.

Version 1:

Reviewer comments:

Referee #1

(Remarks to the Author)

Overall, the authors have spent considerable effort on updating this manuscript and should be commended for these improvements. Below are a series of concerns that still remain in my opinion. Some of these are simply addressed via text or figure changes. However, an underlying concern does still remain about the overall novelty of the findings and the impact to which the TrackerSeq experiments truly convey the (strong) messaging of the title. This, I believe, is more of an editorial decision.

1. In response to my original comment (point #10, reviewer 1) about functional validation of PLAGL, the authors performed a series of overexpression and KO studies in mouse RGCs (shown in Extended Fig. 2). However, these studies did not show that PLAG genes impact the same genes that ZR normally turns on. In addition, KO of PLAGL1 at the same time of ZR tumor initiation did not impact tumor initiation.
The authors do state in their rebuttal that “We conclude from these functional experiments that the Plagl1 TF protein may not contribute significantly to ZR tumor development”. However, in the main text there remains language suggesting a direct involvement of PLAG in ZR tumor initiation. For example, in lines 345-347 (“Considering the role of PLAGL1 and PLAGL2 in brain development and disease, the ability of ZR to maintain the PLAG/L motif accessible beyond the embryonic stage may be an important mechanism in EPN initiation.”) There is also additional language about ZR engaging PLAG/L to “initiate oncogenesis” in lines 337-339, which is not borne out by the new mechanistic experiments.
In the rebuttal the authors nicely state that this lack of effect on tumor initiation may be because of differences in the structure of ZR vs. PLAGL1, with ZR being created by a gene fusion, giving it disordered protein regions that form bimolecular condensates, however these limitations are surprisingly absent in the main text and deserve mention. These additional studies also raise the question of whether the accessibility of PLAGL motifs is merely coincidental in ZR-EPN.

2. The authors should show the proportion of annotated cell types that harbor the dominant barcode LB-1 in Fig. 5-6. Are there cell type-specific biases? This would add to the novelty of the work, as the TrackerSeq results that the authors currently show are not entirely surprising (glial and neuronal lineage programs emerging from cycling progenitor-like cells).

3. Lines 284-286, Fig. 4, and Fig. 1: The authors state that compared to normal development, ZR in human tumors may maintain chromatin accessibility of PLAG/L family motifs that are normally repressed during neuronal and glial differentiation. However, according to Fig. 1K in the mouse model, PLAG/L motif activity was significantly higher in astrocytes vs. neurons, where activity was indeed strongly repressed. This does not appear to be a species-specific effect either as Fig. 1L suggests that PLAGL2 motif activity is higher in astrocytes compared to the neuronal lineage suggesting that PLAG/L motif activity remains active in astrocytes during normal development at the same time when it is repressed in neurons. Can the authors comment on this difference between the glial vs. neuronal PLAG/L activity?

In addition, PLAGL/L motif activity in Fig. 1 is displayed as a numerical score, whereas in Fig. 4 is binned into “high”, “middle”, and “low”, making comparisons between human tumors and normal development rather difficult. I recommend using the same method in both figures. I’m also a bit unclear on how the PLAG/L motif activity in human tumors was divided into high-medium-low, as I could not find clear descriptions regarding this in the Methods section.

Referee #2

(Remarks to the Author)
The authors have added a great deal of new analyses and clarifications that have addressed my concerns.

Referee #3

(Remarks to the Author)
In their revised manuscript, Kardian and colleagues have thoroughly addressed all my comments by including several new datasets (e.g. mouse E12-P6 multi-ome, Tracker-Seq), computational analyses, and clarifications in the text. As a result, their claims have solidified and are presented in greater clarity. I am in support of publication.

Version 2:

Reviewer comments:

Referee #1

(Remarks to the Author)
I am satisfied with the authors' responses and updates to the manuscript. This work is in suitable state for publication in my opinion.

Response to Reviewer comments

Referee #1 … Pg.1-16

Referee #2 … Pg.17-24

Referee #3 … Pg.25-37

Referee #1 (Remarks to the Author):

In this manuscript, Kardian, Sun and colleagues examine pediatric supratentorial ependymomas using murine models and patient samples to show that ZFTA-RELA gene fusions may maintain chromatin accessibility of PLAGL motifs that are otherwise repressed during typical neurogenic differentiation. The techniques used are cutting-edge (scMultiome, lineage tracing), the manuscript is well-written, and the figures are beautifully presented. However, functional/mechanistic studies into the role of the PLAGL gene family during normal development and tumorigenesis in EPNs are missing. Furthermore, some of the in vitro and in vivo murine models have not been fully characterized, control groups are missing, and some of the correlational data in Fig. 2 and Fig. 3 may be overstated. Overall, this group already implicated several years ago (PMID: 33741710) PLAGL motif binding in ZFTA-RELA tumors. The novelty in this new work relates specifically to the findings of lineage specific commitment, and this component of the work requires additional substance. Concerns are highlighted below:

Major concerns:

Reviewer #1 – Point #1

Although these concerns are largely chronologically ordered, one of my main issues is with the lineage tracing component of the work. TrackerSeq is a powerfully applied tool, but this component of the work seems quite underdeveloped. The methods section is scant on details, and little information is provided in terms of how clones were identified (for example, minimum UMI counts required per barcode). In perusing the raw data in table 7, it appears that many "clones" are questionably called, where within a single putative LB clone multiple cells may have only one overlapping barcode with several others differing. For this to be an effect of under sequencing would need to be proven. Along these lines, the clones aren't really shown on UMAPs with the exception of the dominant LB1. Finally, these experiments are performed on a small number of animals (n = 3 I believe) and it appears one of those (P4) has low tumor purity. Essentially, my concern is that this part of the data analysis is critical to the main conclusions but lacks rigor currently. The authors could take many additional steps to validate their clone calling pipeline, convince the readers of the integrity of the system, add additional animals, and present the data in a convincing manner. I think it's a beautiful experiment, no matter the findings, but as presented it is the major weakness of the manuscript in my opinion.

Response:

We thank the reviewer for their thoughtful and detailed feedback regarding the lineage tracing analysis using the Tracker-Seq methodology. We fully agree that this component is an important part of our study and appreciate the opportunity to improve the clarity and rigor around these experiments. Below, we outline the steps that we have taken in the revised manuscript to address these comments, and presented in **Response Figure 1, Main Figure 5**.

1) Expanded Sample Size: We have increased the number of samples from **n = 3** to **n = 11** analyzed to strengthen the robustness of our conclusions, and where possible included technical replicates of Tracker-Seq

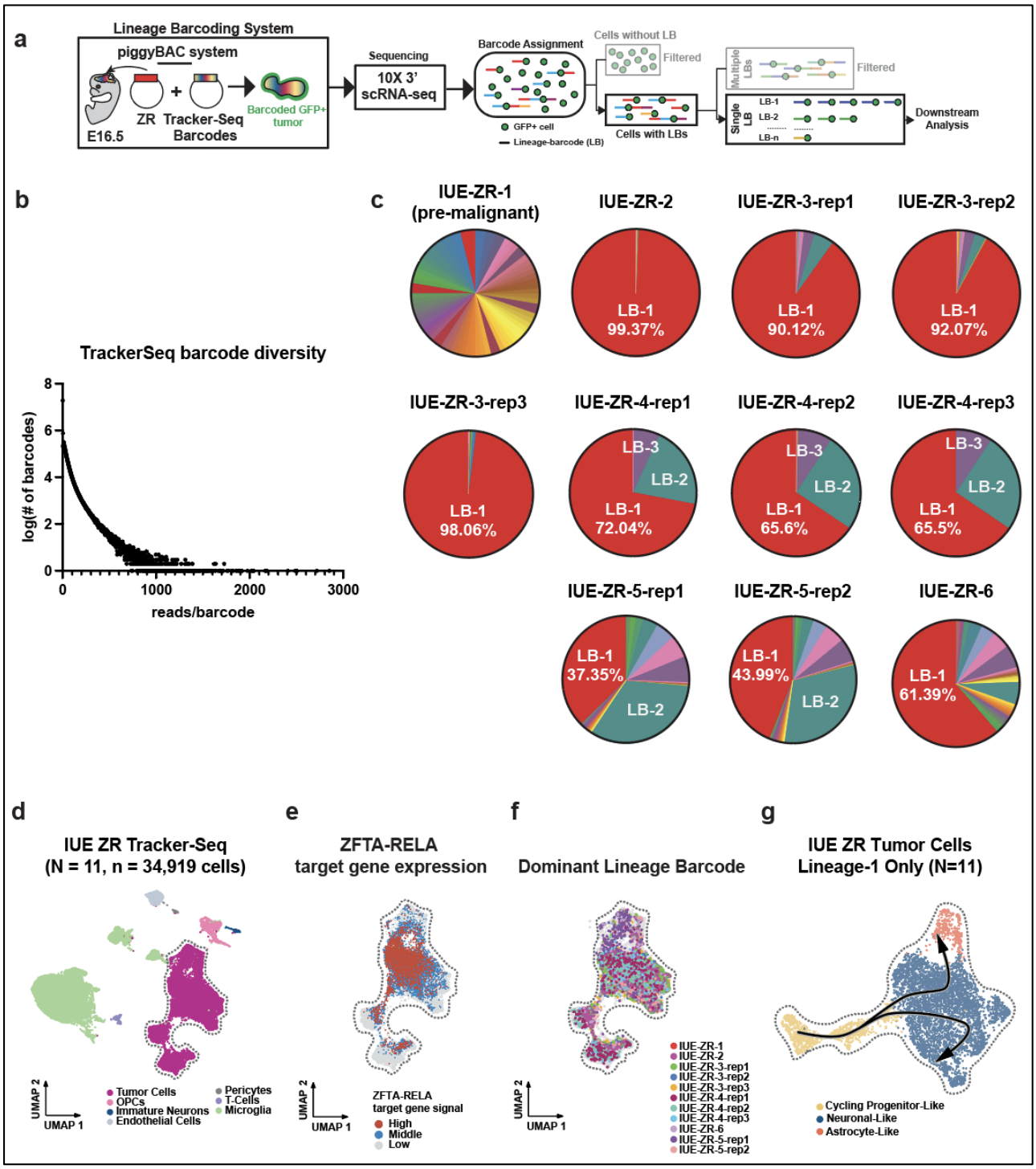
scRNA-seq experiments. We agree with *Reviewer #1* that the P4 (IUE-ZR-1) timepoint is likely low tumor purity. We apologize for the lack of clarity. This sample was of ZR transformed cells seven days post IUE versus brain tumors taken at neurologic endpoint and expected to contain significant normal tissue upon macro-dissection of the tumor at end stage. We anticipated this early neoplastic lesion to have more clones detected as compared to the other Tracker-Seq tumors and wanted to include this sample as a positive control to validate the library diversity *in vivo*. We also have included relevant details on the Tracker-Seq library diversity which contains approximately 30 million unique barcodes (**Response Figure 1, Extended Data Figure 9**).

2) Improved Methodological Clarity: We have substantially revised and expanded the Methods section to provide more detailed and transparent descriptions of our lineage barcode (LB) processing pipeline, with the inclusion of quality control (QC) metrics and explicit filtering criteria. Furthermore, the methods presented in our revised manuscript is more stringent, restricting analyses to cells with only one molecular barcode detected to represent a single lineage. This methodology is also consistent with the approach described in the original manuscript that describes the Tracker-Seq methodology[1].

3) Visualization of Lineage Assignments: As suggested, we have updated new **Main Figure 5** (**Response Figure 1**) to include a UMAP annotated with dominant LB-1 assignments. These visualizations now provide a clearer representation of LB-demarcated cells in transcriptomic space, addressing the reviewer's comment that lineage assignments were not clearly visualized.

In summary, we agree with *Reviewer #1* that Tracker-Seq is a powerful lineage mapping tool, and we value *Reviewer #1's* suggestions to improve the clarity and rigor of these experiments as applied to brain tumor initiation. Our revisions aim to present a more cautious and transparent interpretation of the data. We believe that, even with conservative assumptions, the lineage tracing experiments reveals the emergence of dominant clone(s) that harbor diverse transcriptional states mirroring normal (but incomplete) differentiation programs.

We thank *Reviewer #1* for guiding us towards a more rigorous presentation of the cell lineage tracing experiments.



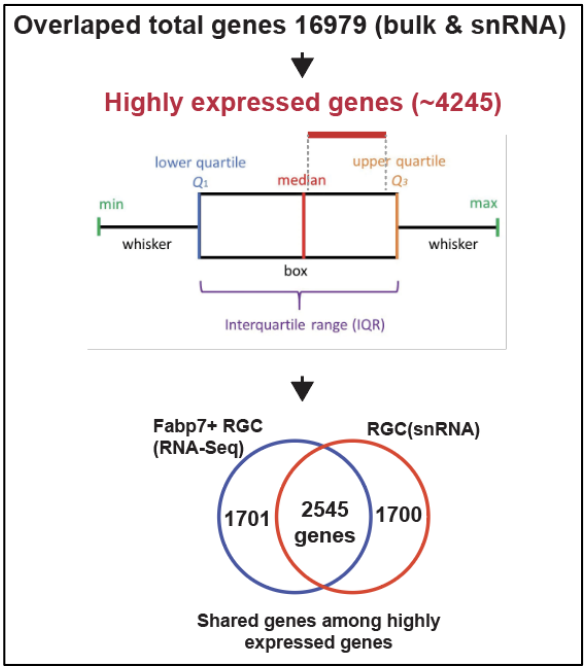
Response Figure 1: (a) Schematic of piggyBAC TrackerSeq lineage barcoding system and raw single-cell RNA data processing workflow. GFP+ IUE cells are isolated and sequenced, and cells with a single lineage barcode are used for downstream analysis. (b) TrackerSeq barcode diversity. (c) Lineage barcode abundance across six IUE biological replicates. Samples IUE-ZR-3 to -5 include technical replicates. Sample IUE-ZR-1, derived from a P4 mouse brain, shows high diversity in the TrackerSeq library shortly after IUE. (d) Integrated UMAP of all 11 IUE TrackerSeq datasets, highlighting tumor cell clusters defined by ZR-fusion target gene expression and enclosed with a dotted line. (e) UMAP visualization highlighting tumor cell clusters with ZR-fusion target gene signal. (f) Projection of cells harboring dominant lineage barcodes from different samples onto tumor cell clusters in the UMAP, assessing barcode distribution across samples. (g) UMAP reconstruction was performed using tumor cells harboring dominant lineage barcodes. Lineage trajectories were inferred by Slingshot based on Seurat cluster assignments. Arrows denote two distinct differentiation trajectories identified through lineage analysis.

Reviewer #1 – Point #2

The authors should characterize the RGCs isolated from Fabp7-eGFP mice. Were the Fabp7+ RGCs biased toward specific subtypes of RGCs? Do those previous studies that the authors cite suggest whether specific subtypes of RGCs are more likely to be involved in EPNs? Fabp7+ cells can mark some subsets of immature OPCs and astrocyte-like cells. This is particularly apparent in the dotplot in Ext. Fig. 1E where OPC expression of FABP7 is quite high. The lack of changes in chromatin accessibility between ZR-expressing vs. control RGCs could be attributed to some of these cell type-specific selection biases of the model.

Response:

We thank *Reviewer #1* for this comment. In response, we compared the degree of transcriptional similarity between Fabp7-eGFP+ labeled RGCs and RGCs from our developmental atlas. To this end we determined the highest expressed genes from Fabp7+ cells and overlapped these with the highest expressed genes in RGCs from the snRNA developmental atlas (**Response Figure 2, Extended Data Figure 1**). We found a significant degree of transcriptional similarity between our *ex vivo* cultured RGCs and those of the developmental atlas. An interesting point that *Reviewer #1* raises is whether specific subsets of RGCs are involved in EPN pathogenesis. While we did not observe any biases towards any subsets of RGCs, this could be a potential reflection of the differences between mouse and human brain development such as increased outer radial glial cell expansion seen in humans[2-4].



Response Figure 2: Highest quartile of genes from Fabp7+ cells was compared to the highest expressed quartile of genes in RGCs from the snRNA developmental atlas. 2545 of 5946 were shared between these two data sets.

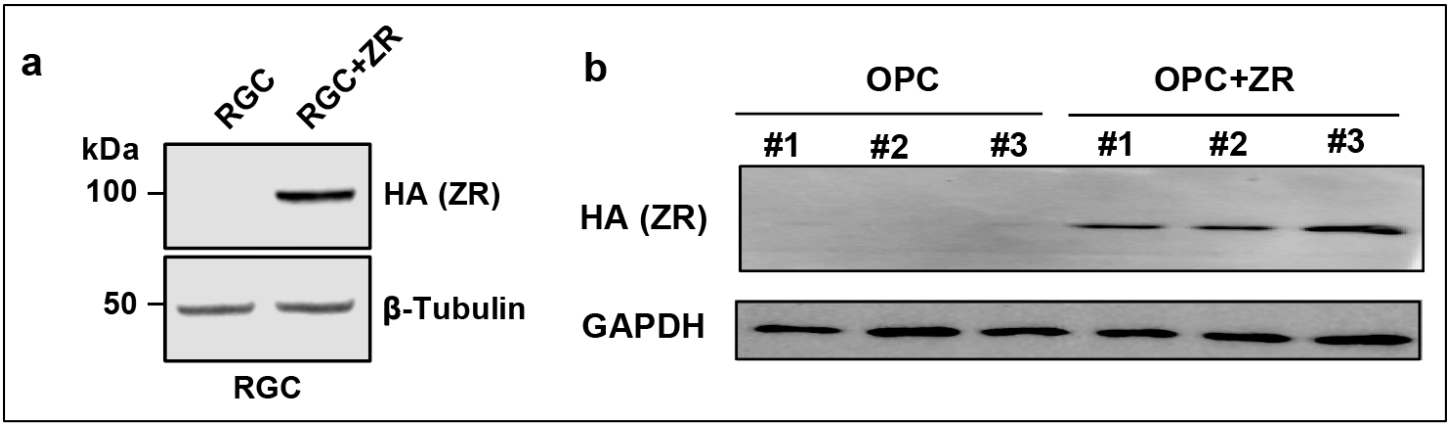
We further appreciate the comment from *Reviewer #1* about the expression of *Fabp7* in other cell types, such as OPCs and astrocytes. One of the major challenges in restricting expression to the RGC lineage is that many RGC markers gradually diminish with development and/or stay expressed (albeit at lower levels) in more differentiated cell types. Identifying promoters that behave closer to an 'on' / 'off' switch between cell types has been very difficult. For example, other radial glial markers are also not exclusively expressed in this cell type, such as GLAST (also astrocytes[5, 6]), PAX6 (neuroectoderm [7]), favoring neuronal fate[8], mature astrocytes[9], or SOX2 (OPC lineage[10], inner cell mass[11], some neurons and glia[12]) are also not exclusively expressed in RGCs. While *Fabp7* promoter activity is not binary, it is known to be expressed in the embryonic brain ventricular and subventricular zone[13-15], which are the cells our IUE method targets from a technical perspective. As an alternate approach, to control for cell type selective effects, we also expressed ZR in isolated embryonic E14.5 derived OPCs and observed very similar patterns that: 1) ZR target gene expression is not driven by significant changes in chromatin accessibility, and 2) ZR expression in OPCs cannot activate the full complement of oncogenic targets seen in mouse ZR tumors. We appreciate *Reviewer #1* for these important comments that have led to new experiments to increase the scientific rigor of our findings.

Reviewer #1 – Point #3

Please show the appropriate Western blots confirming ZFTA-RELA expression following infections.

Response:

We have included western blot analysis showing successful expression of HA-tagged ZR in both mouse RGCs and OPCs in the new functional experiments provided (**Response Figure 3, Extended Data Figure 1).** We apologize for this oversight.



Response Figure 3: Western blots to show ZR expression in (a) RGC and (b) OPC cells.

Reviewer #1 – Point #4

From Fig. 1G, it is evident that the PLAGL1 motifs are also accessible in the OPC lineage, although this cluster is relatively small at E14.5 in the mouse forebrain. What roles do OPCs play in EPNs? Is there also accessibility of ZR-specific DNA binding sites in OPCs?

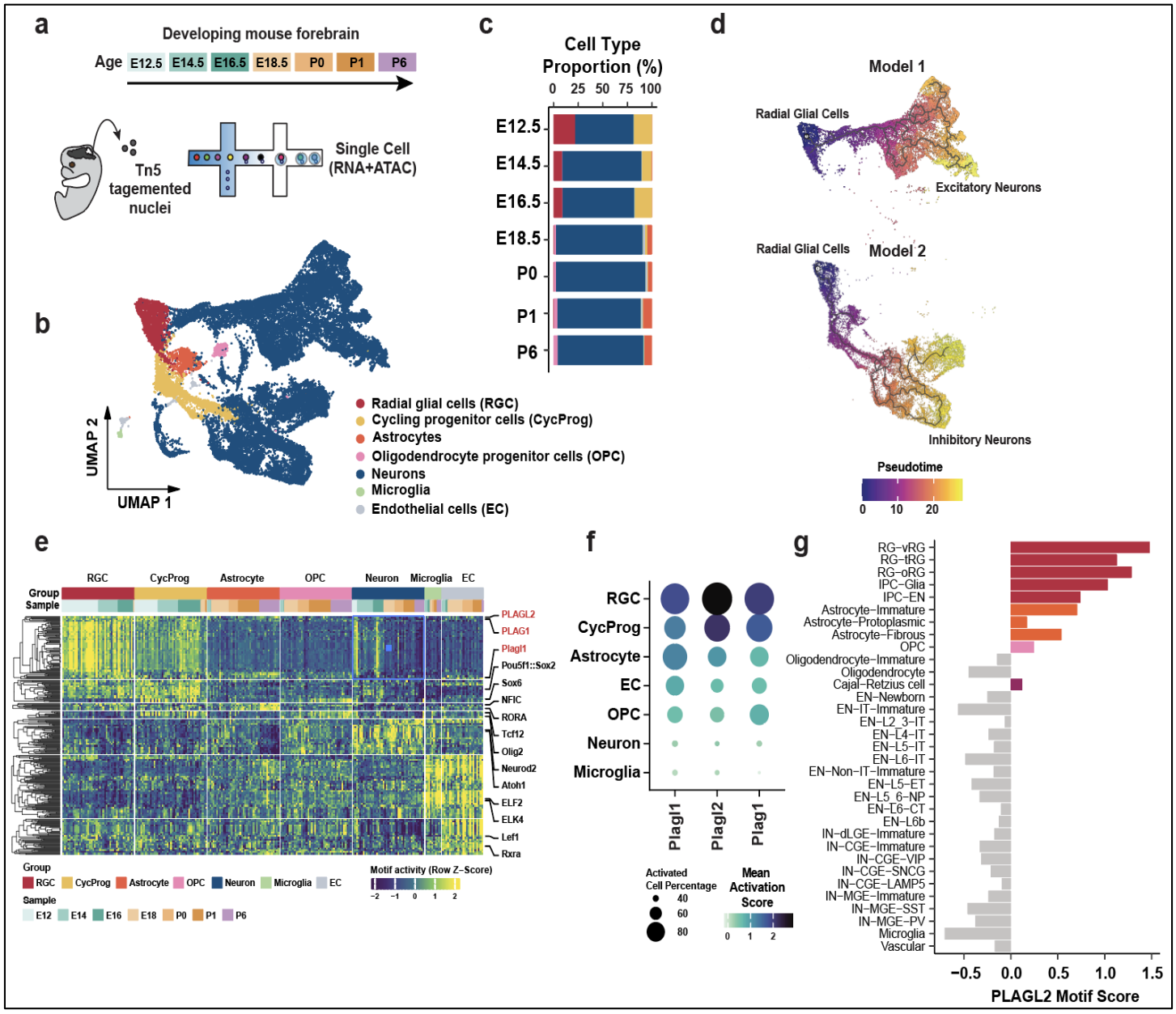
Response:

We thank the reviewer for this very insightful comment. To our knowledge, there have not been any functional studies that implicate OPCs in EPN, and from an immunohistochemical perspective EPNs stain negative for oligodendrocyte precursor cell (OPC) and mature oligodendrocyte markers[16] *(Communications – David Ellison MD PhD, Neuropathologist*). *Reviewer #1* is correct to point out that while Plagl1/2 TF motifs have the greatest chromatin accessibility in radial glial cell and cycling progenitor cells, this diminishes in OPCs, then to a much greater extent following mature neuronal differentiation. This data is represented in a new and expanded snMultiome (ATAC+RNA)-seq dataset of mouse forebrain development that we have now included (**Response Figure 4, Main Figure 1**). Supporting these findings we have also searched datasets on human fetal cortical brain development[3] and observe very similar trends with respect to PLAGL2 TF motif accessibility during human brain development (**Response Figure 4g, Main Figure 1l**).

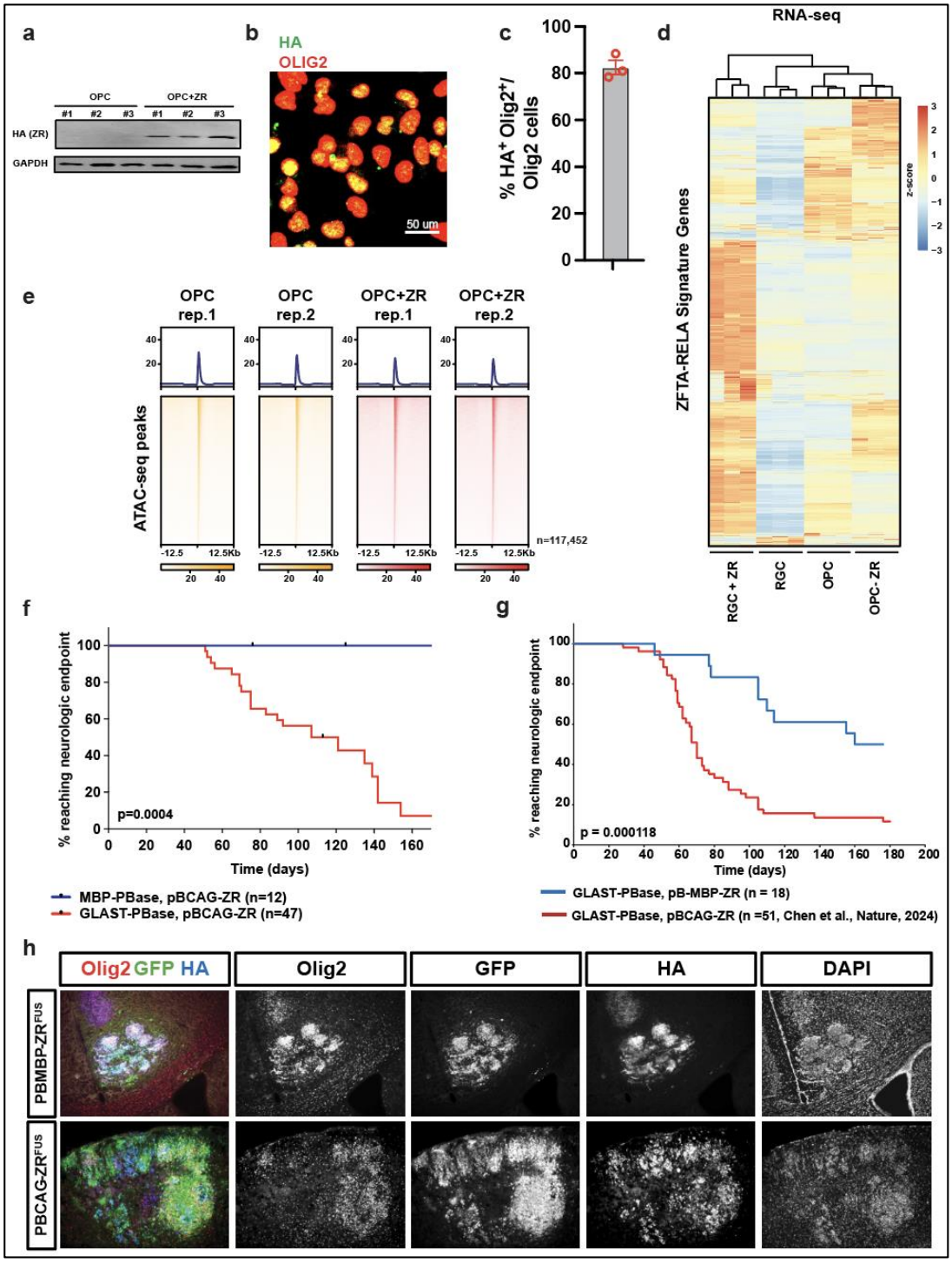
Based on these expanded observations, and suggestions by *Reviewer #1*, we performed new functional experiments to express ZR in E14.5 isolated mouse OPCs (**Response Figure 5, Extended Data Figure 2**). Despite some changes in gene expression programs and activation of ZR target genes, we did not observe marked global changes in chromatin accessibility by ATAC-seq (**Response Figure 5, Extended Data Figure 2**). Furthermore, ZR expression in OPCs could not activate the full extent of ZR target programs seen in transformed RGCs (**Response Figure 5d, Extended Data Figure 2**). These experiments were then extended to our IUE model expressing ZR in the oligodendrocyte lineage using the GLAST piggyBAC transposase and ZR driven by the MBP promoter, which is active in the OPC lineage during these developmental stages. ZR driven in the oligodendrocyte lineage significantly delayed tumor growth, leading to brain tumors with expression of OPC markers such as Olig2 (**Response Figure 5e-g, Extended Data Figure 2e-g**). These experiments were complemented by additional *in vivo* experiments in which the piggyBAC transposase was expressed under the MBP promoter, and ZR under the CAG promoter which completely failed to develop tumors; likely because MBP expressing cells first appear in the hindbrain and not in the ventricular zone until several days after birth, well after the IUE surgery is performed[17].

These findings suggest that ZR expression engages Plagl1/2 TF motifs early in brain development during neurogenesis and the expansion of radial glial and cycling progenitor cells. Supporting this hypothesis is evidence that: 1) EPNs infrequently express oligodendrocyte lineage markers (such as OLIG2)[16], 2) The presence of neuronal-like tumor cells in mouse and human ZR EPN, and 3) OPCs are largely absent from both mouse and human ZR EPN snRNA-seq profiles **(Main Figure 2e,f; 4a-d)**.

We are grateful for this reviewer comment that has led to improvements in our understanding of how ZR oncogenic programs intersect with underlying epigenetic networks during brain development.



Response Figure 4: (a) Schematic depicting the methodology for generating snMultiome data from E12.5 to P6 normal mouse forebrain. (b) UMAP plot illustrating the distribution of annotated cell types following integration of the snMultiome data. (c) Proportions of cell types identified in each of seven normal mouse forebrain samples. (d) Pseudotime reveals two neuronal lineage trajectories from RGCs to excitatory and inhibitory neurons. (e) Landscape of cell type–specific motif activation. (f) PLAG family motif activation scores across cell type. Dot size represents the percentage of activated cells within each cell type. (g) PLAGL2 motif scores across human developing brain cell types, derived from data published by Wang et al. (2015).



Response Figure 5: (a) Western blot gel image showing expression of HA-tagged ZR in OPC-ZR cells compared to control OPCs. GAPDH serves as the loading control. (b) Immunofluorescent staining of OPCs showing HA (green, tags ZR fusion) and Olig2 (red). (c) Quantification of HA+/Olig2+ cells in immunofluorescent staining. (d) Heatmap of ZR 93 gene expression within RGC, RGC+ZR, OPC, and OPC+ZR cell lines. (e) Global ATAC-seq peak profiles of OPC WT and OPC ZR-fusion samples, based on two technical replicates in a mouse brain cell line. (f) Survival curve for MBP-PBase, pBCAG-ZR (blue, n=12) GLAST-PBase, pBCAG-ZR (red, n=47) IUE mice (p=0.0004). (g) Survival curve for GLAST-PBase, pB-MBP-ZR (blue, n=18) GLAST-PBase, pBCAG-ZR (red, n=51, Chen et al. Nature 2024) IUE mice (p=0.000118). (h) Immunofluorescent staining of GLAST-PBase, pB-MBP-ZR (top row) and GLAST-PBase, pBCAG-ZR (bottom row) tumors.

Reviewer #1 – Point #5

Can the authors explain why the IUE experiments were performed slightly later (at E16.5) than the experiments in Fig. 1, where they characterized chromatin accessibility in the developing mouse forebrain (at E14.5)? Could some of the differences related to cell type-specific PLAGL1 motif activity between normal forebrain development and ZR-EPN mouse tumors be attributed to a difference in developmental time of the experimental paradigms?

Response:

Thank you for your insightful comment. We fully agree that our examination of one timepoint during development was not sufficient. In response we have expanded our snMultiome (ATAC+RNA) analysis of the developing forebrain from n = 1 to n = 7 timepoints, from **E12.5** to **P6** (**Response Figure 4, Main Figure 1**). Our findings are largely consistent that Plagl1 and 2 TF motif activity is elevated during the expansion of radial glial and cycling progenitor cells during the embryonic period and diminishing upon differentiation to post-mitotic neurons. The effects of reduced chromatin accessibility specifically at ZR binding sites are also observed during neurogenesis at various embryonic timepoints (**Response Figure 4, Figure 1**). Finally, we have expanded our analysis to validate these findings in the developing human fetal cortex, where very similar trends are observed with respect to PLAGL2 TF motif activity which is reduced upon neuronal differentiation (**Response Figure 4, Figure 1**).

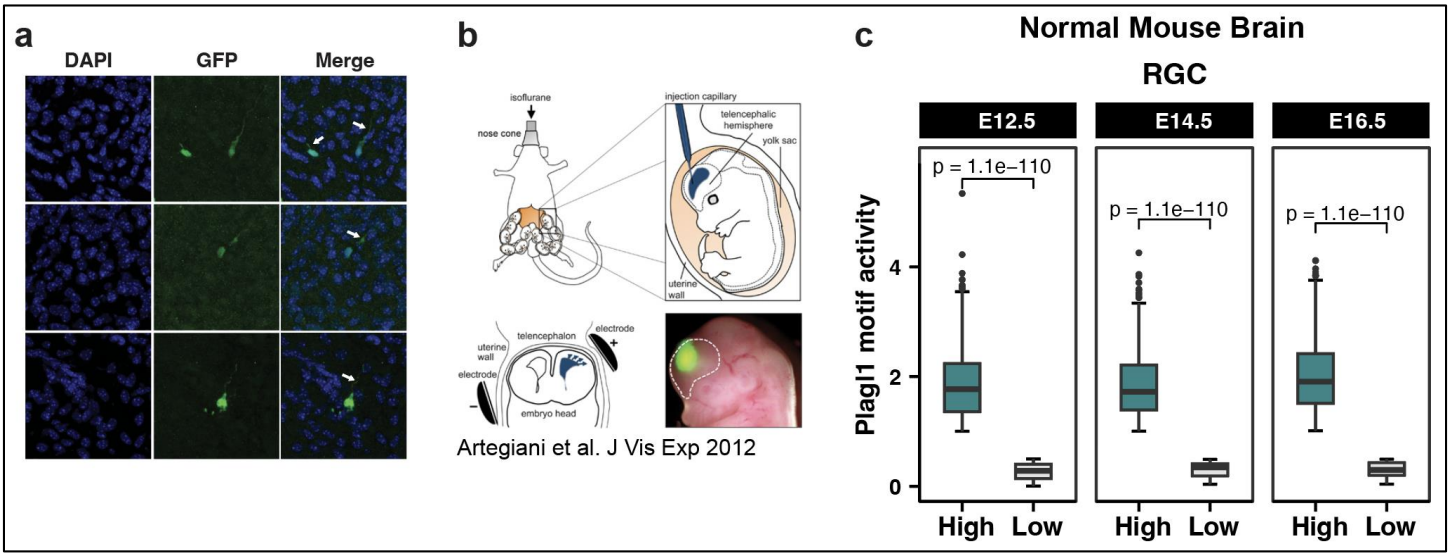
While Plagl1 and 2 TF motif activity is elevated - as a whole - across RGC and cycling progenitor cells during mouse embryonic brain development, (while likely not temporal) there appears to be some cell-type heterogeneity with a small percentage of cells harboring low-no Plagl1/2 TF motif activity (**Response Figure 6**). We are careful to speculate about the potential function of these cells with lower Plagl1 and 2 TF activity and the impact on tumor initiation in the absence of validated promoters that can specifically target these cells. Such efforts are currently being pursued as part of future efforts to better refine the ZR mouse model as a reflection of the human disease. We thank the reviewer for these constructive suggestions that have led to an improved understanding of the Plagl1/2 epigenetic programs during cortical brain development.

Reviewer #1 – Point #6

The authors used the GLAST promoter to target RGCs; however, like most early promoters this is not completely unbiased and leans towards gliogenic progenitors. Have the authors confirmed cell type-specific integration of ZR?

Response:

We thank the reviewer for this comment. Our rationale for preferential targeting of the RGC compartment is based on the IUE technique which incorporates plasmids in cells lining the targeted ventricle, comprising predominantly RGCs during embryonic brain development[18], **Response Figure 6**). As an example, we have performed immunofluorescence staining of developmental cells targeted by IUE, demonstrating morphological features consistent with expression of GFP plasmids in radial glial cells (**Response Figure 6**). The inclusion of GLAST-transposase provides further control of expression within this RGC population through cell-type preferential expression. We agree that we cannot absolutely exclude the possibility of other gliogenic progenitor cells targeted, such as those which arise from RGCs. We have included in the discussion section a description of these limitations, however we would also like to emphasize that our manuscript focuses more so on the Plag/l



Response Figure 6: (a) Immunofluorescence for GFP in P2 mice after GFP IUE. Arrows denote radial fibers typical of RGCs. (b) Schematic of IUE adapted from Artegiani et al J Vis Exp 2012. Mice are injected with GFP plasmid using a capillary pipet. The positive electrode of the tweezzertrode is oriented above the cortex so these cells are targeted for electroporation. (c) In the developing forebrain when RGCs are present (E12.5, E14.5, E16.5), there appears to be some cell-type heterogeneity with a small percentage of cells harboring low-no Plagl1 TF motif activity.

1/2 epigenetic programs accessible in early developmental lineages, and less so on the precise cell types that may (or may not) give rise to EPN. We greatly appreciate the comment here by *Reviewer #1*.

Reviewer #1 – Point #7

For the experiments in Fig. 2, a control (non ZR-expressing) group is missing, but may be critical to understand how ZR-induced tumorigenesis impacts cell type distribution, PLAGL1 motif activity, etc. in this model.

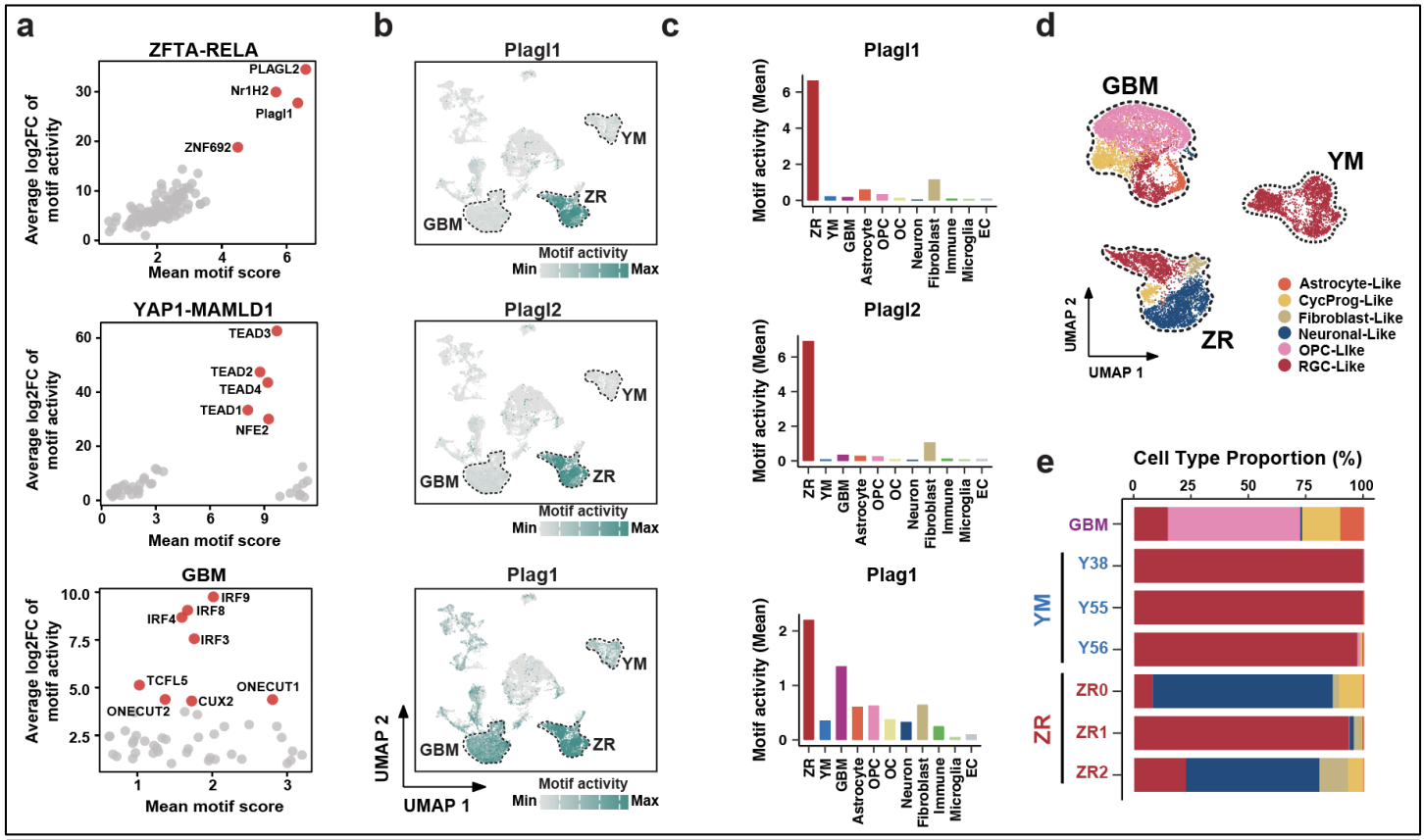
Response:

In response, we have included new data of other EPN subtypes driven by a *YAP1-MAMLD1* gene fusion[19, 20], as well as a GBM model, all profiled by snMultiome analysis (**Response Figure 7, Main Figure 2**). Following integration of the different brain tumor models, we found that ZR tumors continue to engage the Plagl1/2 TF motifs specifically past embryonic brain development. This is in sharp contrast with *YAP1-MAMLD1* tumors enriched with the expected TEAD family of TF motifs (**Response Figure 7, Main Figure 2**). In new comparisons, we found that while ZR tumors often progress through some level of differentiation giving rise to neuronal-like and astrocyte-like cells in mice, this contrasts with GBM tumors that expand the OPC state. Interestingly, *YAP1-MAMLD1* EPN tumors that appear to be relatively blocked in the RGC state (**Response Figure 7, Main Figure 2**). We appreciate this insightful comment from the reviewer regarding the inclusion of disease specific controls, revealing new insights into the transcriptional heterogeneity of EPN and GBM.

Reviewer #1 – Point #8

The inferCNV data in Ext. Fig. 2A-B suggest high variability in CNV patterns between the three ZR-EPN mouse tumors. In addition, the CNV data for the single GBM mouse tumor do not display the classical chr 7 amplification. How do the authors explain these findings?

Response:

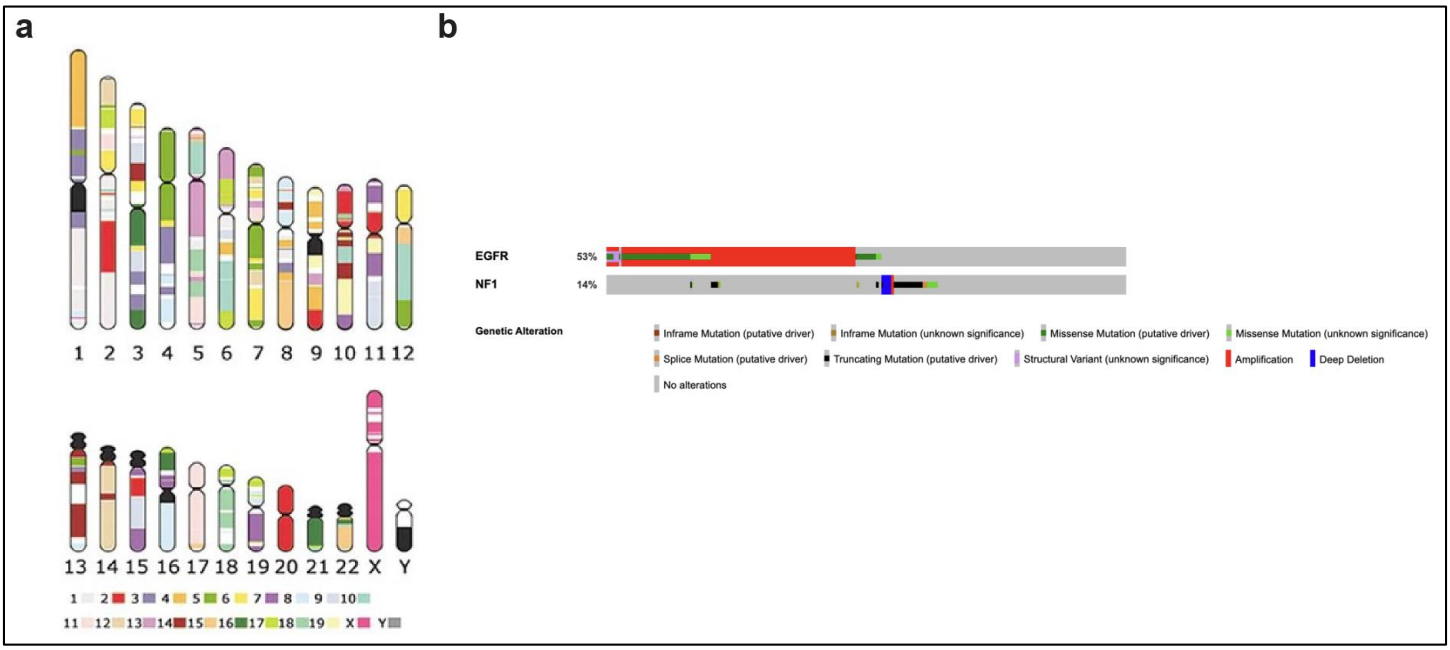


Response Figure 7: (a) Scatter plot illustrating tumor type–specific motif differences relative to other tumor types. The x-axis denotes the mean motif score, while the y-axis represents the average log2 fold-change in motif activity. Highly differential motifs are highlighted in orange and labeled. (b) Activity states of PLAG family motifs across all cell types, with tumor cell groups indicated. (c) PLAG family motif activity across cell types in tumor (ZR, YM, GBM) and non-tumor cell groups. (d) Comparison of cell cycling signal score (y axis) to ZR-fusion target gene expression signal score (x axis) across the various tumor cell types. (e) Reconstructed UMAP based on tumor cells and annotated tumor cell types. (f) Distribution of tumor cell types across individual tumor samples.

We acknowledge the CNV variability observed among the three ZR-EPN mouse tumors (**Extended Data Fig. 2A-B**), which likely reflects intra-tumoral heterogeneity. Such variation is consistent with the complex and diverse genomic landscape characteristic of EPNs. Regarding the GBM model, the absence of classical chromosome 7 amplifications can be attributed to species-specific genomic architecture. Mouse and human genomes differ in both chromosome number and synteny; regions homologous to human chr 7 are dispersed across multiple mouse chromosomes (**Response Figure 8**). As a result, canonical CNV patterns observed in human GBM may not manifest natively in mouse models.

More specifically, *Reviewer #1*, may be asking an important question about why EGFR gain/amplification is not seen in the mouse GBM model. In glioblastoma (GBM), alterations in the PI3K, RAS, and p53 signaling pathways are frequent and play a central role in tumor initiation, invasion, and therapeutic resistance. One common mechanism involves activating mutations of receptor tyrosine kinases, most notably EGFR, or the loss of negative regulators such as NF1, both of which converge on aberrant RAS activation (TCGA Network, 2008). The PI3K–AKT–mTOR axis represents a second major pathway, often dysregulated through activating mutations in *PIK3CA* or loss of the tumor suppressor *PTEN* (TCGA Network, 2008[21]). Finally, disruption of cell cycle control occurs through *CDKN2A* deletion or *TP53* mutation. Large-scale genomic analyses, including The Cancer Genome Atlas (TCGA), have shown that approximately 88–90 percent of GBMs harbor alterations in the RAS pathway (TCGA Network, 2008[21]). Within these data, *CDKN2A* and *TP53* mutations are largely mutually exclusive, as are *PTEN* loss and *PIK3CA* mutations. By the same logic, *EGFR* amplification or mutation and *NF1* loss would also be predicted to be mutually exclusive, as both events achieve the same functional outcome

of driving RAS activation. To evaluate this hypothesis, we queried the TCGA GBM dataset (n = 592) available through cBioPortal [22]. The results confirmed that NF1 and EGFR alterations are indeed mutually exclusive, with an odds ratio of –2.106 and strong statistical support ($p < 0.001$, $q < 0.001$) (**Response Figure 8b**). Together, these findings indicate that in our experimental model, *NF1* loss would obviate the need for concurrent *EGFR* activation, consistent with patterns observed in patient tumors. We thank the reviewer for allowing us to clarify this point, as it highlights an important but often underappreciated principle of redundancy within RAS pathway signaling, especially in contrast to the more widely recognized exclusivity of *PTEN/PIK3CA* and *CDKN2A/TP53* alterations.



Response Figure 8: (a) Mouse and human genomes differ in both chromosome number and synteny. (b) Query of the TCGA GBM dataset shows that NF1 and EGFR alterations are mutually exclusive, with an odds ratio of –2.106 and strong statistical support ($p < 0.001$, $q < 0.001$).

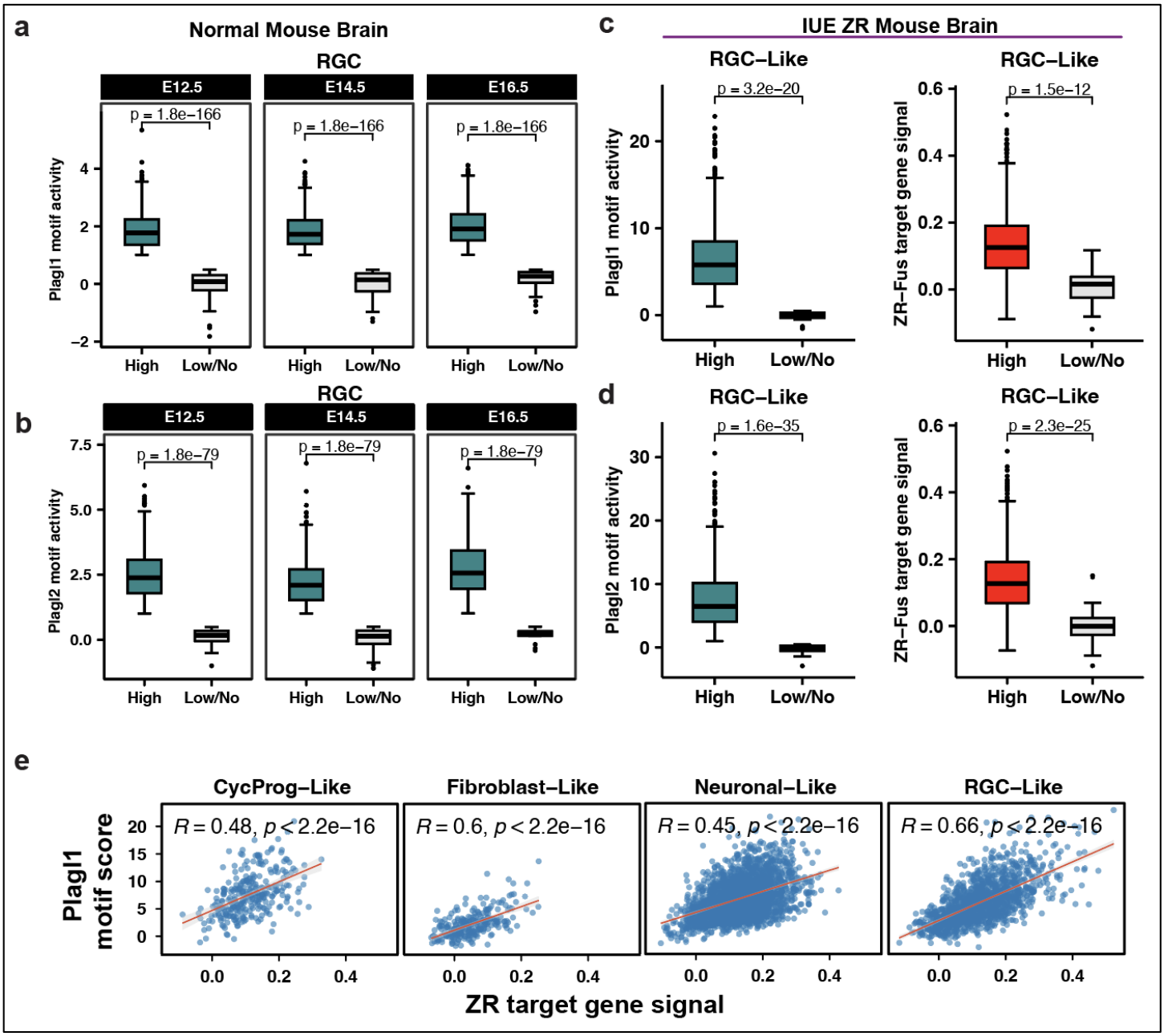
Reviewer #1 – Point #9

From Fig. 2E, it seems as though a subset of the RGC-like cluster has very high PLAGL1 activity, compared to other RGC-like cells within the same cluster, which may drive the differences between normal brain development and the ZR-EPN tumors. The authors could consider sub-clustering the RGC-like cluster to resolve these differences – perhaps some subtypes of RGCs are more likely to harbor high PLAGL1 motif activity in ZR-EPN tumors? Or perhaps this is related to earlier critiques on using GLAST as a promoter, performing IUE experiments later in developmental time, compared to experiments in Fig. 1, and missing a non ZR-expressing control group assayed at the same developmental time as the ZR-EPN tumor-expressing mice?

Response:

We apologize to *Reviewer #1* about the confusing aspects of timing of IUE (at E16.5) and when the developmental map is presented at E14.5. In response, and as described above, we have generated snMultiome maps of the developing mouse forebrain from E12.5 to P6, covering 6 additional timepoints. Our results demonstrate that the Plagl1/2 TF motif is preferentially accessible in RGCs and cycling progenitor cells during the embryonic stage and diminishes during neurogenesis. The reviewer also raises a nice point about heterogeneity of Plagl1/2 TF motif activity in RGC-like cells. While generally elevated compared to other cells, there is some degree of variability of the Plagl1/2 TF motif in RGC-like cells (**Response Figure 9**). We think this

is likely due to variability of ZR activity in cells, with ZR maintaining chromatin accessibility at Plagl 1/2 TF motifs to varying degrees. We thank *Reviewer #1* for this insightful comment.



Response Figure 9: (a-d) Based on Plagl1/2 motif activity, RGCs from normal and tumor IUE brains were divided into high and low/no activity groups. ZR target gene signaling differences between these subgroups were assessed in tumor IUE samples. (e) Correlation between Plagl1 motif score and ZR target gene signal.

Reviewer #1 – Point #9

Fig. 2G-H: The authors state that the correlation between ZR and PLAGL1 target activity is observed preferentially in neuronal-like EPN cells, however, from 2G-H this is not necessarily true: correlation with neuron-like cells may display a lower p-value (greater statistical significance), but the r-values – which denote the strength of the correlation – are slightly greater in RGC-like cells (0.6) vs. neuron-like cells (0.5). Given this and the previous critique, it is unclear if the differences in correlation between RGC and neuron-like cells are biologically significant.

I have a similar comment for Fig. 3H-I, where, in fact, RGC-like and C1-neuron-like clusters show a largely similar correlational "r" value for PLAGL1, although the "r" value for nIPC clusters is slightly higher. Thus, it may be incorrect to state that the "positive correlation between ZR signature and PLAGL family motif activity were detected largely in neuronal-like cells". On examining Fig. 3H-I in more detail, it is also apparent that other neuron-like clusters (C2 & C0), in fact, show a significantly smaller "r" value, suggesting that neuron-like clusters differ in their ZR signature / PLAGL1 motif activity correlations. The authors could assess how these three neuronal subclusters differ at the transcriptomic and functional levels.

Response:

We apologize for the inclusion of this confusing representation; our revised manuscript has removed these sub-panels. The reason for removal of this data was: 1) Its confusing interpretation, and 2) Difficulty in accessing the statistical rigor of those correlations, especially for smaller cell populations in the tumor.

Reviewer #1 – Point #10

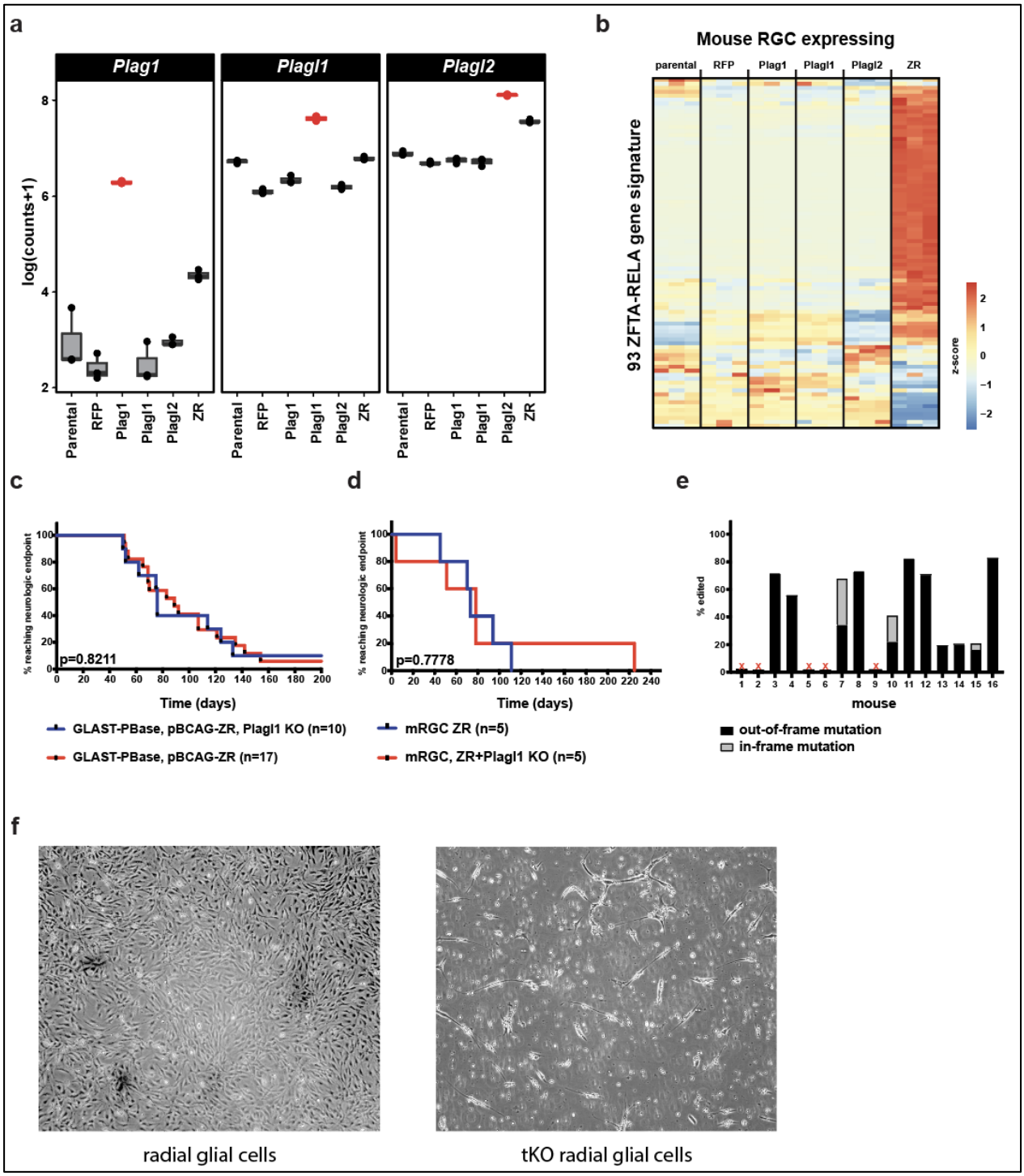
The functional studies in this manuscript are rather limited. The authors accurately state and nicely show that their data suggests that "ZR expression maintains chromatin accessibility of PLAGL family motifs that are normally repressed during neuronal differentiation". However, the consequences of keeping PLAGL family genes active during normal development, within RGCs or within cells along the neuronal lineage would also be important to examine as a part of this manuscript. The authors could also study the mechanistic role of PLAGL following transduction with ZR-expressing lentiviral vectors, as was done in the earlier part of the manuscript.

Response:

We thank *Reviewer #1* for this comment on expanding the functional evaluation of PLAG/L TF proteins and their impact upon oncogenic transcription and tumorigenesis. In response, we have performed a series of gain-of-function studies to over-express *Plag1, Plagl1, Plagl2*, and *ZR* in mouse radial glial cells to determine the impact on ZR oncogenic programs (**Response Figure 10, Extended Data Figure 2**). We hypothesized that expression of Plag/l TFs would lead to convergent activation of ZR transcriptional programs given the predicted binding of the Plagl TF motif. However, expression of *Plag1*, *Plagl1*, *Plagl2* could not significantly activate oncogenic transcriptional programs targeted by ZR (**Response Figure 10, Extended Data Figure 2**). We postulate that this could be due to not only aberrant DNA binding to Plagl TF motifs, but also through gene fusion, the acquisition of necessary disordered regions critical for increased recruitment of transcriptional activation complexes. These concepts relate to our recent finding that ZFTA fusion oncoproteins form biomolecular condensates important for gene activation and acquire disordered regions necessary to mediate low-complexity multi-valent protein interactions[23].

As an alternate hypothesis, we asked whether the Plagl1 TF co-ordinates with ZR to promote tumor progression. To this end, we leveraged our IUE ZR model to perform *in vivo* knock-out (KO) studies of Plagl1 at the same time of ZR tumor initiation (**Response Figure 10, Extended Data Figure 2**). However, we did not observe a significant impact upon tumor progression following Plagl1 KO (**Response Figure 10, Extended Data Figure 2**). We repeated this experiment using a model of ZR transformed mRGCs implanted into adult nod-scid gamma (NSG) mice, which mirrored our IUE results (**Response Figure 10, Extended Data Figure 2**). While we also attempted these experiments with simultaneous knock out of Plagl1 and Plagl2, we found that: 1) We could not achieve efficient dual-editing of both Plagl1 and Plagl2 in our tumors and that 2) Simultaneous KO of these genes in mRGCs was lethal (14% survival by day 7 post transfection, **Response Figure 10**), consistent with previous work concerning this TF family[24]. We conclude from these functional experiments that the Plagl1 TF protein may not contribute significantly to ZR tumor development, and that KO of both Plagl1/2 within RGCs would lead to death rather than facilitate oncogenesis. However, our data are in line with a model in which these Plag/l TF motifs are highly accessible during embryonic brain development in radial glial and cycling progenitor cells,

placing them at direct risk of ZFTA-RELA transformation. We thank *Reviewer #1* for suggesting these experiments that have strengthened the manuscript through new functional studies.



Response Figure 10: (a) Confirmation of *Plag1*, *Plagl1*, and *Plagl2* overexpression in mRGCs by RNA-seq. (b) Heatmap of ZR 93 gene signature for parental, *RFP, Plag1, Plagl1, Plagl2*, and *ZR* expressing RGCs by RNA-seq. (c) Survival curve for GLAST-PBase, pBCAG-ZR (red, n=17) vs GLAST-PBase, pBCAG-ZR, *Plagl1* KO IUE mice (blue, p=0.8211). (d) Survival curve for mRGC ZR+ (blue, n=5) vs mRGC ZR+ *Plagl1* KO (red, n=5) implanted mice (p=0.7778). (e) Confirmation of *Plagl1* KO for mice within survival curves. Red X denotes a mouse with no knockout which was removed from analysis. (f) Simultaneous KO of *Plag1*, *Plagl1* and *Plagl2* in mRGCs. This was lethal, with a 14% survival 7 days post transfection.

Minor concerns:

Reviewer #1 – Point #11

In addition to the CNV heatmaps shown by sample (on the Y-axes) in Ext. Fig. 2, can the authors also display heatmaps of the EPN and GBM tumors by cluster identity on the Y-axes, so the readers can fully visualize which clusters were annotated as malignant vs. non-malignant?

Response:

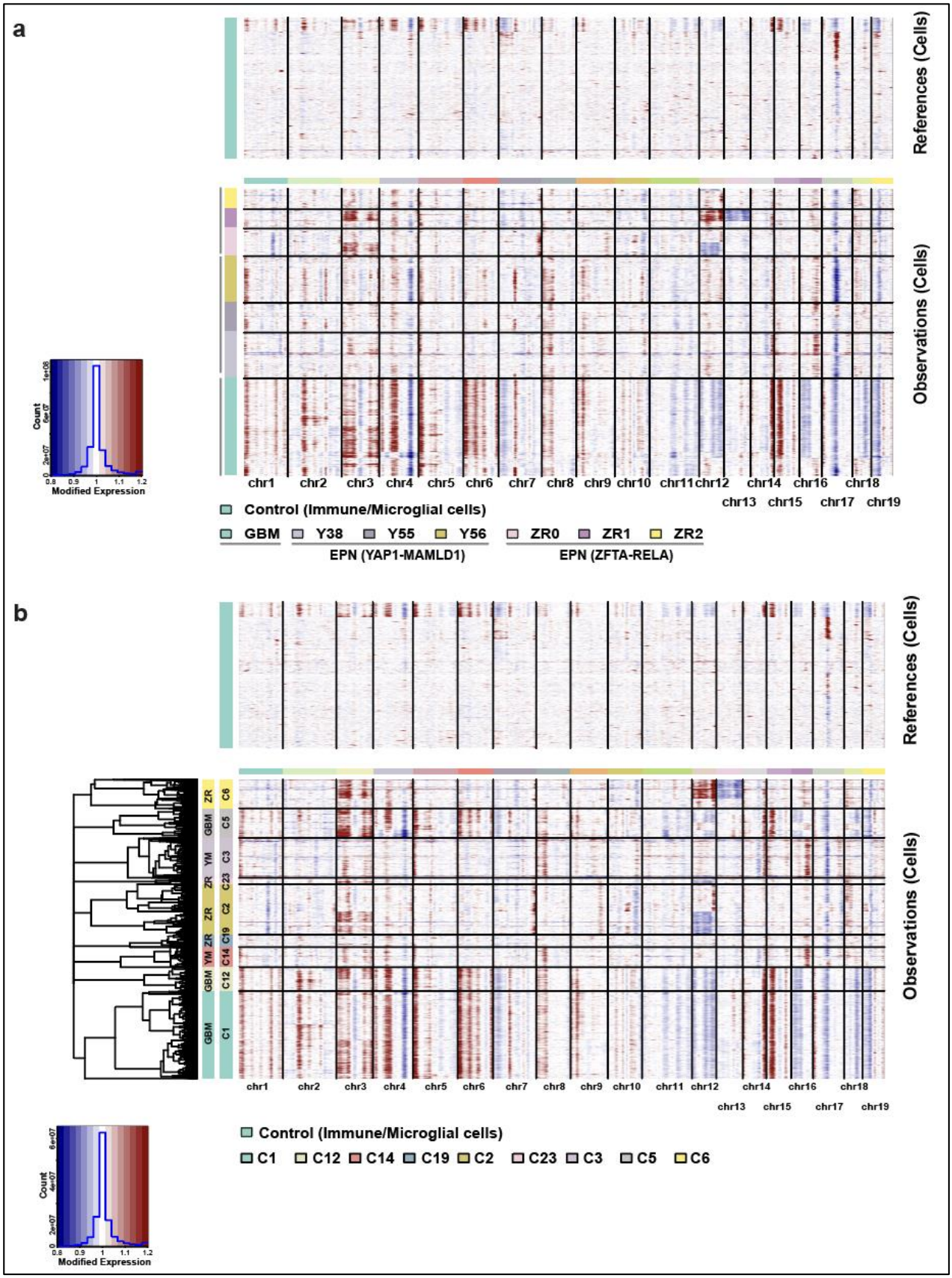
Thank you for your insightful suggestion. In response, we have generated two sets of CNV heatmaps: one organized by individual samples (**Response Figure 11a, Extended Data Fig. 2**), and a new set organized by cluster identity, now included in **(Response Figure 11b, Extended Data Figure 3b**). We would also like to clarify that malignant cells were predicted at the single-cell level rather than the cluster level. As a result, individual clusters often contain a mixture of both malignant and non-malignant cells, making it difficult to definitively label entire clusters as malignant or non-malignant.

Reviewer #1 – Point #11

The authors should discuss in detail their observation that RGCs are not the dominant cell types within murine ZR-EPN tumors (as depicted in the barplots of Ext. Fig. 2E) in the context of their statements in the text citing prior work suggesting that RGCs may be "putative EPN cells of origin".

Response:

We thank the reviewer for this insightful comment. In response, we have added a new paragraph in the discussion about the apparent discrepancy between our observation that RGCs are not the dominant cell type in murine ZR-EPN tumors and prior reports suggesting RGCs as putative EPN cells of origin. We posit that RGCs may initiate tumorigenesis in mouse development but may not persist as dominant populations within fully developed tumors. This is possibly due to the known role of RGCs as they transition to neuronal precursors in the developing brain, and potentially why neuronal-like malignant cells are the predominant cell type observed in mouse and human ZR EPN.



Response Figure 11: CNV heatmap for EPN and GBM IUE tumors organized by (a) individual sample and (b) cluster identity.

Referee #2 (Remarks to the Author):

In this study, Kardian and colleagues investigate the susceptibly of developmental neural cells to transformation and elucidate the mechanism employed by fusion oncoproteins to drive tumorigenesis in pediatric supratentorial (ST) ependymoma (EPN). Using combined single cell ATAC and RNA sequencing in mouse and human EPN they build upon their previous study and find that accessible motifs in progenitor cells are leveraged by the oncogenic transcription factor ZFTA-RELA. They demonstrate that the accessibility program in radial glial cells is found to be enriched in PLAGL family DNA motifs which are open at oncogenic loci known to play a role in ependymoma progression. The study then confirms the EPN tumor cell hierarchy and demonstrates the clonal evolution of EPN.

This is a beautiful and important paper that contributes key insights to mechanistic understanding of ST-EPN.

I have the following recommendations to improve an already excellent paper:

Response:

We thank *Reviewer #2* for their supportive comments and appreciate the recommendations that have led to improvements in the clarity and rigor of our scientific findings.

Reviewer #2 – Point #1

In Figures 1h-j, the motif activity score, DNA and chromatin accessibility are compared between RGC and neurons. As the RGC progenitor population is compared to mature neurons that harbor condensed chromatin, it would be interesting to look at this analysis comparing to a different progenitor cell type to further understand intrinsic vulnerabilities. OPCs appear to have accessible Plagl family TF motifs, how do these differ to the RGCs?

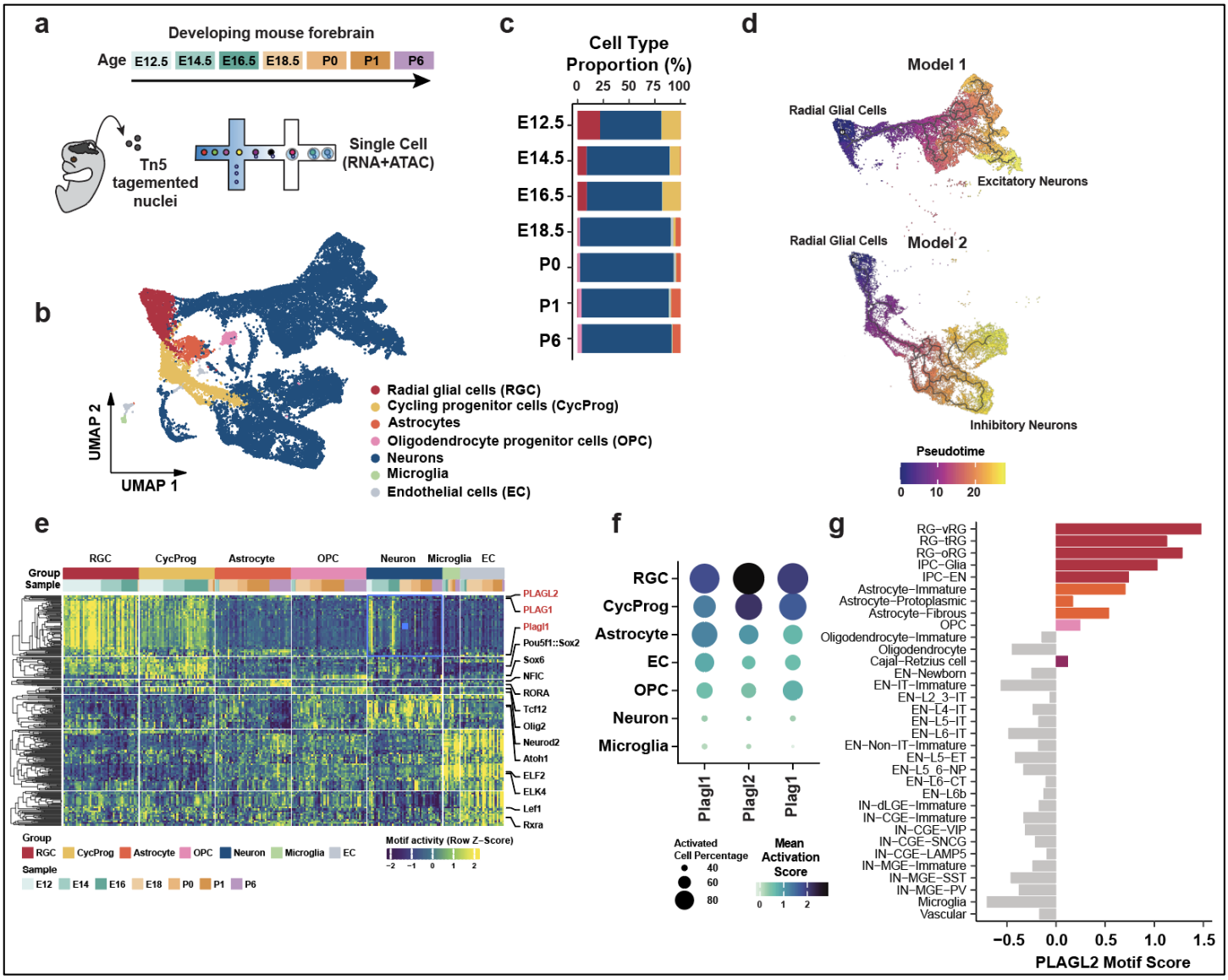
Response:

We thank *Reviewer #2* for this important comment. In our revised manuscript, we provide an expanded developmental snMultiome (RNA+ATAC) signature of the developing mouse forebrain from E12.5 to P6 (**Response Figure 1, Main Figure 1**). This has enabled a more comprehensive view of the Plagl TF family motifs and their chromatin accessibility in developing brain cells as a function of early mouse brain development. Consistent with our original findings, we demonstrate that RGC and cycling progenitor cells have elevated Plagl1 and Plagl2 TF motif activity, which diminishes in the OPC compartment, and then condensed more prominently in mature neurons (**Response Figure 1, Main Figure 1**). Furthermore, we have investigated the human developing fetal brain and observe very similar trends with respect to PLAGL2 TF accessibility, and lower accessibility in the OPC compartment (**Response Figure 1, Main Figure 1**).

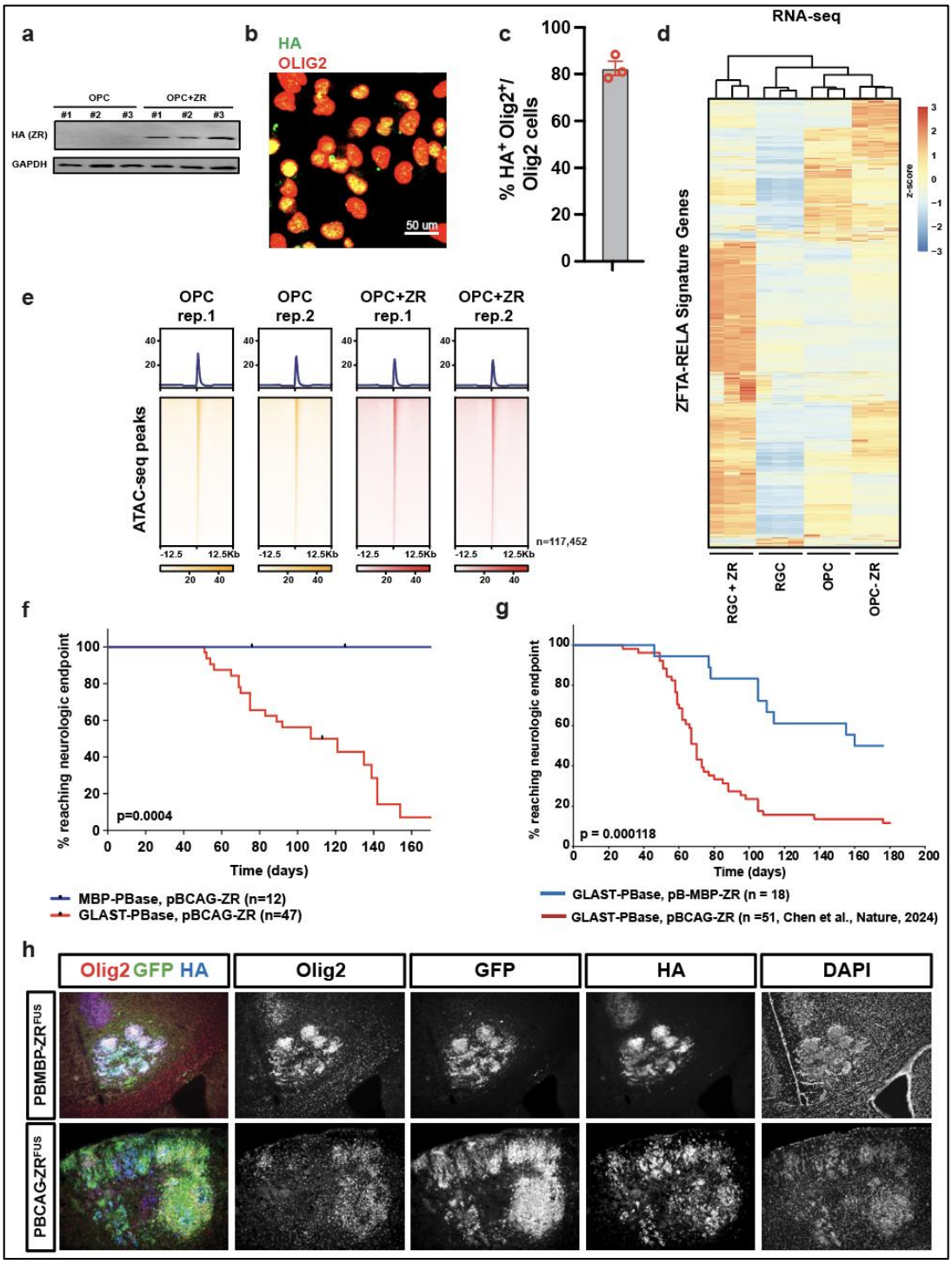
To further understand the role of ZR and its impact on Plagl TF engagement in OPC, we performed a series of *ex vivo* experiments to express ZR in OPCs derived from E14.5 mouse brains (**Response Figure 2, Extended Figure 5**). Consistent with our findings in RGCs we found that the expression of ZR does not result in a marked change in chromatin accessibility as measured by ATAC-seq analysis. Interestingly, we find that expression of ZR in OPCs cannot activate the full extent of ZR gene expression signatures (**Response Figure 2, Extended Figure 2**). Finally, we tested whether ZR expression in OPCs could initiate brain tumors in mice by integration of a new piggyBAC construct that expresses ZR under the MBP promoter (**Response Figure 2, Extended Figure 2**). We demonstrate that brain tumors form significantly later when expressed within the OPC lineage and express OPC markers such as Olig2, typically not frequently observed in mouse and human ZR EPN. To

complement these *in vivo* studies, we also expressed the PB-transposase under the MBP promoter and ZR under the CAG-promoter that abrogates tumor initiation.

We conclude from these experiments that ZR can still engage open Plag/l TF motifs in the OPC compartment to initiate tumorigenesis. However, the ability of ZR to activate the full extent of oncogenic programs is diminished leading to significantly delayed tumor formation. These findings coupled with knowledge that EPN rarely express oligodendrocyte lineage markers[16], leads us to speculate that ZR transformation is restricted by developmental epigenomic programs in the OPC lineage; in sharp contrast with drivers in pediatric and adult glioma that significantly expand the OPC compartment[25].



Response Figure 1: (a) Schematic depicting the methodology for generating snMultiome data from E12.5 to P6 normal mouse forebrain. (b) UMAP plot illustrating the distribution of annotated cell types following integration of the snMultiome data. (c) Proportions of cell types identified in each of seven normal mouse forebrain samples. (d) Pseudotime reveals two neuronal lineage trajectories from RGCs to excitatory and inhibitory neurons. (e) Landscape of cell type–specific motif activation. (f) PLAG family motif activation scores across cell type. Dot size represents the percentage of activated cells within each cell type. (g) PLAGL2 motif scores across human developing brain cell types, derived from data published by Wang et al. (2015).



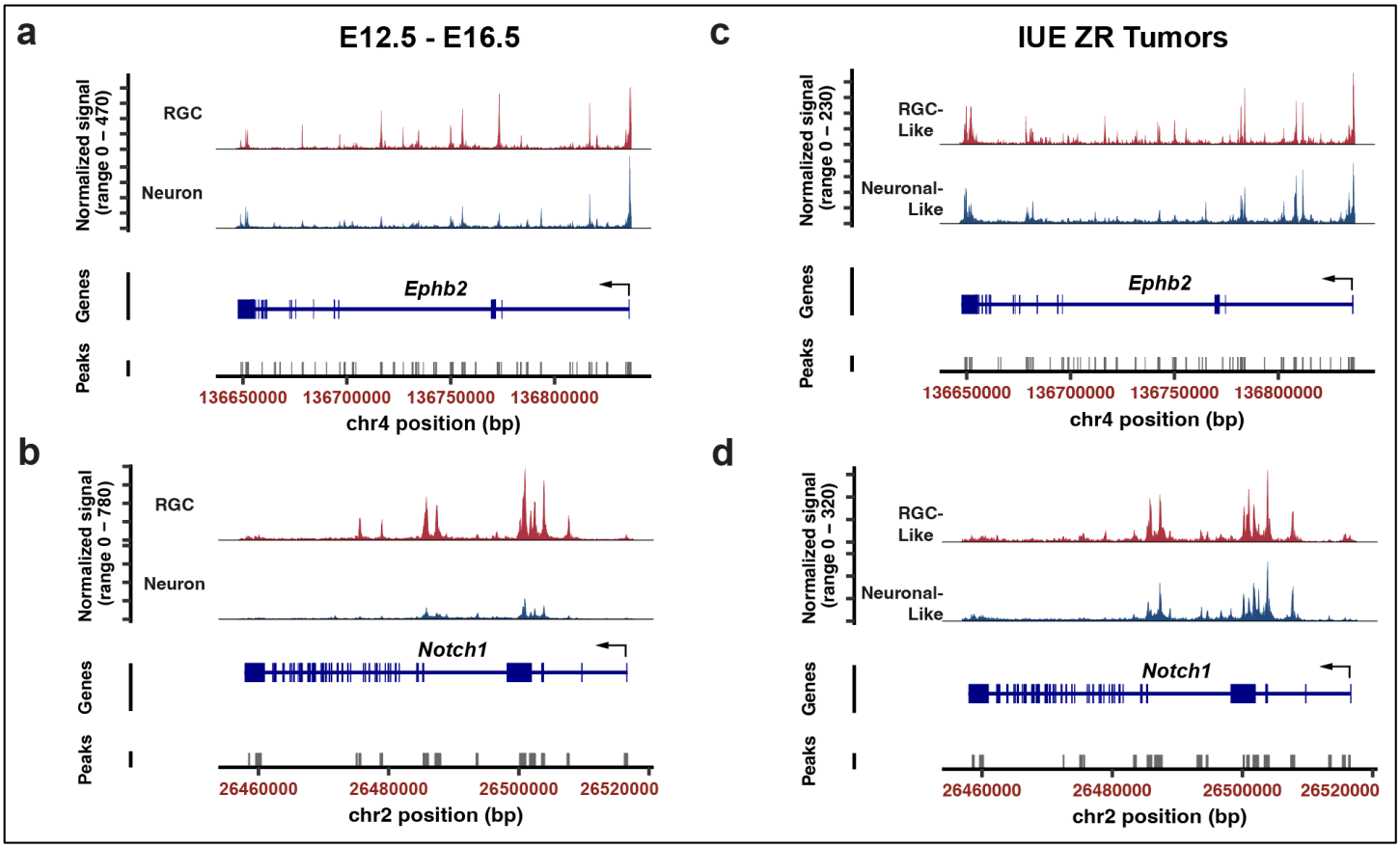
Response Figure 2: (a) Western blot gel image showing expression of HA-tagged ZR in OPC-ZR cells compared to control OPCs. GAPDH serves as the loading control. (b) Immunofluorescent staining of OPCs showing HA (green, tags ZR fusion) and Olig2 (red). (c) Quantification of HA+/Olig2+ cells in immunofluorescent staining. (d) Heatmap of ZR 93 gene expression within RGC, RGC+ZR, OPC, and OPC+ZR cell lines. (e) Global ATAC-seq peak profiles of OPC WT and OPC ZR-fusion samples, based on two technical replicates in a mouse brain cell line. (f) Survival curve for MBP-PBase, pBCAG-ZR (blue, n=12) GLAST-PBase, pBCAG-ZR (red, n=47) IUE mice (p=0.0004). (g) Survival curve for GLAST-PBase, pB-MBP-ZR (blue, n=18) GLAST-PBase, pBCAG-ZR (red, n=51, Chen et al. Nature 2024) IUE mice (p=0.000118). (h) Immunofluorescent staining of GLAST-PBase, pB-MBP-ZR (top row) and GLAST-PBase, pBCAG-ZR (bottom row) tumors.

Reviewer #2 – Point #2

Similar to the data shown in Fig.1i and Ext Data Fig.1i for the mouse embryo forebrain analysis, how does the cell type specific accessibility alter for known EPN oncogenes (e.g. EphB2, Notch1) in the presence of ZR in the IUE mouse model?

Response:

In response, we have examined the cell type–specific chromatin accessibility of known EPN oncogenes, specifically *EphB2* and *Notch1*, in the presence of ZR in the IUE mouse model. We present the single cell chromatin accessibility profiles for these genes in both normal forebrain tissue and ZR-expressing IUE samples. Here, we observe that most of the active regulatory elements in normal and mouse ZR EPN are conserved, further highlighting our putative model that ZR engages cis-regulatory programs in development and maintains activity throughout tumorigenesis (**Response Figure 3, Extended Data Figure 1**). We thank *Reviewer #2* for this very helpful comment.



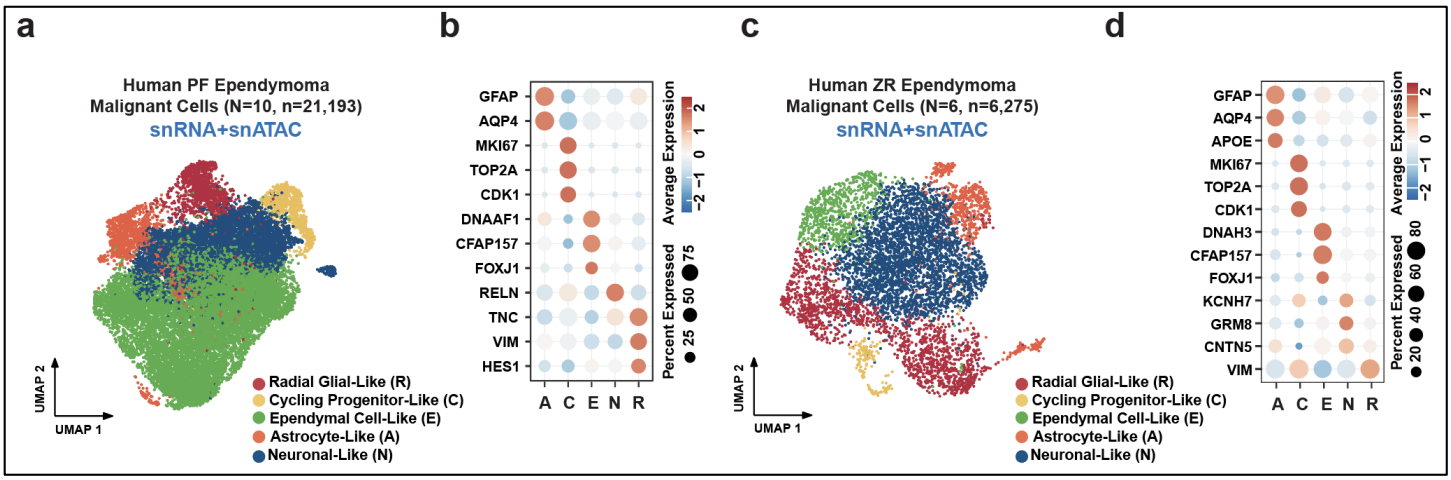
Response Figure 3: Chromatin accessibility of RGC and neurons from snATAC-seq at known ZR target genes *Ephb2* and *Notch1* in development (a,b) and IUE tumors (c,d).

Reviewer #2 – Point #3

Given that ependymal-like cells are a known cell state in EPN and have been identified in previous transcriptomic analyses of ST-EPN (Gojo et al., Cell 2020), it is striking that they aren't present in any of the ST-EPN samples analyzed. The authors should discuss/clarify why that may be the case. Are ependymal cell markers present in the cell-type reference gene list used to characterize these samples?

Response:

We thank *Reviewer #2* for pointing out this discrepancy. Our initial process for cell annotation separately utilized different cell marker lists from developmental posterior fossa (PF) and cortical brain cell types. As a result, in human ZR EPN, ependymal cell types were mis-annotated as neuronal-like cells. Our revised manuscript presents an updated approach with one comprehensive reference dataset that is applied to both ZR EPN and PF EPN tumors samples, including the provision of a cell annotation tool for reproducibility of our findings and extension to future studies. Using this updated approach, we were able to identify ependymal-like cells in our ZR EPN dataset, and these findings are now reflected in the revised **Figure 4** and **Response Figure 4**. We thank *Reviewer #2* for identifying this discrepancy and prompting our efforts to develop a more comprehensive cell annotation methodology, now included in the revised manuscript.



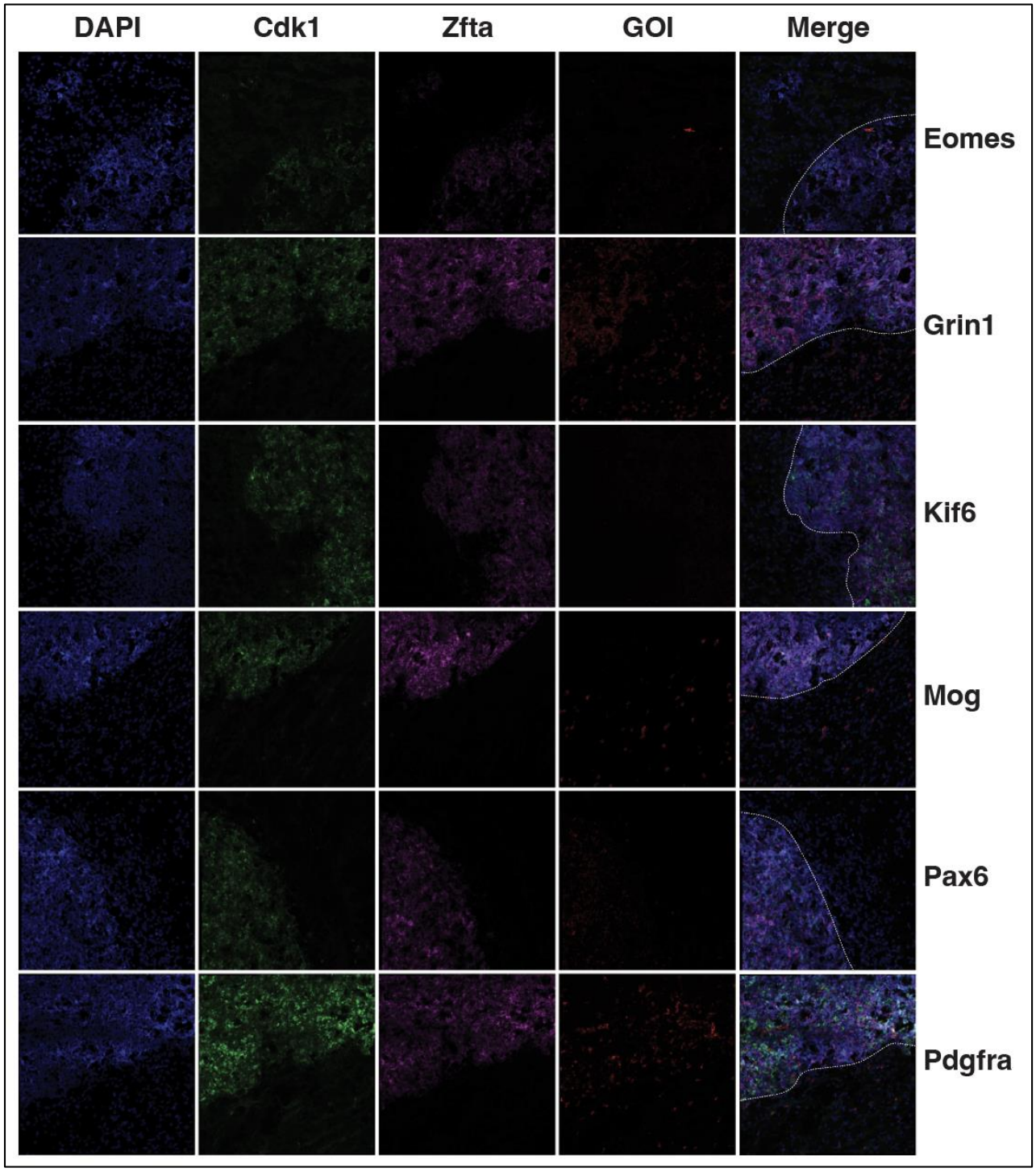
Response Figure 4: (a,c) Annotated cell types among malignant tumor cells from human PF and ZR snMultiome data. (b,d) Expression of known marker genes for each malignant tumor cell type in PF and ZR tumors.

Reviewer #2 – Point #4

Given the ependymal-like cell state is not present for the ST-EPN samples analyzed here, the authors should perform complimentary staining of the mouse model (and, if possible, human ST-EPN tissue) to validate the cell states identified by sequencing.

Response:

In response, we have revised our cell annotation methodology described in **Reviewer 2, Point 3,** to detail the annotation of ependymal-like cells, as demarcated by genes such as *FOXJ1*, and dynein family related motor-proteins. There are species-related differences, however. While tumor cell markers in our ZR IUE mouse model validate with RNAscope using a custom ZFTA probe, we have not observed ependymal-like cells in our IUE ZR mouse model as measured by *Kif6* negativity (**Response Figure 5, Extended Data Figure 4**). We thank Reviewer #2 for this important comment.



Response Figure 5: Validation of cell type markers in IUE tumors via RNAscope. Eomes=early excitatory neurons, Grin1=neurons, Kif6=ependymal cells, Mog=oligodendrocytes, Pax6=RGCs, Pdgfra=OPCs

Reviewer #2 – Point #5

The reference gene lists used for cell-type annotations is not clear. For example, Line 150, the developmental mouse brain atlas reference [23] is La Manno Nature 2021, yet the corresponding figure Ext Data Fig 1b is labeled as Bella Nature 2021 (should be Di Bella?). This is not included as a referenced paper. Similarly, line 239 reference [33] is Braun Science 2023, yet the Ext data Fig 4k states Trevino Nature 2021. Again, this is not included as a referenced paper.

Response:

Thank you, Reviewer #2, for your careful review and for pointing out these inconsistencies. We apologize for the confusion caused by the incorrect and unclear referencing. To clarify, the gene lists used for cell-type annotation are available in our GitHub repository (https://github.com/combiozone/CellTypeEstimate), where we have provided detailed references for each set of cell-type markers, including PMIDs for transparency and traceability. We have also corrected the citation errors in the updated manuscript—specifically, we have revised the mislabeled previous figures to reflect the appropriate references (such as *Di Bella et al.* and *Trevino et al.*). Thank you again for helping us improve the clarity and accuracy of our manuscript.

Human Brain Cell Type Annotation Markers – References:

PMID: 35803925, PMID: 32663469, PMID: 37824650, PMID: 10409504, PMID: 11566863, PMID: 15582153, PMID: 16957075, PMID: 17122047, PMID: 24259518, PMID: 25784853, PMID: 26060301, PMID: 26060302, PMID: 28817799, PMID: 29545511, PMID: 30089906, PMID: 31327527, PMID: 31624095, PMID: 32663469, PMID: 34140698, PMID: 34390642, PMID: 34616062, PMID: 34983929, PMID: 37704762, PMID: 9348346

Mouse Brain Cell Type Annotation Markers – References:

PMID: 32404418, PMID: 32848611, PMID: 34912118, PMID: 30089906, PMID: 30266827, PMID: 30382198, PMID: 30096314, PMID: 30677129, PMID: 15634788, PMID: 32679030, PMID: 33560373, PMID: 34163074, PMID: 34321664, PMID: 34846534, PMID: 35609197, PMID: 35609197, PMID: 37034735, PMID: 32769974

Reviewer #2 – Point #6

On Line 177, the cell states that are specified include "cycling neural intermediate progenitor cells (IPC)", is this referring to the CycProg-Like group displayed in the figure? The IPC cell state is later discussed for Figure 4 (Line 243), where it is termed as nIPC-like. As this is the first time using nIPC, the authors should write the full term, or adjust the abbreviation in the previous line. Are these CycProg-Like and nIPC-like cell states identical as suggested on Line 258?

Response:

Thank you for pointing out the inconsistency in cell type annotations. We apologize for the confusion in the previous version. In the revised manuscript, we have improved our methodology and refined the cell-type annotations, which has led to a more accurate classification of cell types. Previously, the term "nIPC-like" referred to a population enriched in cell cycle-related genes such as TOP2A and MKI67, as labeled by a previous study[26]. We felt that given the lack of functional evaluation of IPC state in EPN tumors, that a less biased label of cycling progenitor-like ("CycProg-like") more accurately reflected its transcriptional profile and proliferative characteristics. To clarify, the "CycProg-like" and previously termed "nIPC-like" populations refer to the same group of cycling progenitor-like cells. We have updated the manuscript accordingly to ensure consistent and accurate terminology throughout.

Reviewer #2 – Point #7

For the three neuronal-like cell states identified in ZFTA-RELA EPN (Figure 4f), are the markers for these cell-states suggestive of any specific neuronal types?

Response:

Thank you for your insightful question. In the updated analysis, we refined our methodology and improved cell-type annotation, which led to a more accurate classification of the neuronal-like population. As a result, we no longer observe a separation into multiple neuronal-like cell states as previously reported. Notably, a portion of the population previously identified as neuronal-like is now more accurately classified as ependymal-like based on updated marker expression and trajectory analysis.

Minor Comments:

Reviewer #2 – Point #8

All figures must be displayed in the order that they are discussed in the results.

Response:

Thank you for your helpful comment. We have revised the figure order to ensure that all figures are now presented in the same sequence as they are discussed in the Results section, in accordance with Nature's guidelines.

Reviewer #2 – Point #9

Ext data Fig.1f is not referenced in results.

Response:

Thank you for pointing this out. We have now revised the manuscript and removed the uncited Results section. This ensures consistency between the text and figures and improves clarity for the reader.

Referee #3 (Remarks to the Author):

Kardian and colleagues present a manuscript on ZFTA-RELA-driven ependymoma. Ependymoma are among the more common malignant childhood brain tumors, and the ZFTA-RELA-driven subtype makes up for a relatively small fraction. Patient outcomes are poor. The authors apply single-cell multiome profiling to study human and mouse tumors, as well as the developing mouse forebrain (E14.5). In the second part of the study, the authors combine their previously established in utero electroporation mouse model with a molecular barcoding strategy to distinguish malignant clones. Using advanced computational analyses on resulting genomic datasets, the authors conclude that specific chromatin states during brain development are vulnerable to transformation by ZFTA-RELA, and that differentiation hierarchies of malignant cells are conserved between tumors collected from humans and the mouse model. Using molecular barcoding coupled to single cell sequencing, the authors show that the entire hierarchy of malignant cells is present within a single clone.

The presented work is a continuation of a previous study by the same senior authors that investigated the effect of ZFTA-RELA on ependymoma formation (Arabzade, Cancer Discovery, 2021). The mechanisms by which ZFTA-RELA transforms a specific cell type during normal development is of interest to better understand the biology of these tumors and to motivate novel therapeutic approaches. I believe this kind of research can be very impactful and is much needed in the field of childhood cancer research. The authors apply a number of state-of-the-art experimental approaches (mouse modeling, single-cell genomics, molecular barcoding). Similar analyses have recently been performed in other types of cancer and the conceptual/methodological novelty is rather small, making the study less relevant for the broader research community. The manuscript is well written with some room for clarification. A number of main figure panels are not required for the narrative and could be made more concise. Overall, I find the study to be interesting and of decent quality, but I struggle to see novelty that would mandate publication in Nature.

Response:

We appreciate *Reviewer #3's* comments, and in the revised manuscript, include new genomic and functional experiments to further strengthen the scientific rigor and novelty of our findings.

While efforts have been made to pinpoint the precise cellular origins of EPN, understanding the epigenetic basis (specifically patterns of chromatin accessibility) that underlies this risk of transformation has not investigated. A challenge has been the relative paucity of dual single nucleus ATAC+RNA (snMultiome) sequencing datasets of the developing mouse and human embryonic brain. We appreciate the guidance from *Reviewer #3* to expand this early developmental snMultiome map of the developing mouse forebrain (from E12.5 to P6) (n = 7) as a foundation to investigate the Plag/l TF motif activity as a risk factor for ZR driven transformation. These concepts lead us to hypothesize that other pediatric oncogenic drivers (i.e fusion oncoproteins) may have similar lineage-associated epigenetic programs that facilitate tumor initiation.

Patterns of single cell chromatin accessibility and transcriptional networks have also not been described in EPN, or similar *Plag/l* gene fusion driven brain tumors. We are the first to investigate these signatures in both mouse and human EPN, and first to analyze these datasets in a cross-species fashion. We believe that our snMultiome dataset comprising n = 28 tumors serves as a valuable resource to the brain tumor community, while also allowing us to make important inferences about: 1) Cycling progenitor-like (including radial glia-like) cells that may establish a putative EPN cell hierarchy that undergoes incomplete differentiation, and 2) ZFTA-RELA transformation compatibility with distinct developmental epigenetic states requiring precise 'goldilocks' levels of fusion oncoprotein expression. We feel that these are novel findings that shed light on important mechanisms of ZFTA-RELA directed gene control, as well as provide a foundation to investigate differentiation strategies in the future as a potential treatment paradigm.

Finally, we provide molecular barcoding combined with transcriptional characterization studies of natively forming *ZFTA-RELA* mouse EPN; concepts which have not been investigated in EPN or widely across pediatric or adult brain tumors. These experiments shed light on tumor lineage relationships and emergence of dominant clones that establish the entire EPN cellular hierarchy reflecting gliogenic and neurogenic differentiation programs. In our opinion, these findings provide new insights on the contribution of diverse developmental states and the potential impact upon therapeutic resistance.

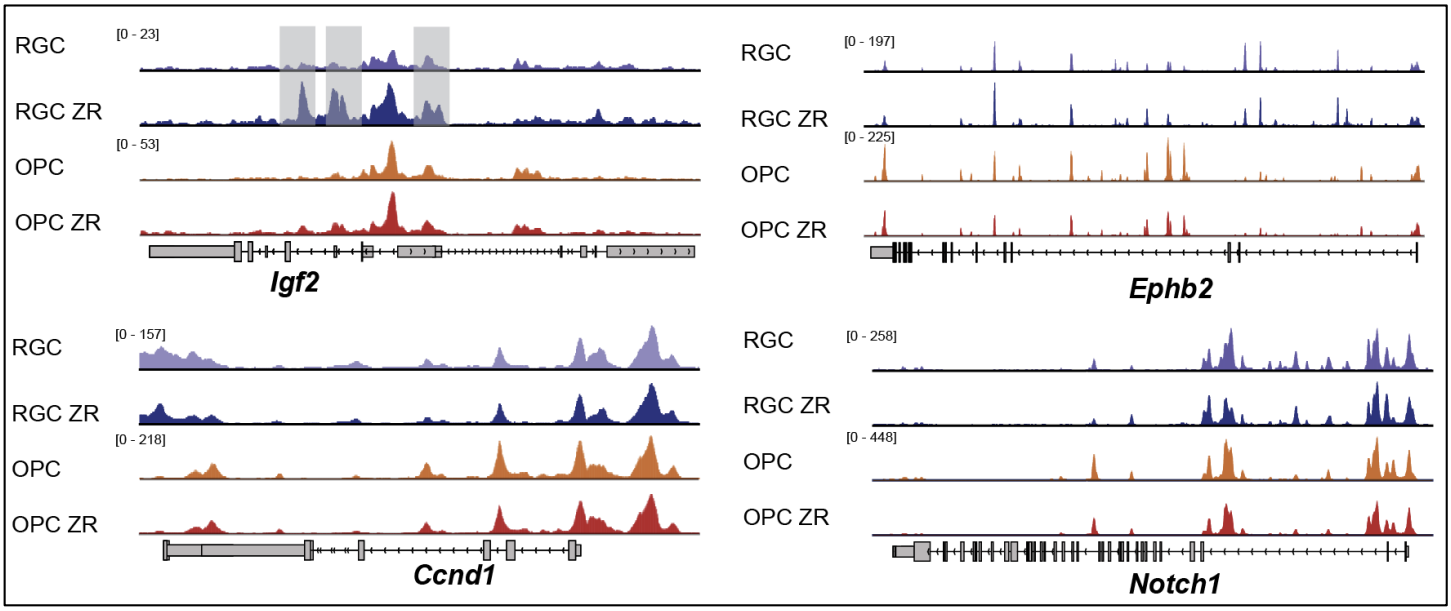
Together, we feel that our manuscript reveals several novel observations on the role of epigenomic programs in development, engagement by pediatric oncogenic drivers, and the resulting impact on lineage programs and transcriptional diversity in EPN. We greatly appreciate *Reviewer #3's* comments and suggestions to add new experimental and functional experiments as detailed in our response letter to follow.

Reviewer #3 – Point #1

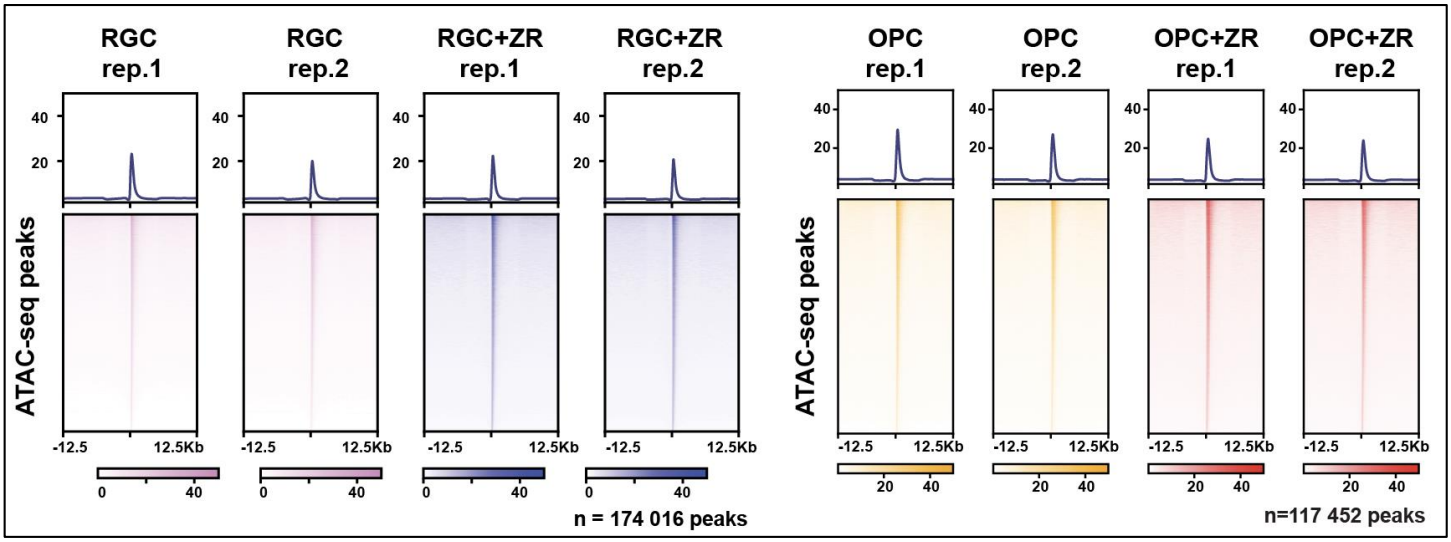
Figure 1b shows that there are only minimal changes in global accessibility profiles between ZFTA-RELA (ZR) expressing radial glial cells and empty vector controls, suggesting that "patterns of chromatin accessibility were already established in specific lineage programs and subsequently commissioned by ZR", a central claim of the manuscript. This claim is not supported by the violin plot showing the total number ATAC-seq peaks (without any statistical tests). An analysis like the one performed on the corresponding RNA-seq dataset shown in EDF1a, which shows major differences, needs to be performed.

Response:

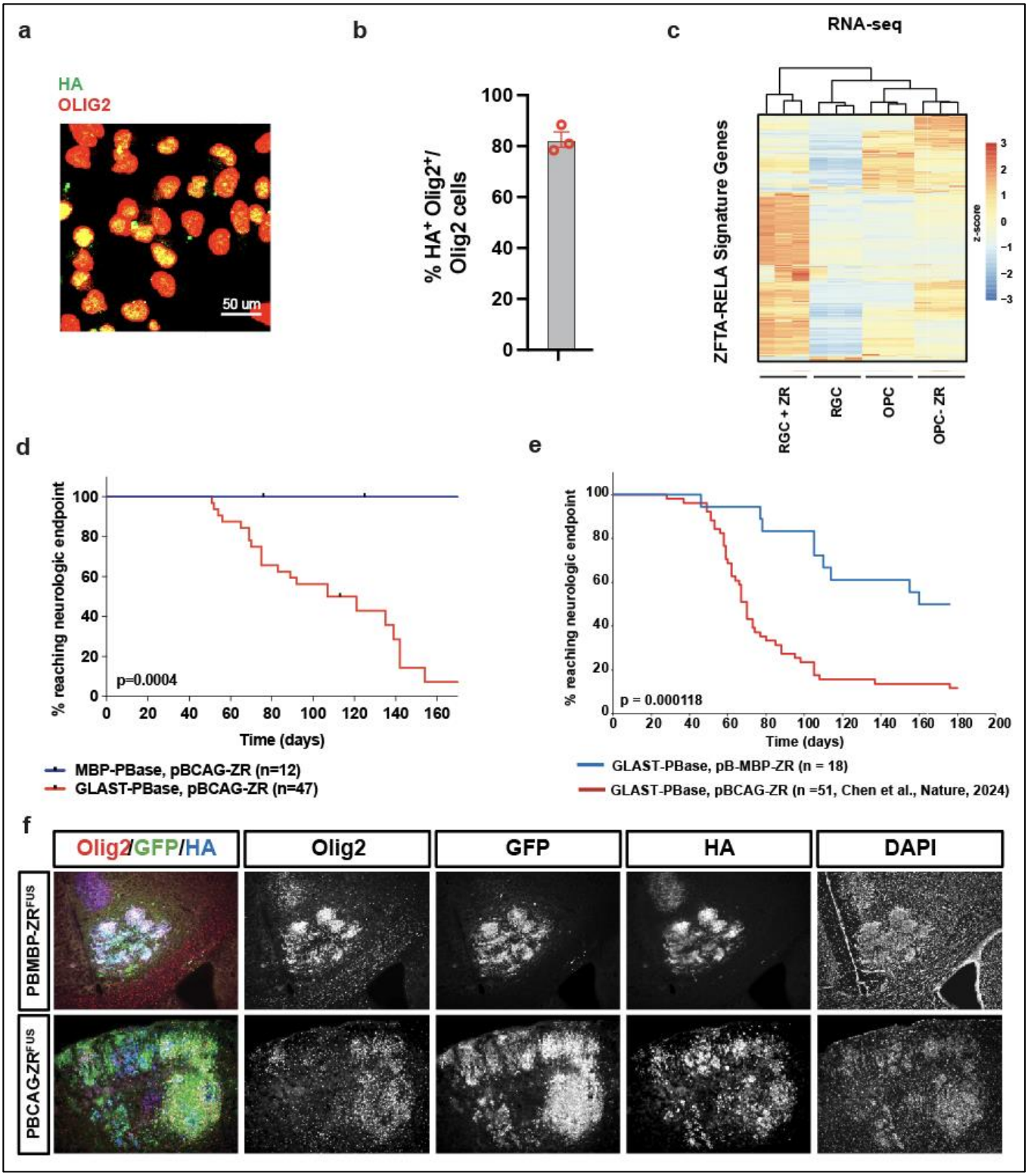
We thank *Reviewer #3* for this important comment and would like to clarify that while there are some changes in patterns of chromatin accessibility (i.e. at imprinted genes *IGF2*), however the vast majority of ZR binding sites are already accessible in RGCs, including at downstream ZR targets such as *Ephb2* and *Notch1* (**Response Figure 1, Extended Data Figure 1**). This is in sharp contrast to n = 5355 genes upregulated by ZR, and the thousands of sites that it engages across the epigenome. We also appreciate the reviewer's emphasis on scientific rigor. In response, we have repeated the ATAC-seq experiments in mouse RGCs and observe reproducible findings in independent replicates (**Response Figure 2, Extended Data Figure 5**). Finally, we have performed the same series of experiments to express ZR in oligodendrocyte progenitor cells (OPC), that have some (albeit lesser) degree of chromatin accessibility of the Plagl1/2 TF motif than RGCs and cycling progenitor cells during mouse and human brain development (**Response Figure 3, Extended Data Figure 5**). We demonstrate in OPCs, that ZR driven gene activation is not correlated with comparable changes in patterns of chromatin accessibility (**Response Figure 3, Extended Data Figure 5**). Furthermore, more restricted Plagl1 and 2 TF motif accessibility is associated with impairment of ZR target gene activation (**Response Figure 3, Extended Data Figure 5**). Finally, we performed a new set of in utero electroporation (IUE) experiments to express ZR in the oligodendrocyte compartment, leading to Olig2+ tumors (very rarely observed in the human EPN disease[16], and a significant delay in tumor initiation (**Response Figure 3, Extended Data Figure 5**). We provide these new genomic and functional experiments to further support our hypothesis that ZR engages an epigenomic program already accessible within the progenitor cell lineage programs that may give rise to EPN.



Response Figure 1: Chromatin accessibility patterns from ATAC-seq at known target genes *Igf2*, *Ephb2*, *Ccnd1*, *Notch1* across RGC, RGC+ZR, OPC, OPC+ZR samples.



Response Figure 2: Global ATAC-seq peak profiles of RGC, RGC+ZR, OPC, and OPC+ZR mouse brain samples, with two technical replicates per condition.



Response Figure 3: (a) Immunofluorescent staining of OPCs showing HA (green, tags ZR fusion) and Olig2 (red). (b) Quantification of HA+/Olig2+ cells in immunofluorescent staining. (c) Heatmap of ZR 93 gene expression within RGC, RGC+ZR, OPC, and OPC+ZR cell lines. (d) Survival curve for MBP-PBase, pBCAG-ZR (blue, n=12) GLAST-PBase, pBCAG-ZR (red, n=47) IUE mice (p=0.0004). (e) Survival curve for GLAST-PBase, pB-MBP-ZR (blue, n=18) GLAST-PBase, pBCAG-ZR (red, n=51, Chen et al. Nature 2024) IUE mice (p=0.000118). (f) Immunofluorescent staining of GLAST-PBase, pB-MBP-ZR (top row) and GLAST-PBase, pBCAG-ZR (bottom row) tumors.

Reviewer #3 – Point #2

I am unclear about the EWSR1-PLAGL1 (EWP) tumors that function as a contrast for the ZR tumors in the single cell analysis of patient samples in Figure 3. Important background/references are missing on these tumors. Do they reflect histologically diagnosed ependymoma that form their own DNA methylation class of PLAGL1-fused neuroepithelial tumors (e.g., Sievers et al, 2021)? The comparison to ZR tumors is not sufficiently motivated considering presumed differences in cellular origins. Also, methylation array profiling can only suggest the presence of these fusion events.

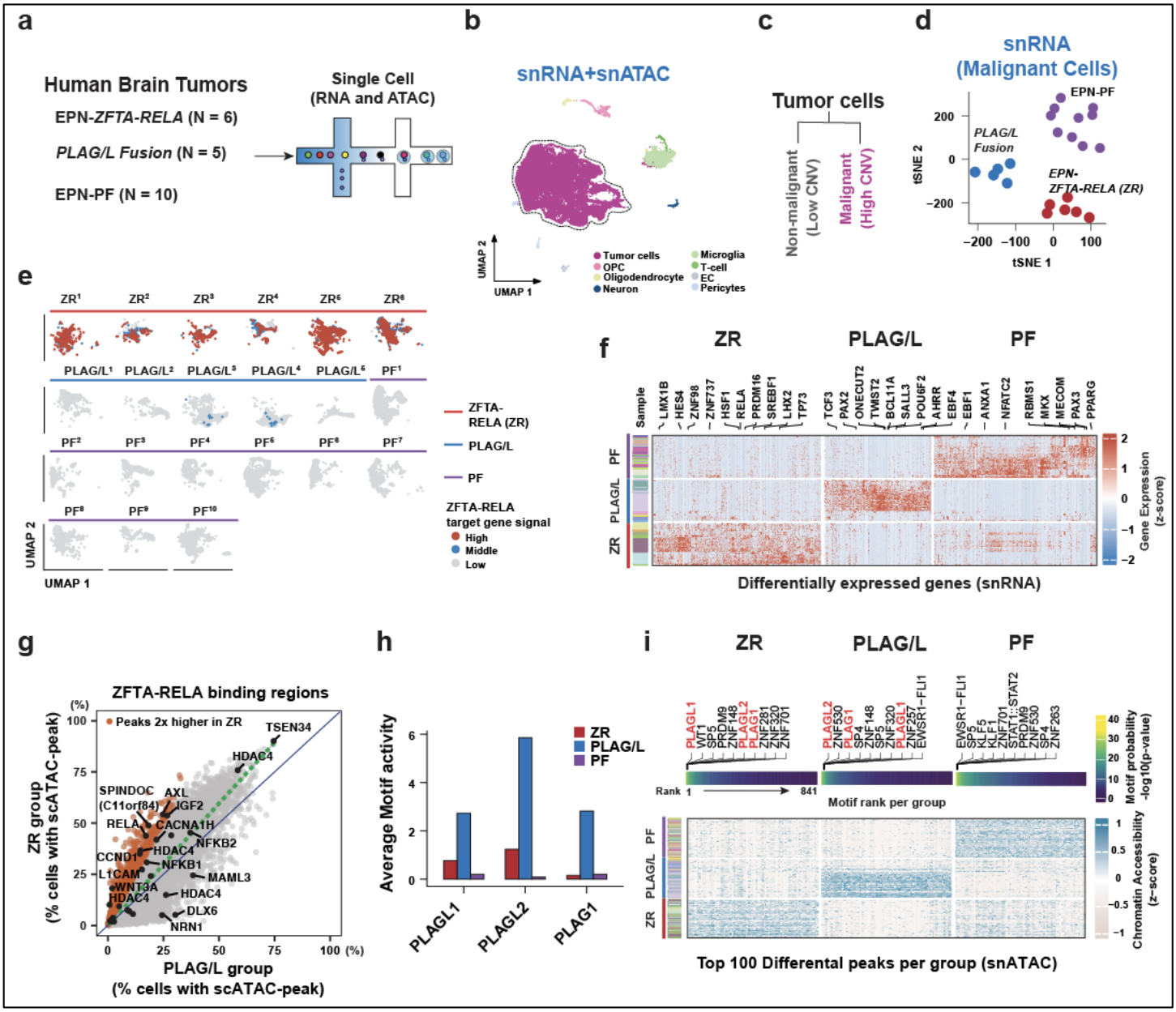
Response:

We thank *Reviewer #3* for this comment and recognize that the initial presentation of the comparative analysis lacked clarity. Our goal was to compare ZR EPN tumors to Posterior Fossa (PF) EPN within the same disease. Here we demonstrate that ZR and other Plag/l fusion driven tumors consistently engage the Plagl1 and 2 TF motif as compared to PF-EPN (**Response Figure 4, Main Figure 3**).

The purpose of including *EWSR1-PLAGL1* tumors was to demonstrate that other CNS malignancies share similar patterns of convergence upon the Plagl1 and 2 family of TF motifs. Notably, in our resubmission we have added additional *EWSR1-PLAGL1* and *PLAG-TCF4* fusion driven tumors all profiled by snMultiome sequencing and re-labeled this group *PLAG/L* fusion (**Response Figure 4, Main Figure 3**). While the cellular origins of *EWSR1-PLAGL1* tumors is not clear, they have both similar and divergent histological features from EPN and have disparate DNA methylation signatures[27-29]. Our revised data supports similar epigenetic patterns of convergence of ZR and PLAG/L fusion tumors upon PLAGL1/2 motifs but also differences in transcriptional programs; potentially reflecting different cells-of-origin (**Response Figure 4, Figure 3**).

Reviewer #3 is correct that DNA methylation patterns can be incredibly useful as a diagnostic tool[30-33], but can only infer disease subtypes and their associated gene fusions enriched in these tumors. In response, we have performed bulk-RNA sequencing on available tissue and have confirmed the presence of gene fusions (**Supplementary Table 1**). This has also led to the identification of one tumor with a *BCOR* alteration that was included in the original cohort, which has since been removed from the manuscript.

We appreciate *Reviewer #3* for these comments that have improved the clarity and rigor of our scientific findings.



Response Figure 4: (a) Schematic of human snMultiome data generation from 21 patients, including ZFTA-RELA fusion–driven ependymoma (N=6), PLAG/L fusion–driven tumors (N=5), and posterior fossa ependymoma (N=10). (b)Integrated snMultiome data from 21 patients with initial cell type annotation. UMAP was generated by joint analysis of snRNA and snATAC data using the weighted nearest neighbor (WNN) method. (c) Tumor cells were categorized into non-malignant and malignant types according to their degree of copy number variation. (d) Analysis of malignant tumor cells using tumor-specific gene expression markers classified patients into three molecular groups (EPN-PF, PLAG/L Fusion, and EPN-ZFTA-RELA). Specific group characteristics were further validated by DNA methylation analysis. (e) ZR fusion signal in malignant cells was assessed across samples by expression of the 93-gene signature. (f) Heatmap showing genes with significant differential expression among malignant cells predicted within the three tumor groups. Transcription factors with significant expression differences are highlighted. (g) Comparison of the proportion of cells exhibiting open chromatin at the ZR binding region between the ZR and PLAG/L groups. Orange dots indicate regions where the proportion of cells with open ZR binding sites is more than twofold higher in the ZR group compared to the PLAG/L group. Black-highlighted dots represent ZR fusion target genes. The green dotted line represents the regression line. (h) PLAG family motif activity in each tumor type. (i) Heatmap displaying the top 100 differential snATAC peaks identified for each group. Motif enrichment was predicted separately for each group based on its top 100 peaks, with the top 10 enriched motifs highlighted alongside their associated gene names. PLAG/L family motifs are highlighted in red.

Reviewer #3 – Point #3

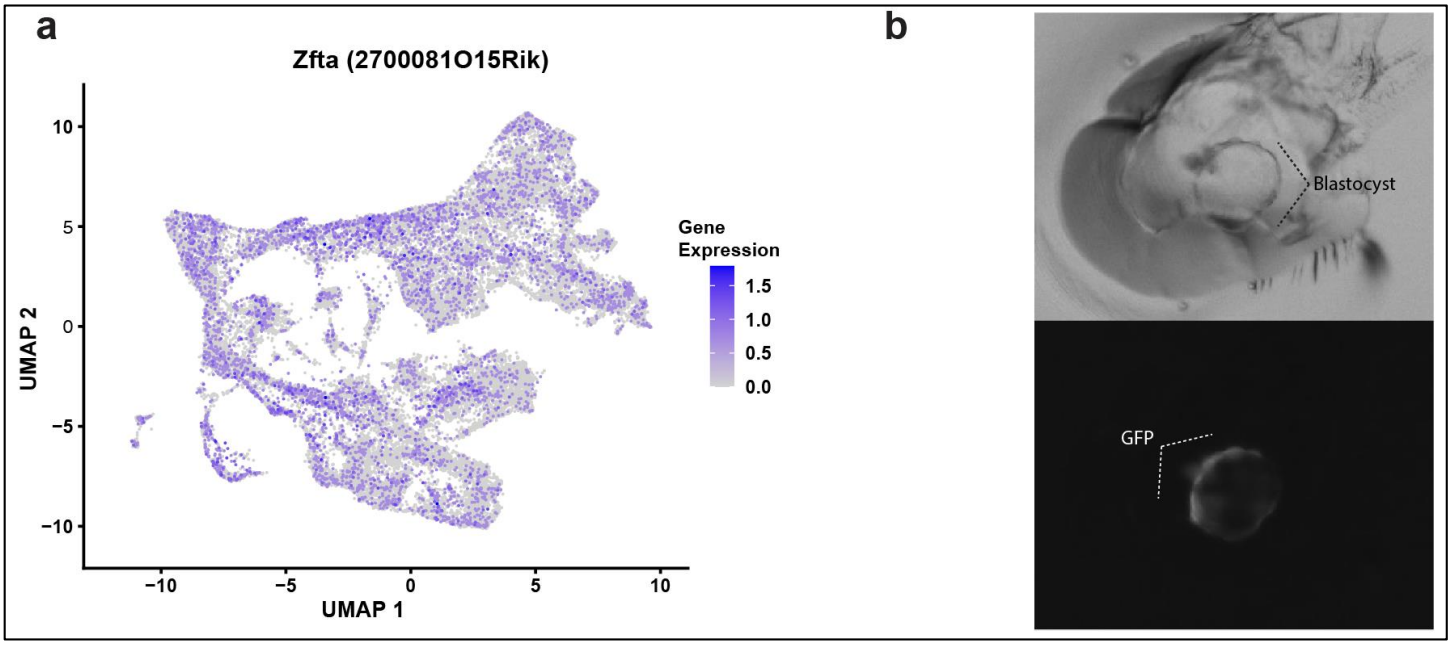
A main concern I have about the mouse model is the choice of the GLAST promoter for expression of the fusion oncogene. While this promoter is active in radial glia and other neural cell types, it might show different activity than the endogenous promoter driving ZR expression in patient tumors. This is important when studying the effect of the oncogene transformation or in regulating malignant cellular hierarchies. For example, expression of ectopic ZR might occur in cell types in which the fusion would not be expressed in the natural context. At a minimum, this shortcoming needs to be clearly stated and potential implications discussed in more detail. Expression of wild-type ZFTA should be investigated in the mouse developmental dataset. A more ambitious approach would be to model the ZR fusion by CRISPR-guided deletion of the genomic region (1.5Mb) between ZFTA and RELA leading to the fusion oncogene.

Response:

We thank *Reviewer#3* for this comment and the limitation of our model in capturing the cell-type and temporal selectivity of the *ZFTA* promoter and cis-regulatory elements. We have included this short-coming and caveats of this model, and its potential implications in the discussion section of the manuscript.

An important comment by *Reviewer #3* was to expand the developmental dataset (*see Reviewer #3, Point 4*). We fully agree with this comment as this is one of the only snMultiome (ATAC+RNA) datasets of the developing mouse forebrain. Within this dataset from E12.5 to P6, we examined the expression of *Zfta* across early cortical brain development and could not identify any cell type restricted patterns that might help pinpoint a precise cell type of origin, potentially owing to its relatively low level of gene expression (**Response Figure 5)**. From this new data, we have mapped the Plagl1/2 TF motifs and revealed its accessibility enriched in the RGC and cycling progenitor cell compartment, supporting our hypothesis that ZR can engage many of these 'open' sites in the RGC-cycling progenitor cell lineage during embryonic development (**Response Figure 6, Main Figure 1**). These findings are also supported by the inclusion of snATAC-seq data from the developing human fetal cortex which reveals similar patterns of Plag/l TF motif activity (**Response Figure 6, Main Figure 1**).

We agree that an elegant and complementary approach would be to perform a CRISPR-deletion between *Zfta* and *Rela*. In response we electroporated sgRNAs targeting *Zfta* and *Rela* in CD-1 mouse embryos at the morula stage (2.5 dpc) (**Response Figure 5)**. As a control, we expressed dual sgRNAs targeting the rosa26 locus. Embryos were then transferred to pseudo-pregnant females approximately 24 hours later. Unfortunately, these pregnant females failed to generate any live pups. We're currently not certain whether this embryonic lethality is due to the effects of CRISPR-editing, aberrant ZFTA-RELA expression (which can induce cell death in non-stem/RGC cell types), or other biological/technical issues. Thus, while we fully agree that this is an elegant experiment, modeling this genomic lesion in our hands has been very challenging in mice, and respectfully, is beyond the scope of this manuscript. We appreciate the reviewer for this comment, as we work towards more faithful models of the disease. We will continue to pursue experimentation of CRISPR-driven ZR fusion modeling in the future as we fully appreciate the importance of this line of experiments.



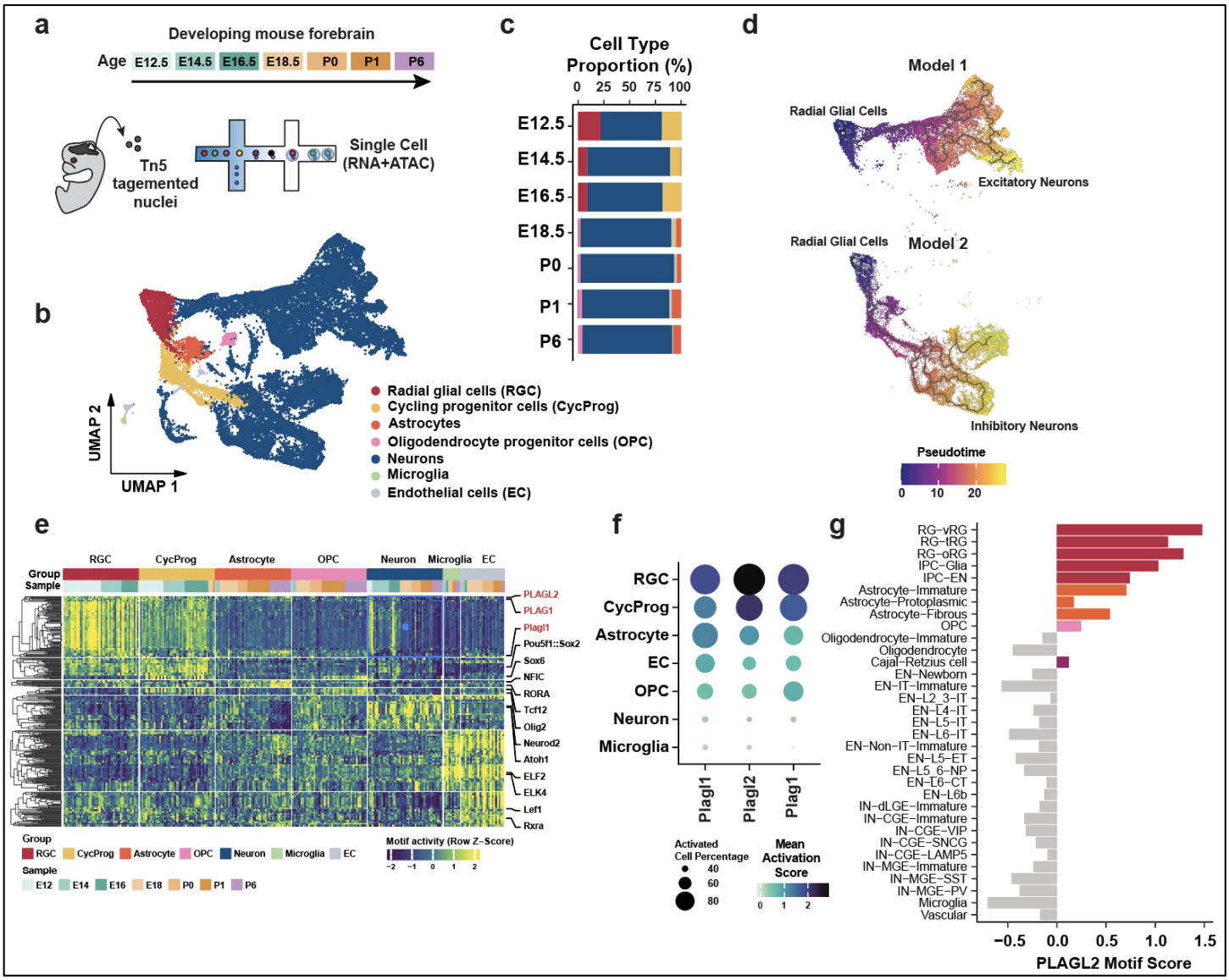
Response Figure 5: (a) Expression of *Zfta* across mouse forebrain developmental dataset UMAP. (b) Electroporation of sgRNAs targeting *Zfta* and *Rela* in CD-1 mouse embryos. These were imaged for GFP at the blastocyst stage.

Reviewer #3 – Point #4

Several other datasets of the developing mouse brain have been published in recent years. Relying on a single time-point seems like a missed opportunity to map the developmental correlates of ZR-driven ependymoma with greater accuracy.

Response:

We thank *Reviewer #3* for this comment. In our original submission, there had been no snMultiome data sets available to study the developing mouse forebrain. We fully agree that relying one single timepoint was a missed opportunity to examine the Plag/l TF motif expression and activity across an expanded window across brain development. In the revised manuscript, we have expanded the normal mouse forebrain snMultiome dataset across multiple developmental time points (***E12.5, E14.5, E16.5, E18.5, P0, P1, P6***) **(Response Figure 6, Main Figure 1)**. We demonstrate that the Plagl 1/2 TF motifs exhibit increased chromatin accessibility in RGC and cycling progenitor cells broadly, and this accessibility sharply decreases during post-natal development and neuronal differentiation (**Response Figure 6**). Furthermore, we have validated these findings by leveraging snATAC-seq data from the human fetal cortex, revealing the enrichment of Plagl TF motif accessibility in RGC and intermediate/cycling progenitor cell (IPC) populations. Finally, our new developmental atlas has enabled the expanded analysis of ZR binding sites during embryonic brain and the earliest stages of post-natal development **(Response Figure 6, Main Figure 1).** We appreciate this comment, that has led to improvements in our understanding of epigenomic patterns (i.e. Plag/l TF 1/2 motifs) at risk of transformation during brain tumor development



Response Figure 6: (a) Schematic depicting the methodology for generating snMultiome data from E12.5 to P6 normal mouse forebrain. (b) UMAP plot illustrating the distribution of annotated cell types following integration of the snMultiome data. (c) Proportions of cell types identified in each of seven normal mouse forebrain samples. (d) Pseudotime reveals two neuronal lineage trajectories from RGCs to excitatory and inhibitory neurons. (e) Landscape of cell type–specific motif activation. (f) PLAG family motif activation scores across cell type. Dot size represents the percentage of activated cells within each cell type. (g) PLAGL2 motif scores across human developing brain cell types, derived from data published by Wang et al. (2015).

Reviewer #3 – Point #5

The molecular barcoding approach is interesting, but the number of samples are too small and the analyses are too premature to yield conclusive results. Most findings are rather obvious, and it is unclear what could have been an alternative finding. For example, it can be expected that the clonal diversity is higher in early neoplastic lesions. It needs to be clarified if the non-tumor cells in Figures 5f and 5i carry the dominant lineage barcode (I assume not?). The concluding paragraph in the discussion seems to be a bit of an overstatement ("lineage tracing experiments demonstrated the emergence of dominant, often single clones", and "conventional therapies may miss single aggressive tumor clones capable of driving tumor recurrence" – how can it be both?).

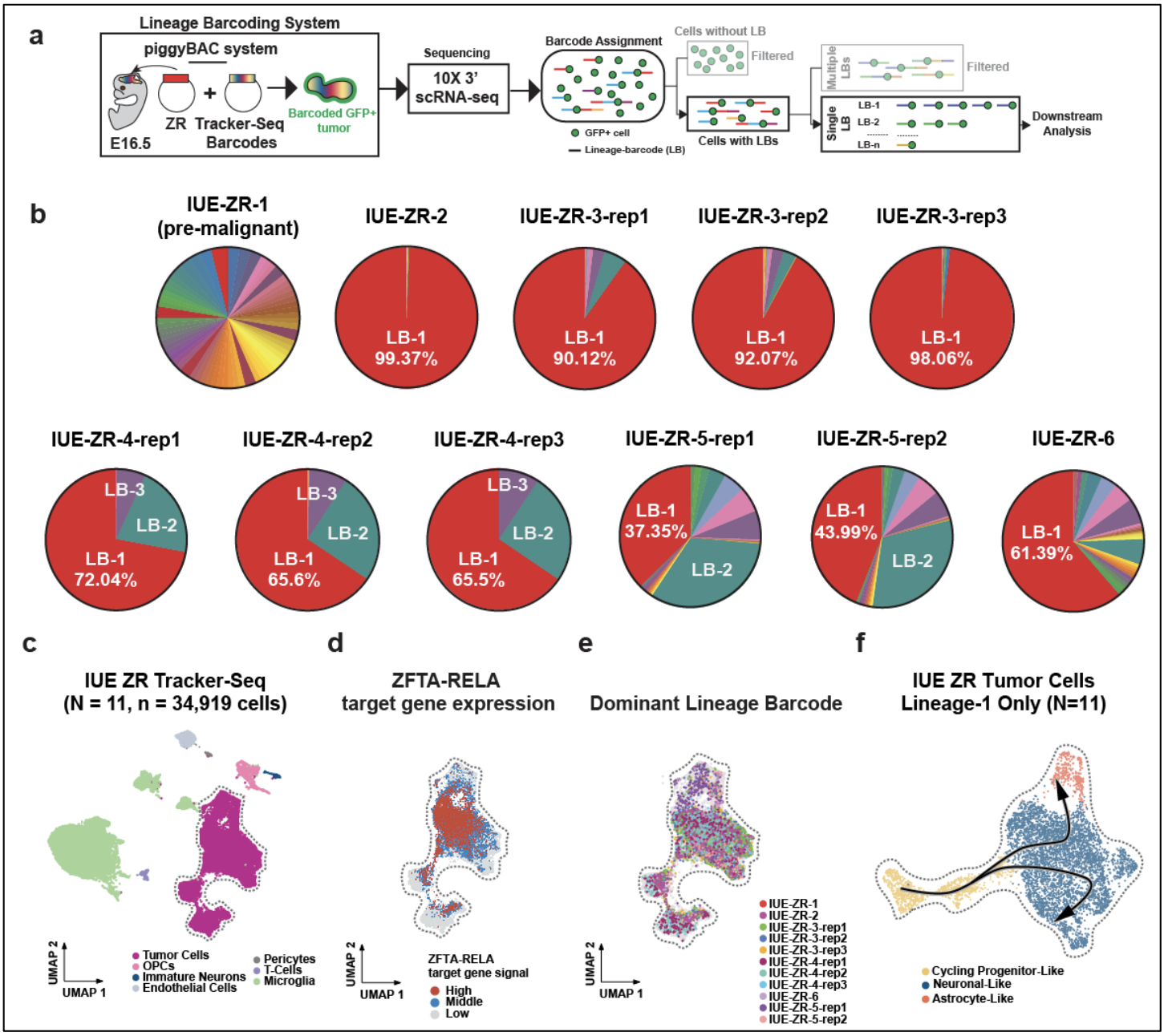
Response:

We thank the reviewer for the thoughtful and detailed feedback regarding the lineage tracing analysis using the Tracker-Seq methodology. We fully agree that this component is central to our study and appreciate the opportunity to improve its clarity and rigor. Below, we outline the major steps we have taken in the revised manuscript to address these concerns, and presented in **Response Figure 7, Main Figure 5**.

We have increased the number of samples from **n = 3** to **n = 11** analyzed to strengthen the robustness of our conclusions, and where possible inclusion of replicates of scRNA-seq experiments. These types of molecular barcoding analysis of EPN tumors paired with scRNA-seq transcriptional profiling has not been performed to this date. Before performing these studies, we had the opposite hypothesis that diverse clones would emerge and that increased clonal complexity would correlate with therapeutic resistance. With this expanded analysis, we observe the presence of a dominant clone capable of recapitulating the transcriptional heterogeneity seen in our snMultiome analysis. However, in some cases we do observe the increased prevalence of sub-clones raising the question of how these different clones respond to therapies such as radiation and chemotherapy, which is currently being pursued in future studies.

We apologize for the lack of clarity of the Tracker-seq approach in the original submission. Because the Tracker-seq barcode system barcodes the developing normal brain at the same time of tumor initiation, it's expected that molecular barcodes will be present in non-neoplastic cells. When Tracker-seq tumors are fully formed, the tumors are micro-dissected and enzymatically dissociated and sorted using flow cytometry of GFP+ and live/dead cells. As a result of this harsh process to extract GFP+ tumor cells, we encounter a significant degree of cell death, consistent with FACS based sorting experiments before scRNA-seq analysis. This process leads to the release of RNA from dying/dead cells which can introduce background and molecular barcodes detected in non-neoplastic cells. As a result, our computational workflow has been established to be highly stringent in controlling for the effects of background ambient RNA and molecular barcode detection. In response to *Reviewer #3* we have mapped the dominant lineage barcodes in both malignant and non-malignant cells, excluding microglia/macrophages that phagocytose tumor debris (**Response Figure 8**). Here, we confirm the predominance of the dominant lineage barcode (*LB-1*) in malignant cells versus non-malignant cells such as OPCs, endothelial cells, pericytes, and neurons. We appreciate *Reviewer #3's* feedback which has led to a clearer and more rigorous approach to the quality control and analysis of our Tracker-Seq data.

Finally, based on *Reviewer #3's* comment we have pulled back on the overstated conclusions and clarified our interpretation of our Tracker-seq findings. We wanted to clarify that despite the emergence of often single dominant clones, these tumors are characterized by distinct transcriptional signatures reflecting embryonic brain, albeit incomplete, developmental programs. These findings are consistent with cellular models of cancer that detail stem-/progenitor- like cells capable of reforming the entire tumor hierarchy and driving resistance to anti-cancer therapy[34-40]. We believe that our expanded Tracker-seq dataset provides an integrated and more rigorous description of these cellular programs that combines clonal tracing with single cell transcriptional profiling. We appreciate *Reviewer #3's* comments that have added to the scientific rigor and clarity across this line of experiments.



Response Figure 7: (a) Schematic of piggyBAC TrackerSeq lineage barcoding system and raw single-cell RNA data processing workflow. GFP+ IUE cells are isolated and sequenced, and cells with a single lineage barcode are used for downstream analysis. (b) Lineage barcode abundance across six IUE biological replicates. Samples IUE-ZR-3 to -5 include technical replicates. Sample IUE-ZR-1, derived from a P4 mouse brain, shows high diversity in the TrackerSeq library shortly after IUE. (c) Integrated UMAP of all 11 IUE TrackerSeq datasets, highlighting tumor cell clusters defined by ZR-fusion target gene expression and enclosed with a dotted line. (d) UMAP visualization highlighting tumor cell clusters with ZR-fusion target gene signal. (e) Projection of cells harboring dominant lineage barcodes from different samples onto tumor cell clusters in the UMAP, assessing barcode distribution across samples. (f) UMAP reconstruction was performed using tumor cells harboring dominant lineage barcodes. Lineage trajectories were inferred by Slingshot based on Seurat cluster assignments. Arrows denote two distinct differentiation trajectories identified through lineage analysis.

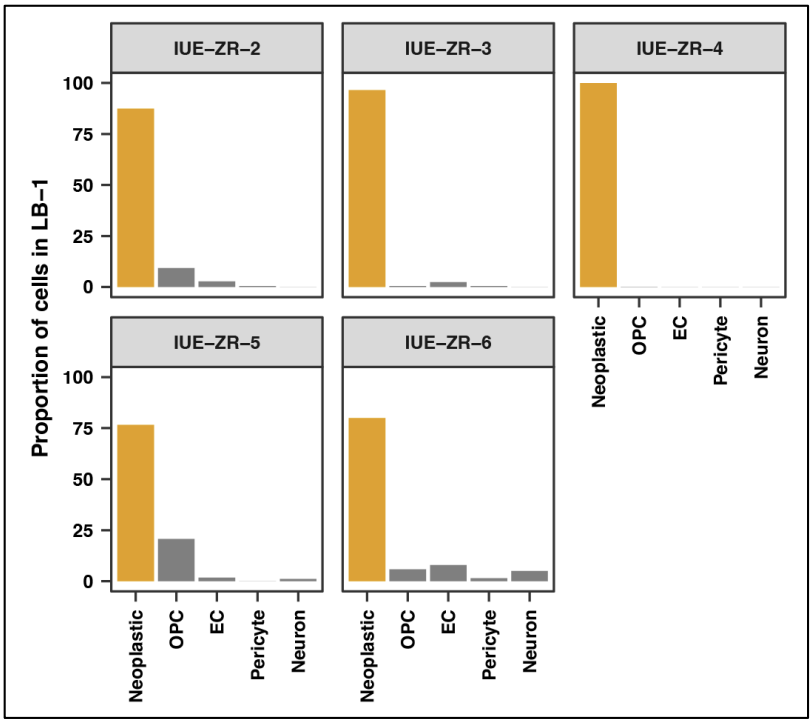
Reviewer #3 – Point #6

I am a bit confused by the proposed cellular hierarchy of ZR-driven ependymoma. Pseudotime inference analysis resulted in a model in which cycling nIPC cells give rise to gliogenic and neurogenic differentiation programs. How does this relate to the UMAP presented in Figure 4b? Where do RGC-like cells fall? Ideally such analyses should be shown in individual samples as cell types and their abundance may vary between patient tumors.

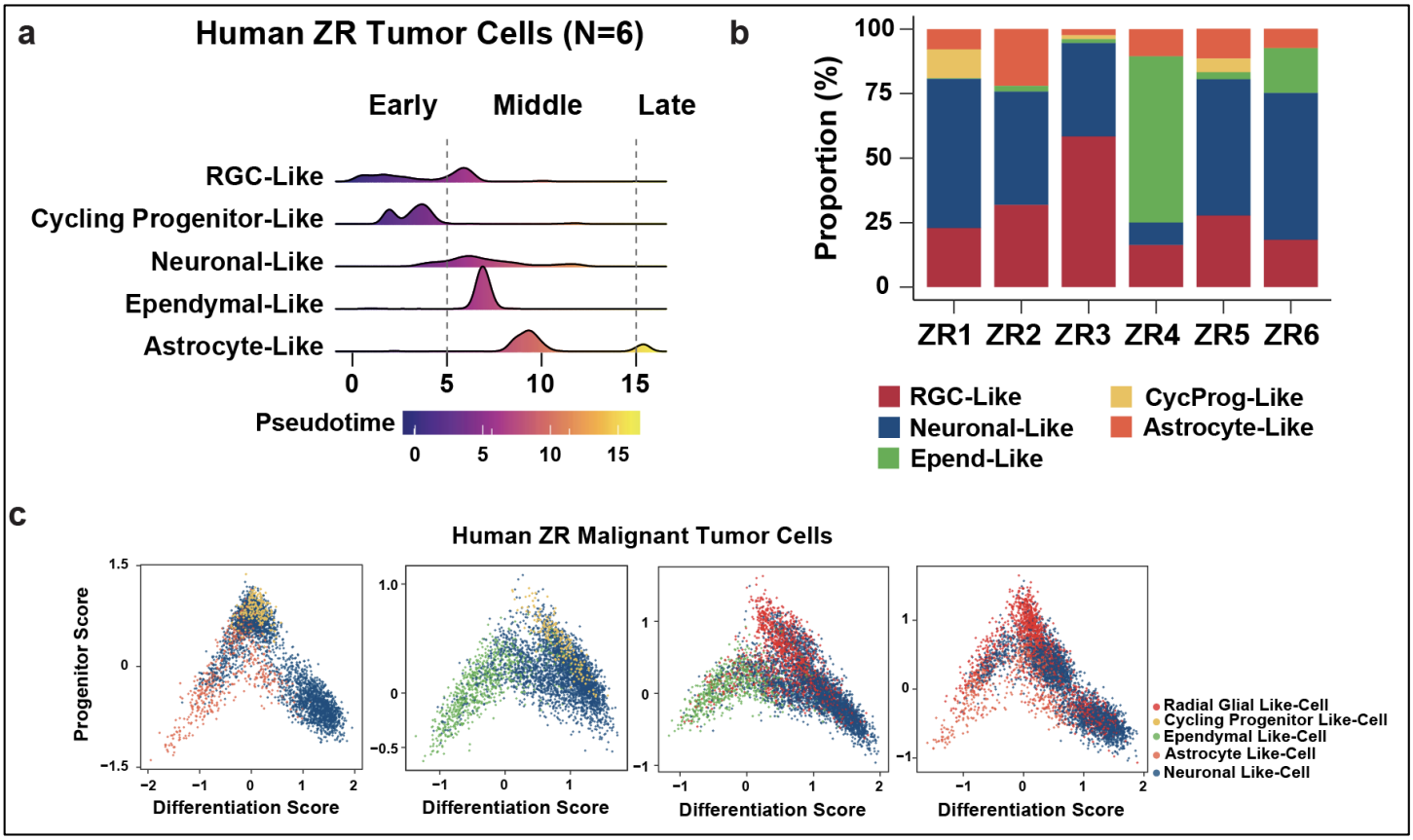
Response:

We thank Reviewer #3 for this comment in the revised manuscript, we have expanded the sample set and improved our analysis and cell type annotation, with the former nIPC population now refined and renamed as cycling progenitor (CycProg) cells according to additional guidance from *Reviewer #2*. Importantly, we now present all tumor cell types (RGC-Like, CycProg-Like, Astrocyte-Like, Epen-Like, Neuronal-Like) in Pseudotime density figure from human ZR-driven ependymoma, which aligns well with the UMAP shown in the updated **Figure 4** (**Response Figure 9, Main Figure 6b**). RGC-like cells cluster near CycProg, consistent with their developmental relationship. Consistent with mouse tracker-seq results, CycProg cells are positioned centrally and giving rise to distinct gliogenic and neurogenic differentiation programs. As suggested, we have included analyses of cell types and their abundance in individual samples to highlight patient-specific variability and tumor heterogeneity **(Response Figure 9b, Extended Data Figure 8)**. These updates provide a clearer and more comprehensive depiction of the potential cellular hierarchy and developmental dynamics of EPN tumors and their dominant clones.

We appreciate *Reviewer #3* for all of their insightful and constructive comments that have allowed us to improve our manuscript.



Response Figure 8: Proportions of dominant lineage barcodes in both malignant and non-malignant cells within Tracker-Seq tumors, excluding microglia/macrophages that phagocytose tumor debris.



Response Figure 9: (a) Pseudotime density figure from human ZR-driven ependymoma including RGC-, Neuronal-, Ependymal-, Cycling Progenitor-, Astrocyte-like cell types. (b) Cell types and their abundance in individual samples. (c) Malignant tumor cell trajectories predicted by progenitor and differentiation scores demonstrate a transition from progenitor-like cells (RGC-/CycProg-like) to glial-like and neuronal-like cell types in human ZR ependymomas.

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Response to Reviewer #1 comments

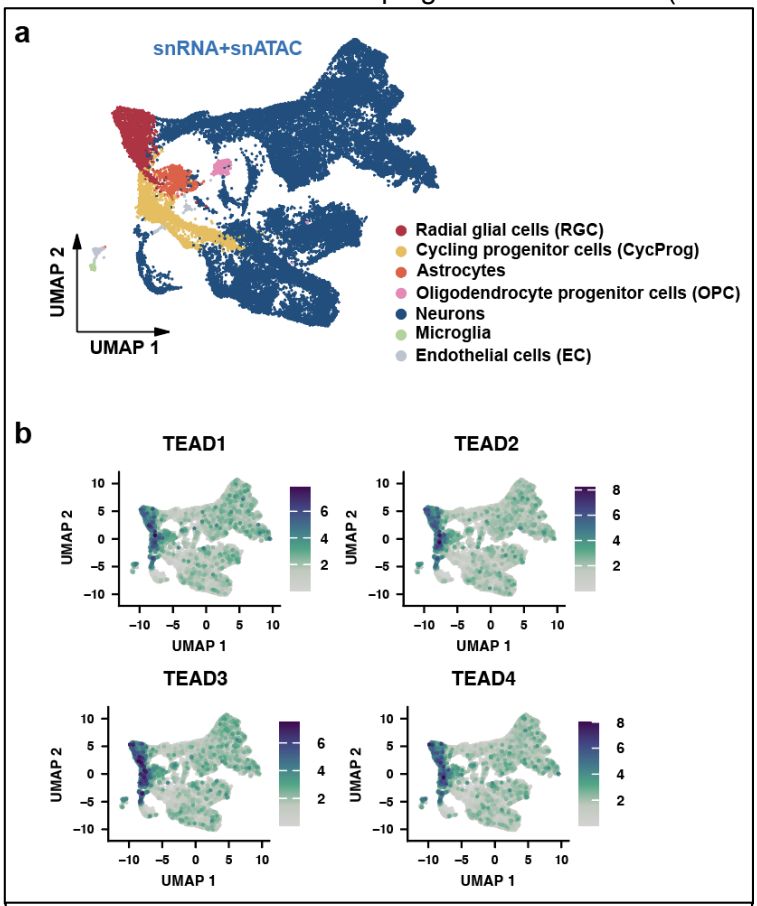
Referee #1 (Remarks to the Author):

Overall, the authors have spent considerable effort on updating this manuscript and should be commended for these improvements. Below are a series of concerns that still remain in my opinion. Some of these are simply addressed via text or figure changes. However, an underlying concern does still remain about the overall novelty of the findings and the impact to which the Tracker-Seq experiments truly convey the (strong) messaging of the title. This, I believe, is more of an editorial decision.

We thank the reviewer for their time and positive feedback on our revised manuscript and appreciate their continued efforts to ensure its scientific rigor and novelty. The key contributions of our work can be summarized in three areas:

1) Epigenetic programs as risk factors for cancer initiation

Although prior studies have sought to identify the cellular origins of EPN, the epigenetic basis—particularly chromatin accessibility patterns—underlying this risk has not been explored. A major limitation has been the scarcity of dual single-nucleus ATAC+RNA (snMultiome) datasets from developing mouse and human embryonic brain. Our study addresses this gap by providing snMultiome data from the developing mouse forebrain (E12.5 to P6; n = 7). This resource enabled us to examine Plag/l transcription factor (TF) motif activity during normal development as a potential risk factor for ZR-driven transformation. Interestingly, we observed very similar findings in the context of accessibility of Tead TF motifs highly-accessible in RGCs during normal brain development, that diminishes upon neuronal and glial differentiation. *Yap1-Mamld1* gene fusion in EPN lead to constitutive activation of the Yap signaling pathway, and persistent activation of the Tead TF motifs past embryonic development (**Response Figure 1**). These findings lead us to hypothesize that other pediatric oncogenic drivers (e.g., fusion oncoproteins) may similarly exploit lineage-associated epigenetic programs to initiate tumors.



Response Figure 1 (a) uMAP plot of scATAC+RNA data representing the mouse embryonic brain development atlas. **(b)** Depiction of Tead1-4 motif activity enriched in RGCs and cycling progenitor cells during embryonic brain development.

2) Oncogenic chromatin accessibility networks in mouse and human ependymal tumors

Single-cell chromatin accessibility and transcriptional networks have not previously been characterized in EPN or other Plag/l fusion-driven brain tumors. We are the first to analyze these signatures in both mouse and human EPN and to perform cross-species comparisons. Our snMultiome dataset (across n = 28 tumors) provides a valuable resource for the brain tumor community

and supports key insights: i) Identification of cycling progenitor-like cells (including radial glia-like) that may form a hierarchical structure in EPN with incomplete differentiation, and ii) Evidence that ZR transformation requires compatibility with specific developmental epigenetic states and precise “goldilocks” levels of fusion oncoprotein expression. These findings reveal novel mechanisms of ZR–mediated gene regulation and lay the groundwork for exploring differentiation-based therapeutic strategies in future studies.

3) Tumor cell lineage trajectories that shape ependymoma heterogeneity

We combined molecular barcoding with transcriptional profiling to study lineage relationships in natively forming ZR mouse EPN. While one group has examined clonal dynamics in mouse glioma[1], their study relies on an experimental model that expresses an oncogene rarely observed in disease. Our experiments uncover lineage relationships and dominant clones that establish the EPN cellular hierarchy, reflecting both gliogenic and neurogenic differentiation programs—findings not previously reported. These insights highlight the role of diverse developmental states in therapeutic resistance and open avenues for functional studies to characterize these states and lineage programs and their contribution to disease progression.

We greatly appreciate the additional *Reviewer #1* comments and the opportunity to further improve this manuscript.

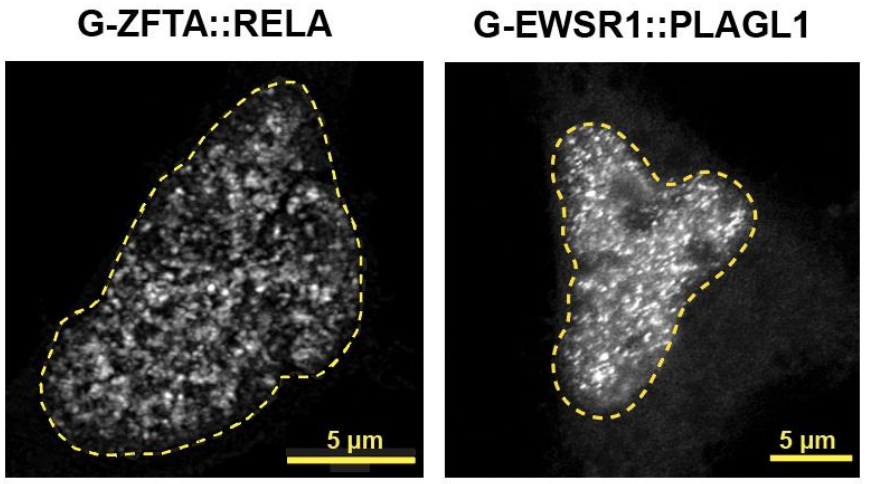
Reviewer #1 – Point #1

In response to my original comment (point #10, reviewer 1) about functional validation of PLAGL, the authors performed a series of overexpression and KO studies in mouse RGCs (shown in Extended Fig. 2). However, these studies did not show that PLAG genes impact the same genes that ZR normally turns on. In addition, KO of PLAGL1 at the same time of ZR tumor initiation did not impact tumor initiation. The authors do state in their rebuttal that “We conclude from these functional experiments that the Plagl1 TF protein may not contribute significantly to ZR tumor development”. However, in the main text there remains language suggesting a direct involvement of PLAG in ZR tumor initiation. For example, in lines 345-347 (“Considering the role of PLAGL1 and PLAGL2 in brain development and disease, the ability of ZR to maintain the PLAG/L motif accessible beyond the embryonic stage may be an important mechanism in EPN initiation.”) There is also additional language about ZR engaging PLAG/L to “initiate oncogenesis” in lines 337-339, which is not borne out by the new mechanistic experiments. In the rebuttal the authors nicely state that this lack of effect on tumor initiation may be because of differences in the structure of ZR vs. PLAGL1, with ZR being created by a gene fusion, giving it disordered protein regions that form bimolecular condensates, however these limitations are surprisingly absent in the main text and deserve mention. These additional studies also raise the question of whether the accessibility of PLAGL motifs is merely coincidental in ZR-EPN.

We sincerely thank *Reviewer #1* for their constructive comments, and we apologize for any lack of clarity in our initial interpretation of the results. Based on the data generated during the first round of revisions, we explored the potential of the PLAG/L family of proteins to activate oncogenic transcriptional programs shared with ZFTA-RELA (ZR). Our findings indicate that PLAG/L transcription factors cannot upregulate the full complement of target genes regulated by ZR.

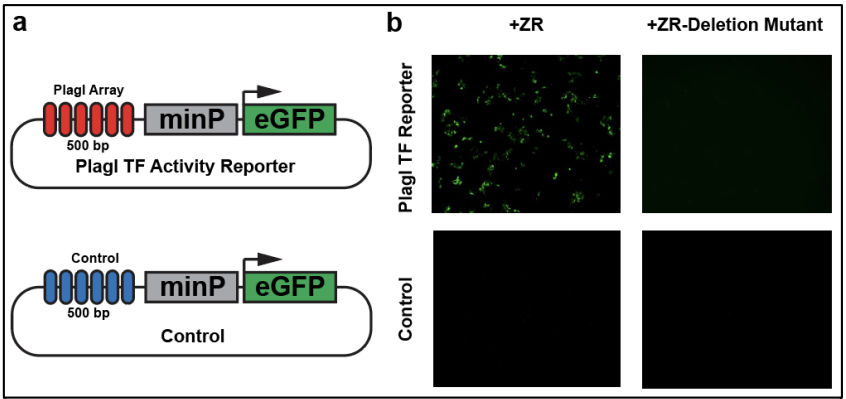
We hypothesize that this limitation may stem from the strong transcriptional activity acquired during gene fusion events, such as those involving ZR and *EWSR1-PLAGL1*. This activity is possibly driven by the acquisition of

disordered regions that promote biomolecular condensate formation—a feature common to many fusion oncoproteins[2]. This hypothesis is supported by new super-resolution microscopy data demonstrating that EWSR1-PLAGL1 fusion oncoproteins exhibit condensate-forming properties similar to ZR (**Response Figure 2**). As part of future studies, we are working to characterize the disordered regions of EWSR1-PLAGL1 and have included this point in the revised manuscript discussion, as you suggested.



Response Figure 2. Super-resolution imaging of GFP tagged ZFTA-RELA left panel (Arabzade et al., NCB, 2025), as compared to GFP-tagged EWSR1-PLAGL1 (right panel, data unpublished).

Regarding the role of PLAG/L proteins in cancer initiation, our experiments suggest that accessibility of PLAG/L motifs in the developing mouse brain may play an important role in ependymoma pathogenesis (rather than the PLAG/L TFs themselves). This is supported by our observations that: i) ZR expression in neural progenitor cells does not induce PLAG/L TF accessibility (i.e., ZR does not have pioneering activity), ii) ZR preferentially binds PLAG/L TF motifs through cognate site identification assays, iii) PLAG/L TF motifs are highly accessible in radial glial cells and cycling progenitors—the likely cells of origin for EPN—compared to differentiated neurons, and iv) Many ZR target oncogenes are preferentially accessible in RGCs versus differentiated neuronal populations. We agree that accessibility alone does not guarantee ZR binding or gene activation. To address this point more directly, we developed a reporter system containing an array of PLAG/L TF motifs, compared to an equal-length random DNA sequence, cloned into a vector with a minimal promoter (minP) driving eGFP (**Response Figure 3**). HEK293 cells co-transfected with ZR selectively activated the PLAG/L TF reporter and expressed GFP, while failing to activate GFP in the control sequence or when deletion mutations were introduced in the ZR condensate driving domain (**Response Figure 3**). These results further support our hypothesis that PLAG/L TF accessibility in specific developmental lineages may predispose cells to downstream ZR oncogene activation and transformation.



Response Figure 3. Co-transfection of a Plagl TF reporter vs control with either ZFTA-RELA or ZFTA-RELA-deletion mutant, containing inactivating mutations in the condensate formation domain.

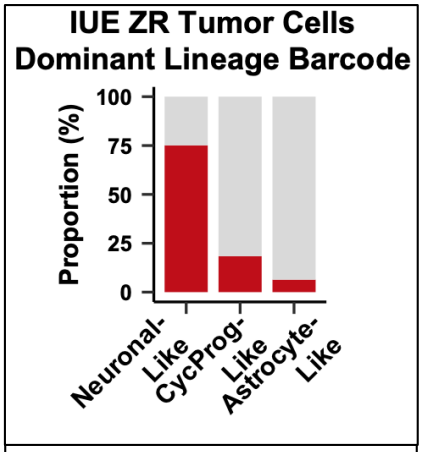
Once again, we thank Reviewer #1 for their valuable feedback. We have revised the manuscript to temper over-stated conclusions and have incorporated additional discussion on the potential role of PLAG/L TF proteins in biomolecular condensate formation and gene activation.

Reviewer #1 – Point #2

2. The authors should show the proportion of annotated cell types that harbor the dominant barcode LB-1 in Fig.

5-6. Are there cell type-specific biases? This would add to the novelty of the work, as the TrackerSeq results that the authors currently show are not entirely surprising (glial and neuronal lineage programs emerging from cycling progenitor-like cells).

We thank *Reviewer #1* for this important suggestion. From the Tracker-seq results we were surprised to observe the emergence of dominant clones capable of capturing the tumor cell heterogeneity and recapitulating normal albeit incomplete differentiation programs. Before these experiments were performed, we had the opposite hypothesis that due to the relatively silent mutational profiles of EPN[3], that diverse clones would predominate with disparate transcriptional and epigenetic signatures – potentially associated with therapy resistance. Furthermore, the ability of cycling progenitor-like cells to give rise to glial and neuronal lineages – while incomplete differentiation – was surprising to us since we were expecting a complete blockade of differentiation and persistent expansion of radial glial cell / cycling progenitor cells beyond embryonic development. Interestingly, this appears to be the case in the context of expression of a different EPN driver gene, *YAP1-MAMLD1*, that correlates with persistent radial glial cell expansion seen in EPN tumors. Why this is also not true in the context of ZR is unclear, and has not yet been described. *Reviewer 1* raises an important point to evaluate in the dominant clones for any cell type-specific biases as a point of novelty. In response, we have quantified the predominant malignant cells in the dominant clones and found that cells are over-represented by neuronal-like cells (**Response Figure 4**). These findings may provide important insight upon the potential chemo-resistant nature of EPN, and that slowly/infrequently cycling neuronal-like populations could be refractory to treatment with anti-proliferative chemotherapies; a finding that has not yet been reported. We thank Reviewer #1 for these comments, that have led to further improvements to the novelty of our manuscript.



Response Figure 4: Proportion of major cell types within the dominant lineage barcode, suggesting a differentiation bias towards neuronal-like cells.

Reviewer #1 – Point #3

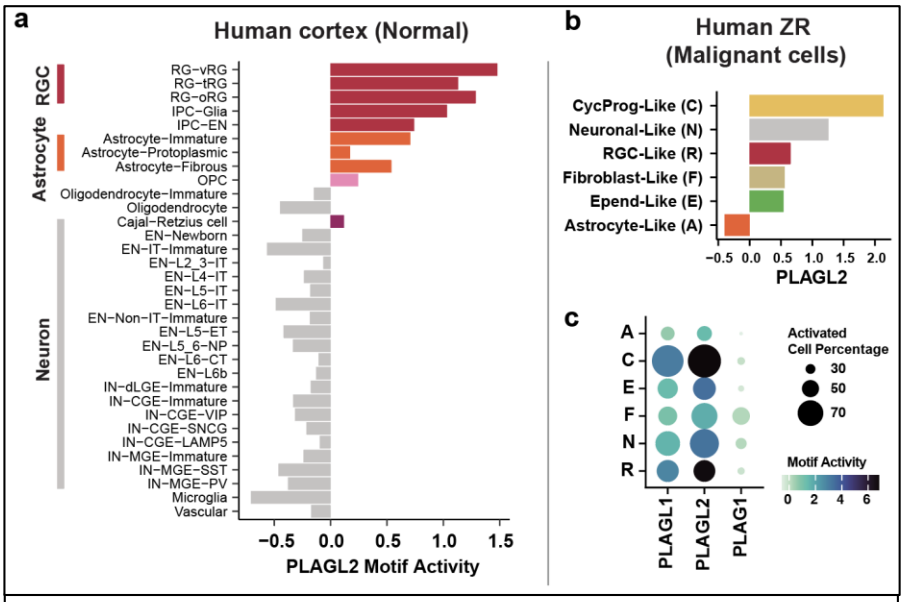
Lines 284-286, Fig. 4, and Fig. 1: The authors state that compared to normal development, ZR in human tumors may maintain chromatin accessibility of PLAG/L family motifs that are normally repressed during neuronal and glial differentiation. However, according to Fig. 1K in the mouse model, PLAG/L motif activity was significantly higher in astrocytes vs. neurons, where activity was indeed strongly repressed. This does not appear to be a species-specific effect either as Fig. 1L suggests that PLAGL2 motif activity is higher in astrocytes compared to the neuronal lineage suggesting that PLAG/L motif activity remains active in astrocytes during normal development at the same time when it is repressed in neurons. Can the authors comment on this difference between the glial vs. neuronal PLAG/L activity? In addition, PLAGL/L motif activity in Fig. 1 is displayed as a numerical score, whereas in Fig. 4 is binned into "high", "middle", and "low", making comparisons between human tumors and normal development rather difficult. I recommend using the same method in both figures. I'm also a bit unclear on how the PLAG/L motif activity in human tumors was divided into high-medium-low, as I could not find clear descriptions regarding this in the Methods section.

We appreciate this relevant point raised by *Reviewer #1*, which we would like to address in a couple of ways:

1) Radial glial cells (RGCs) differentiate directly into astrocytes, whereas several cell types exist between RGCs and neurons such as intermediate progenitor cells. In this way, RGCs and astrocytes are more similar than RGCs and neurons and express similar gene expression programs that makes them difficult to distinguish. This could

in part explain why early astrocytes show more ‘open’ PLAG/L sites than neurons – albeit less than embryonic RGCs and cycling progenitor cells.

2) We would also like to emphasize that our findings are based on comparative analyses between cell types. While astrocytes have some open PLAG/L TF binding sites, there are still fewer open sites than in RGCs and cycling progenitors. We cannot entirely exclude the possibility that, while likely infrequent, progenitor cells that persist through post-natal development could be transformed by ZR and supported by experiments where we transformed OPCs that gave rise to slower growing brain tumors. These observations do lead us to hypothesize that ZR driven adult EPN (while very rare) could arise from transformation of a progenitor-like cell with fewer accessible PLAG/L binding sites that restrains neoplastic transformation.



Response Figure 5: a) PLAGL2 motif scores across human developing brain cell types, derived from data published by Wang et al. (2015). b) PLAGL2 motif scores across human ZR malignant cell types. More PLAGL2 motifs are open in malignant cells compared to development. c) PLAG family motif activation scores across cell type. Dot size represents the percentage of activated cells within each cell type.

3) While PLAG/L TF proteins have not been extensively studied, they are known to be expressed in progenitor cells of development and regulate functions such as cell proliferation[4, 5]. While not yet published[6], prior research also suggests Plagl1 gene expression can increase in Müller glia, a type of astrocyte in the retina, in response to injury. Activated Müller glia undergo reactive gliosis, which includes proliferation/re-entry into the cell cycle[7], which does not happen in differentiated neurons. Maintaining open PLAG/L sites in astrocytes may have important biological functions outside of tumor biology that we do not yet understand.

Finally, we have made the Plag/l motif activity plots consistent across Figures 1 and 4. To improve consistency and facilitate comparison between human tumors and normal development, we have revised Figure 4F to include two complementary visualizations of PLAGL/L motif activity. The first is a dot plot, which provides a more informative representation of the data by including the percentage of active cells, while the second is a bar plot formatted in the same style as **Figure 1L** to ensure visual consistency (**Response Figure 5**).

We thank *Reviewer #1* for these comments to improve the clarity and discussion points of our revised manuscript.

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