nature portfolio

# Peer Review File

# Molecular Cartography of the Human Down Syndrome and Trisomic Mouse Brain

Corresponding Author: Dr Brian Kalish

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

Version 0:

Reviewer comments:

Reviewer #1

(Remarks to the Author)
This study explores the molecular mechanisms behind neurodevelopmental abnormalities and accelerated neurodegeneration in Down syndrome (DS), a genetic cause of intellectual disability. The authors use single-nucleus sequencing, spatial transcriptomics, and proteomics on mid-gestational prenatal human brain tissue to identify altered expression of lineage commitment genes and de-repression of transposable elements in DS neural progenitor cells, suggesting disruptions in neuronal and glial cell fate. Additionally, analysis in a trisomic mouse model (Ts65Dn) provides further insights into the cellular landscape during early life and aging.

However, the following detailed comments need to be addressed by authors:

Study design:
1. The details on how different omics techniques work together to characterize cell phenotypes and spatial architecture are insufficient.
2. The rationale for selecting two specific time points in the Ts65Dn mouse model is not well-explained. The design to include aging Ts65Dn mice seems out of place. If aging is part of your research topic, I recommend incorporating the snRNA-seq of human aging Down syndrome brain and comparing to results from this published paper: Palmer, C. R., Liu, C. S., Romanow, W. J., Lee, M. H., & Chun, J. (2021). Altered cell and RNA isoform diversity in aging Down syndrome brains. Proceedings of the National Academy of Sciences of the United States of America, 118(47), e2114326118.
3. Although the authors highlighted the need to bridge the gap between human and mouse model in studying prenatal DS brain development, this study lacks such cross-species comparisons at key findings. There is a lack of in-depth discussion on the internal connections and comparative analysis between the results in humans and mice, which weakens the logical flow from human to mouse studies and the construction of the cross-species atlas.
4. A significant weakness lies in the limited functional validation of identified pathways and the lack of protein-level confirmation for many findings. The study mainly relies on correlative observations and lacks clear causal relationships and mechanistic insights.

Results:
Line 94-116: While identifying and annotating cell subtypes by markers is acceptable, authors should also provide automatic cell annotation results by label transferring or similar computational approaches from well-annotated and accepted reference datasets to further support your annotation. It's an efficient and reproducible way that incorporate prior biological knowledge, and is recommended by guidelines published in Nature Protocols (https://www.nature.com/articles/s41596-021-00534-0). Same suggestion for annotations in the following analyses.

Line 149-150: oRG and NB ExN show downregulation of genes related to cell cycle, translation, protein homeostasis, and programmed cell death (Fig. 2a). Fig. 2f lacks quantification and wet experiment validation, and it's not clear if other representative markers of related pathways have differences.

Line 171-172: The conclusion about the emergence of senescence in DS NPCs based on the downregulation of chromatin-associated proteins and DNA methyltransferases is not well-supported. The expression of other typical senescence genes in Fig. 3b is not shown.

Line 187-188 and 845-871: Authors should provide more testing/reference supporting the reliability of such customized statistical method comparing spatial organization of cell types (i.e. neighborhood enrichment) between euploid and DS group.

Line 188-189: The analysis mainly focuses on several specific cell types, while other potentially important cell types in DS brain development are less involved. The gestational weeks of the selected samples may not fully cover the key stages of DS brain development, and the impact of sample differences between individuals is not well-addressed, which may affect the universality of the conclusions. The conclusion in Fig. 3d about the functional and positional changes of oRG in the ventricular zone of the DS brain during prenatal development is hasty and lacks ST results or wet experiment validation.

Line 196-197: The Cellchat analysis is considered redundant and does not lead to reliable conclusions. The derivation of conclusions is rather hasty. It is suggested to either conduct in-depth analysis or delete this part. Otherwise, presenting unreliable analysis results will only confuse the readers. Moreover, it is questioned why spatial sequencing samples are not used for cell communication analysis since there are spatial slices available.

Line 223-227: The interpretations of RNA velocity results here seem very subjective. The RNA velocity analyses should be further replicated in subsets of samples and other available datasets published. If applicable, statistical testing should be performed to support the claimed difference between the DS and euploid group. Additional trajectory analyses by other computational methods such as monocle are also needed to support these observations.

Line 231: The results from RNA velocity generated are not sufficient to draw such conclusion. As samples were collected at different PCWs, it might be more informative to incorporate the time stamps. Additional in vitro studies are warranted to test the developmental stages of NPCs in both DS and euploid brain.

Line 239-241: There is no differentiation of cell types in the proteomics analysis, and evidence such as statistical charts or tables for proteomics differences is lacking.

Line 260: The logical relationship between ATR—chronic replication stress—increased gene dosage is not clear, and the markers for the existence of chronic replication stress are not provided.

Line 281-283: The snRNA-seq profiled the transcriptome of TEs. The evidence for the causal relationship between Hsa21 triplication and transposon alteration is mainly correlative. The specific mechanism by which Hsa21 triplication disrupts epigenetic modifications to cause transposon alteration is not clearly described.

Line 294: The limitations of using only S9.6 antibody immunostaining to confirm transposon differences are pointed out, including potential cross-reactions and affected quantitative accuracy. Suggestions are provided to improve the quantification of transposon levels by combining nucleic acid hybridization with quantitative PCR and to use sequencing methods and functional validation in cell/animal models.

Line 302: The specific selection criteria for the 500 genes used in the analysis are not clearly described, and it is not certain whether these genes can comprehensively and accurately cover all important aspects related to DS.

Line 343: It is questioned whether 6mo mice are sufficient to reflect aging signals. The study should increase the description of how gene expression changes translate into cell function changes and affect cell phenotypes and overall brain function during neurodevelopment and aging.

Line 389-391: The relationship between signal transduction changes and the inflammatory environment is oversimplified. The study should consider other factors that may affect the inflammatory response and explore in-depth how these signal transduction changes interact with other factors to affect the formation and development of the inflammatory environment. The use of non-spatial sequencing for cell communication analysis in the mouse model is questioned.

Line 395, 401: I assume these are mis-references of figures.

While this study attempts to advance our understanding of Down syndrome neurobiology through multi-omics approaches, several fundamental limitations cast doubt on the broader applicability and reliability of its findings. The extremely limited sample size, coupled with significant technical constraints in both human and mouse studies, raises serious concerns about the generalizability of the results. The lack of functional validation for key molecular findings, absence of protein-level confirmation, and heavy reliance on correlative observations without clear mechanistic insights significantly weakens the study's conclusions. The methodological gaps in controlling for post-mortem artifacts, limited temporal coverage, and insufficient cross-species validation suggest that many of the reported molecular signatures may reflect technical artifacts or specimen-specific variations rather than true biological phenomena. Furthermore, the absence of thorough documentation and incomplete statistical rigor compromises the reproducibility of these findings. While the study introduces potentially interesting hypotheses about altered lineage commitment and transposable element dysregulation, substantial additional work with larger cohorts, rigorous functional validation, and more comprehensive controls will be necessary before these findings can meaningfully contribute to our understanding of DS neurodevelopment or inform therapeutic strategies. This work ultimately highlights the significant challenges and methodological improvements needed in the field rather than providing definitive insights into DS pathobiology.

(Remarks on code availability)
Codes should be additionally reviewed by specialists.

Reviewer #2

(Remarks to the Author)
Molecular Cartography of the Human and Mouse Down Syndrome Brain - Review
Min Yi Feng….Brain T. Kalish

Overview and Summary:
Down syndrome (DS) presents the dual challenges of abnormal neurodevelopment, and accelerated age- and Alzheimer's disease-related neurodegeneration. How these aberrant processes interact to result in impaired intellect, hyperactive immune responses, and defects in other critical systems in DS is only partially understood. To define neurodevelopmental abnormalities, and map the molecular landscape of trisomy-related changes during perinatal development with cellular resolution, the set of studies by Feng et al used cross-species, integrated single-nucleus RNAseq, spatial transcriptomics and proteomics approaches, with a focused analysis of the maintenance, differentiation and proliferation of neural precursor cells (NPCs). These studies were largely performed using prenatal human DS and euploid brains, with additional support from data generated using Ts65Dn DS model mice.

Alterations in core cellular processes in DS NPCs
SnRNAseq performed with DS and euploid human brain (second trimester) identified five subtypes of NPCs (outer radial glia, oRG; ventricular radial glia, vRG; cycling progenitors, CP; intermediate progenitors fated to an excitatory lineage, IP ExN; and IP fated to an inhibitory lineage, IP InN). These subpopulations of NPCs were spatially defined to the ventricular zone using slide-seqV2.

Significant down-regulation of genes regulating transcription, translation and proteosomal proteolysis was observed in oRG, IP, and newborn excitatory neurons (NB ExN) from DS brain, including genes encoding DNA and RNA polymerases, ribosomal subunits and translation initiation factors. Components of other core cellular processes, such as tRNA and mRNA maturation factors, were also found down-regulated in DS NPCs. Several genetic components of DNA repair machinery and DNA damage checkpoint control were found to be down-regulated in DS NPCs in the current study, consistent with previous findings that elevated oxidative stress and inefficient DNA repair processes contribute to the manifestation of DS. Consistent with nuclear disruption and senescence, LMNB1 was decreased in DS. Genes regulating maintenance of NPC multipotency were also disrupted in DS brain.

Microenvironmental organization
Spatial transcriptomics with slide-seqV2 was used to examine identity changes in the cellular neighborhood proximal to NPCs. In DS brains, a sparsity of oRG was reported in the vicinity of IP cells in the ventricular zone. Since oRG cells differentiate into IP cells, the finding raises the possibility of a defective expansion of the stem cell niche in DS.

The organization of the microenvironment depends on ligand-receptor signaling during neurodevelopment. To investigate potential alterations in cell-cell communication in DS, the authors inferred incoming and outgoing signaling tone by comparing ligand and cognate receptor expression across cell types. In human DS brain, stronger incoming and outgoing signals from inhibitory neurons in cortex and striatum were inferred, whereas stronger signals from excitatory neurons were inferred in euploid brain. The authors used network analysis to infer that BMP and WNT signaling is increased in several cell types in DS brain, including WNT5B signaling from cortical neurons, and BMP7 from astrocytes, to NPCs. On the other hand, decreased SLIT-ROBO, signaling was inferred in DS NPCs, and decreases in ROBO1, SLIT1, were additionally supported by slide-seqV2. Considering the established role of SLIT-ROBO signaling in neuronal migration in the developing brain, this decrease may contribute to architectural disorganization in DS.

Disrupted lineage progression in DS
By comparing the isoform ratios of spliced and unspliced pools of RNA within progenitor cells, lineage progression and rate of differentiation can be predicted (RNA velocity analysis). The authors used the technique to predict that most cell types in DS brain follow a similar pattern of differentiation to controls, but identify exceptions in NPCs, newborn superficial excitatory neurons, and inhibitory neurons in cortex and striatum. Notably, in DS, NPCs were predicted, based on splicing pattern, to undergo decelerated differentiation, while newborn excitatory neurons were predicted to show early termination of differentiation, and/or entrance into senescence; several driver genes of maturation, migration and synaptogenesis were depleted in these cells in DS, including SLIT-ROBO. Taken together, these data predict a slower differentiation of NPCs, and early terminal differentiation of newborn excitatory neurons, in DS.

Proteomics of the prenatal DS brain
The authors performed proteomics by MS with 4 DS and 3 control brains, age-matched with the transcriptomics experiments (13-19 weeks post-conception). Differential protein expression analysis found that of almost 900 significantly altered proteins, 2/3 are up-regulated, and 1/3 down-regulated in DS versus control. In DS, phagocytosis, proteolysis, inflammation and innate and adaptive immune responses were enriched ontology terms; proteins involved in mRNA processing, synapse assembly and cortical migration were down-regulated, consistent with RNA data presented earlier in the manuscript. Some proteins involved in DNA damage and repair were up-regulated in DS (e.g. ATR, CHEK1, CDK2, MCM3-7, RAD9A/17 and ORC), while others were concurrently downregulated (e.g. p53, RAD51C, HMGA1), suggesting an impaired ability to

respond effectively to DNA damage. Neuroimmune dysregulation was supported by proteomics data indicating increased expression of innate and adaptive immune response (including interferon regulatory transcription factor IRF3).

Pronounced de-repression of transposable elements in DS
RNA and protein data from the current study, and others, indicate a down-regulation of chromatin-associated proteins and epigenetic modifiers like methyltransferases. Since such changes have been linked to de-repression of transposable elements, including DNA transposons and retrotransposons, the authors tested a hypothesis that NPCs in DS have aberrantly activated TE expression. Significant enrichment of long interspersed nuclear elements (LINEs) was found in NPCs, newborn and mature excitatory neurons, and vascular cells; this was further evidenced by increased immunostaining for RNA-DNA hybrids, which result from retrotransposon processing and can trigger cellular stress responses.

Mouse: Spatial transcriptomics in the neonatal and mature Ts65Dn forebrain
Coronal sections of P0 and 6 month-old Ts65Dn and euploid mice were processed for MERFISH (n=3 per genotype). A panel of 500 probes was curated to examine proliferation, differentiation, inflammation and immunity, and findings were in accord with changes described herein for human tissue. Ligand-receptor analyses predicted increased signaling by astrocytes, OPCs and excitatory neurons via CX3CL1 to CX3CR1 receptors on microglia in perinatal DS; this elevation persists at 6 months of age, where increased CCL8-CCR3 pro-inflammatory signaling is predicted. Altered WNT, SHH and NOTCH signaling was inferred by MERFISH in NPCs, and systems previously identified as aberrant in human NPCs (translation and transcription regulation, DNA repair and cell cycle checkpoint regulation, self-renewal), were also disrupted in Ts65Dn mice. At 6 months, trisomic NSCs showed elevated transcription of interferon-related signaling and neuroinflammatory responses involving CCL8-CCR2 and CX3CL1-CX3CR1 signaling axes.

Conclusion:
The paper represents an informative and important contribution to understanding the developing DS brain. As such, will be of interest to investigators in DS and, more generally, to those in neurodevelopment. The key limitation, as acknowledged, is that while a large collection of interesting observations with possible inferences is presented, it is only when the suggested molecular pathways and mechanisms have been further investigated will the salience of findings be defined. This is not a criticism but it would be useful to further expand in the discussion as to suggested priorities for future mechanistic studies. The manuscript would also benefit from consideration of the several considerations/ suggestions, below.

Considerations/Suggestions:

1. The authors should consider this small change in title for the paper: “Molecular Cartography of the Brain Developing Human Down Syndrome Brain and the Developing and Mature Ts65Dn Mouse Model Brain”.

2. The findings obtained in the mouse model are broadly consistent with those made in human tissue, and indicate disruptions in core cellular processes like translation, transcription, chromatin dynamics, DNA repair, differentiation, renewal, and migration in NPCs.

The authors predicted decelerated differentiation of NPCs, and premature terminal differentiation in newborn excitatory neurons in human DS. Since the mouse model enables fine temporal studies of NPC lineage progression that are implausible in human, these predictions could be tested empirically in the days/weeks following birth. The cross-species validation of general effects provided here is welcome, but leveraging the mouse model to test these predictions about lineage progression, which are hypothesized from RNA velocity analyses, would have raised the impact of this section of the study.

3. On line 247, the word “assembly” appears twice, and the sentence requires further small adjustment to be complete.

4. The proteomics data from brain lysate partially validates key findings inferred from transcriptomics methods, though lacking single-cell resolution. We appreciate that single-cell proteomics may be out of the scope of the study, but a protein-based, cell-type specific validation (i.e. IHC) of key findings would bolster them, e.g. to confirm or reject predictions about premature terminal differentiation of newborn excitatory neurons with markers of mature synapses, and to validate the predictions about enriched and depleted incoming and outgoing pro-inflammatory signals in glia and neurons.

5. Since chromatin organization defects are described (TE activation, up- and down-regulation of DNA damage repair genes/proteins), what is the status of trisomic chromatin-associated factors such as Usp16, Hmgn1 and Dyrk1a in these cells?

6. In the 6 month-old mouse brain, were transposable elements activated by trisomy, as in neonatal brain? By 6 months, are there other cell types in which transposable elements have been activated?

7. Since TE activation is observed in NPCs, what is the status of cytosolic DNA sensing pathways, e.g. cGAS-STING, in proteomic and transcriptomic datasets? This knowledge may be useful in defining correlates of senescence in this setting.

8. I was struck by the wide differences in gene expression for HSA21 genes, as depicted in S Figure 1. The authors may want to further address what appears to a quite variable pattern demonstrating increases and decreases across cell types. I note in particular that APP gene expression was rarely increased in neurons, an in most cases decreased, a finding at odds with much earlier data in the brains of mouse models. Perhaps the authors could consult with other sources of data in humans and respond.

9. Given the large amount of data, it would help to more frequently reference the source of observations – e.g. when citing the results of proteomic studies, etc. As an example, on page 11 in two paragraphs we read of changes in a number of proteins. Specifically calling out the locus of these data will help.

10. In the legend to Figure 1, the panels for neural progenitors and excitatory neurons are misstated.

11. On pages 16 and 17, the authors reference S Figure 5, but I have no such figure. I believe they mean to reference S Figure 4 panels h through k.

(Remarks on code availability)

Reviewer #3

(Remarks to the Author)
I co-reviewed this manuscript with one of the reviewers who provided the listed reports. This is part of the Nature Communications initiative to facilitate training in peer review and to provide appropriate recognition for Early Career Researchers who co-review manuscripts.

(Remarks on code availability)
I co-reviewed this manuscript with one of the reviewers who provided the listed reports. This is part of the Nature Communications initiative to facilitate training in peer review and to provide appropriate recognition for Early Career Researchers who co-review manuscripts.

Version 1:

Reviewer comments:

Reviewer #1

(Remarks to the Author)
The study has been significantly enhanced through a series of comprehensive revisions, which have significantly strengthened the study through additional validations (e.g., TP53, ROBO1, LINE1-ORF1 immunofluorescence), enhanced analyses (e.g., LOSO, SenMayo GSEA, SoloTE for transposable elements), and improved clarity. The publicly accessible data (GEO: GSE280175, GSE280170, GSE280177; MassIVE: MSV000096108) and code (GitHub: https://github.com/KalishLab/Molecular-Cartography-of-DS-Brain) further enhance its value as a resource for Down syndrome (DS) research. However, to ensure the robustness and interpretability of key findings, I have a few follow-up questions addressing unresolved or partially addressed points from my original comments. These questions aim to refine the manuscript and maximize its scientific impact.

1.Senescence Conclusions (Comment 2, Results p. 10, Fig. 2i, Supplementary Table 2): The addition of the SenMayo gene set analysis (Fig. 2i) and LMNB1 immunofluorescence validation (Supplementary Fig. 2f, g) provides valuable evidence for a senescence-like state in DS neural progenitor cells (NPCs), and the softened conclusions are appreciated. However, the original comment noted that Figure 3b does not show the expression of other canonical senescence genes (e.g., CDKN2A, CDKN1A), which are critical for robustly supporting senescence claims given its complex phenotype. Could you provide the expression data for CDKN2A, CDKN1A, or other senescence markers in NPCs (e.g., oRG, vRG, IP) from Supplementary Table 2, including log2 fold change and FDR values? If these genes were not detected or analyzed, please explain why (e.g., low expression levels, technical limitations) and discuss how this impacts the senescence interpretation.

2.Transposable Element De-repression Mechanism (Comment 6, Discussion p. 29-30, Supplementary Tables 2, 5, 6): The LINE1-ORF1 validation (Fig. 4f, g) and discussion of potential mechanisms (e.g., chromatin accessibility changes, VPS37A downregulation) strengthen the transposable element (TE) de-repression findings. However, the mechanism by which Hsa21 triplication drives TE de-repression remains general, particularly regarding which Hsa21 genes disrupt epigenetic modifications. Supplementary Table 2 shows altered expression of Hsa21 genes like DYRK1A and HMGN1 in DS NPCs, but their role in TE regulation is not discussed. Could you specify which Hsa21 genes (e.g., DYRK1A, HMGN1) might regulate epigenetic modifications (e.g., DNA methylation, histone acetylation) to drive LINE1 de-repression, and provide preliminary data (e.g., correlation analysis between DYRK1A/HMGN1 and LINE1 expression from Supplementary Tables 2 and 6)? If such data are unavailable, please propose a more specific testable hypothesis in the Discussion to guide future studies.

3.Functional Impact of Gene Expression Changes in Ts65Dn Mice (Comment 9, Results p. 24-25, Discussion p. 29, Supplementary Table 8): Re-framing the 6-month Ts65Dn timepoint as a “mature” rather than “aging” brain clarifies the study’s scope, and the detailed differential gene expression (DEG) data (Fig. 6a-c, Supplementary Table 8) provide insights into dysregulated pathways (e.g., WNT, SHH, NOTCH). However, the original comment requested more description of how these DEGs affect cell functon(e.g., NPC proliferation, migration, gliogenesis) and brain functon(e.g., cortical development,

cognition). The Discussion briefly mentions reduced NSC proliferation (citing Chakrabarti et al., 2007), but does not elaborate on how DEGs like Mki67, Dcx (downregulated in P0 NPCs), or Gfap, Aqp4 (upregulated at 6 months) contribute to DS phenotypes. Could you expand on the potential functional impacts of these DEGs on NPC behavior and brain development, supported by relevant Ts65Dn phenotype studies (e.g., Chakrabarti et al., 2007; Stagni et al., 2022)? If this is beyond the current scope, please discuss the potential links between these DEGs and DS neurodevelopmental deficits in the Discussion.

4.Control of Inter-sample Variability and Post-mortem Artifacts (Summary Comment, Methods p. 33-34, Discussion p. 30, Supplementary Table 1): The LOSO analysis, stringent quality control (e.g., mitochondrial gene filtering, SCTransform normalization, Harmony batch correction), and data/code availability significantly enhance result robustness. However, the original comment raised concerns about post-mortem artifacts (e.g., RNA degradation, cell state alterations) in human prenatal brain samples, which could affect gene expression and TE analyses. The Discussion acknowledges potential inconsistencies in post-mortem tissue sectioning but does not detail specific controls for post-mortem intervals (PMI) or RNA integrity. Supplementary Table 1 lacks PMI or preservation condition details. Could you clarify the measures taken to control post-mortem artifacts (e.g., PMI range, RNA integrity number, or storage conditions)? Additionally, while the GitHub code is accessible, has it been reviewed by external specialists to ensure reproducibility, or do you plan to facilitate such a review (e.g., via bioRxiv community feedback)? These clarifications would strengthen confidence in the dataset's reliability.

Addressing these points will further refine the manuscript and solidify its contribution to understanding DS neurodevelopment.

(Remarks on code availability)

Reviewer #2

(Remarks to the Author)
I found the revisions satisfactory. As stated before, I believe it makes a significant contribution to research in DS. I would suggest fixing a few minor typographical errors:

Line 84: modify "single nucleus" to "single-nucleus"
Lines 55-56: modify "high resolution" to "high-resolution"
Lines 995-995: modify "an Vanquish" to "a Vanquish"
Lines 578-579: modify "reveals" to "reveal" to reflect that the subject "dysregulation" is singular

(Remarks on code availability)

Reviewer #3

(Remarks to the Author)
I co-reviewed this manuscript with one of the reviewers who provided the listed reports. This is part of the Nature Communications initiative to facilitate training in peer review and to provide appropriate recognition for Early Career Researchers who co-review manuscripts.

(Remarks on code availability)

Version 2:

Reviewer comments:

Reviewer #1

(Remarks to the Author)
The revisions are satisfactory. It makes a significant contribution to the field of DS research.

(Remarks on code availability)

Point-by-Point Response to Review:

**REVIEWER 1**

**Reviewer Comment:** This study explores the molecular mechanisms behind neurodevelopmental abnormalities and accelerated neurodegeneration in Down syndrome (DS), a genetic cause of intellectual disability. The authors use single-nucleus sequencing, spatial transcriptomics, and proteomics on mid-gestational prenatal human brain tissue to identify altered expression of lineage commitment genes and de-repression of transposable elements in DS neural progenitor cells, suggesting disruptions in neuronal and glial cell fate. Additionally, analysis in a trisomic mouse model (Ts65Dn) provides further insights into the cellular landscape during early life and aging.
However, the following detailed comments need to be addressed by authors: The details on how different omics techniques work together to characterize cell phenotypes and spatial architecture are insufficient.
**Author Response:** Thank you for this suggestion. We have added additional context regarding the spatial and single cell omic approaches to both the Introduction and Discussion.

**Reviewer Comment:** The rationale for selecting two specific time points in the Ts65Dn mouse model is not well-explained. The design to include aging Ts65Dn mice seems out of place. If aging is part of your research topic, I recommend incorporating the snRNA-seq of human aging Down syndrome brain and comparing to results from this published paper: Palmer, C. R., Liu, C. S., Romanow, W. J., Lee, M. H., & Chun, J. (2021). Altered cell and RNA isoform diversity in aging Down syndrome brains. Proceedings of the National Academy of Sciences of the United States of America, 118(47), e2114326118.

**Author Response:** We have revised the Results to include a clearer rationale for the selected Ts65Dn mouse experimental timepoints. Based on the Reviewer's comments, we have removed reference to 'aging', as the purpose of this Resource was not to define the role of aging, as the selected timepoints (and emphasis on human fetal development) would not be appropriate. We have revised the manuscript to more accurately frame experiments as comparing the *developing* (P0) and *mature* (6mo) Ts65Dn brain.

**Reviewer Comment:** Although the authors highlighted the need to bridge the gap between human and mouse model in studying prenatal DS brain development, this study lacks such cross-species comparisons at key findings. There is a lack of in-depth discussion on the internal connections and comparative analysis between the results in humans and mice, which weakens the logical flow from human to mouse studies and the construction of the cross-species atlas.

**Author Response:** Thank you for this feedback. We have expanded upon the end of the Results section to provide more detailed comparative analysis between the results in humans and mice. Given that one of the key findings from this Resource is the de-repression of transposable elements in the human prenatal DS brain, we have performed transposable

element analysis from a previously published Ts65Dn snRNA-seq dataset and added the results to the manuscript.

**Reviewer Comment:** A significant weakness lies in the limited functional validation of identified pathways and the lack of protein-level confirmation for many findings. The study mainly relies on correlative observations and lacks clear causal relationships and mechanistic insights.

**Author Response:** We have performed additional validation experiments in the prenatal human brain to support the findings from omic analysis. As the Reviewer appreciated, the prenatal human brain tissue is a valuable and scarce resource, but we have now validated, using immunofluorescence, key findings related to TP53, ROBO1, and LINE1-ORF1. We have also improved our intersection of the proteomics and snRNA-seq, as those pathways and targets that show significant transcriptional and protein-level changes are most robust.

**Reviewer Comment:** Line 94-116: While identifying and annotating cell subtypes by markers is acceptable, authors should also provide automatic cell annotation results by label transferring or similar computational approaches from well-annotated and accepted reference datasets to further support your annotation. It's an efficient and reproducible way that incorporate prior biological knowledge, and is recommended by guidelines published in Nature Protocols (https://www.nature.com/articles/s41596-021-00534-0). Same suggestion for annotations in the following analyses.

**Author Response:** Thanks for this suggestion. The automatic cell annotation approaches, such as those referenced by the Reviewer, are designed for use in adult human tissue; similar such automated annotation approaches are not readily available for the developing human or mouse brain, which is a significant gap. In the developing brain, there are unique progenitor and migratory cell populations that have not been well incorporated into the automated cell annotation approaches as of yet (e.g. neural stem cells, intermediate progenitors, and the like). This is our primary rationale for manually curating the cell type annotation. We have provided references to the papers used for consistent marker gene utilization, and included marker gene plots (Fig. 1c, Supplementary Fig. 1b, Supplementary Fig. 5a-f) to explicitly justify the cell typing. Further, in response to the Reviewer, we used the automated annotation program CellTypist (as now stated in the Methods) and confirmed our cell type annotations, with minor additions for improved granularity given unique populations in the developing brain.

**Reviewer Comment:** Line 149-150: oRG and NB ExN show downregulation of genes related to cell cycle, translation, protein homeostasis, and programmed cell death (Fig. 2a). Fig. 2f lacks quantification and wet experiment validation, and it's not clear if other representative markers of related pathways have differences.

**Author Response:** Based on feedback from the Reviewer, we have extensively revised the Results and particularly the differential expression section of the manuscript. We have organized findings more closely based on pathways, to improve readability and cohesiveness. With regard to the Reviewer's point about validation of candidates in the pathways we

emphasize (such as cell cycle), we have validated the significant reduction in TP53 expression in NPCs in the human prenatal DS brain, as shown in Fig. 2b, c.

**Reviewer Comment:** Line 171-172: The conclusion about the emergence of senescence in DS NPCs based on the downregulation of chromatin-associated proteins and DNA methyltransferases is not well-supported. The expression of other typical senescence genes in Fig. 3b is not shown.

**Author Response:** The transcriptional signatures of senescence are notoriously challenging to phenotype, particularly by scRNA-seq, but identification of these signatures can provide important insight into disease pathogenesis. This is why the SenMayo gene set approach was developed (see PMID 35974106, Nat Communications 2022). This approach was extensively experimentally validated, and it has the strength of capturing the breadth of an otherwise heterogeneous senescence state. This is why we employed this approach to our dataset. Further, our results are consistent with recent studies showing NPC senescence in Down Syndrome (PMID 34995493), as stated in our Discussion. We also provide experimental validation using LaminB1, a very well-established marker for senescence (see PMID 23934658). However, we appreciate the reviewer's point that the purpose of this manuscript is not to determinate causality and precise mechanisms of senescence in the prenatal DS brain. Therefore, we have significantly softened our conclusions regarding senescence, both in the Results and Discussion.

**Reviewer Comment:** Line 187-188 and 845-871: Authors should provide more testing/reference supporting the reliability of such customized statistical method comparing spatial organization of cell types (i.e. neighborhood enrichment) between euploid and DS group.

**Author Response:** We appreciate the reviewer's concern regarding the reliability of our method. Initially, we considered using Squidpy's neighborhood enrichment analysis. However, this approach relies on permutation testing, which assumes random shuffling of cell types across the tissue. Such permutations may not be appropriate for our purposes as the way the cells are organized in the brain is not random. To address this concern more robustly, we instead implemented a leave-one-subject-out (LOSO) cross-validation approach. Specifically, for each iteration, we left out one subject per group and calculated the cell type composition for the remaining samples. We then computed the mean and standard deviation of effect sizes across all iterations to identify interactions that were consistently divergent or conserved between groups. This method captures inter-subject variability and allows us to assess the robustness and generalizability of spatial interaction differences across samples, rather than relying on theoretical null distributions that may not reflect biological reality. The results of this validation are shown in Fig. 3i-j; Fig 4a-f.

**Reviewer Comment:** Line 188-189: The analysis mainly focuses on several specific cell types, while other potentially important cell types in DS brain development are less involved. The gestational weeks of the selected samples may not fully cover the key stages of DS brain development, and the impact of sample differences between individuals is not well-addressed, which may affect the universality of the conclusions. The conclusion in Fig. 3d about the

functional and positional changes of oRG in the ventricular zone of the DS brain during prenatal development is hasty and lacks ST results or wet experiment validation.

**Author Response:** We have significantly revised the Results of the prenatal human data, broadening the description to other cell types. We fully acknowledge that the gestational weeks of the sample are all early/mid second trimester; this is a logistical constraint given the timing of pregnancy termination and tissue availability. Such prenatal human brain tissue is quite rare, so we feel that this dataset is highly unique in that context. We have acknowledged the limitation of a narrow gestational window in the Discussion. Regarding Fig. 3d, we have implemented the leave one sample out (LOSO) approach as described above and were able to reproduce the significant results. We have also modified the text surrounding the microenvironment analysis to avoid over-stating the conclusions.

**Reviewer Comment:** Line 196-197: The Cellchat analysis is considered redundant and does not lead to reliable conclusions. The derivation of conclusions is rather hasty. It is suggested to either conduct in-depth analysis or delete this part. Otherwise, presenting unreliable analysis results will only confuse the readers. Moreover, it is questioned why spatial sequencing samples are not used for cell communication analysis since there are spatial slices available.

**Author Response:** We agree that differential gene expression analysis and CellChat ligand-receptor analysis can be redundant, as the CellChat analysis relies upon changes in gene expression to infer cellular communication. We have taken the advice of the Reviewer and removed the CellChat analysis from the manuscript (both mouse and human datasets). This has streamlined the manuscript and improved readability.

**Reviewer Comment:** Line 223-227: The interpretations of RNA velocity results here seem very subjective. The RNA velocity analyses should be further replicated in subsets of samples and other available datasets published. If applicable, statistical testing should be performed to support the claimed difference between the DS and euploid group. Additional trajectory analyses by other computational methods such as monocle are also needed to support these observations.

**Author Response:** We appreciate the reviewer's comments. We would like to clarify that RNA velocity methods, including scVelo, are primarily designed to model transcriptional dynamics within individual datasets, rather than for direct comparisons between experimental groups. This may have led to confusion in the original manuscript. RNA velocity, including scVelo, infer cell state transitions and lineage trajectories by leveraging the ratio of unspliced to spliced RNA, and do not provide built-in statistical frameworks for group comparisons. Therefore, we have substantially revised the RNA velocity section to more accurately describe the results as presented. Our interpretation and description is consistent with other recently published papers that employ RNA velocity (see PMID: 33723434, Nature Neurosci 2021). In addition, we strengthened our analysis by examining latent time using scVelo, providing a complementary measure of cell state progression. Importantly, the latent time results, along with driver gene analysis, closely aligned with the visualized embedding streams, supporting the altered trajectories of radial glia and newborn neurons. We have also expanded the discussion of the key driver genes.

While Monocle provides a measure of cell trajectory, this is markedly different than RNA velocity (which is the time derivative of the gene expression state), and thus Monocle is not an

appropriate comparator (see PMID 36495456 for details of how these approaches are not comparable).

**Reviewer Comment:** Line 231: The results from RNA velocity generated are not sufficient to draw such conclusion. As samples were collected at different PCWs, it might be more informative to incorporate the time stamps. Additional in vitro studies are warranted to test the developmental stages of NPCs in both DS and euploid brain.

**Author Response:** As stated above, we have revised the presentation and conclusions of RNA velocity. With respect to developmental stage variation, all samples analyzed were collected during the second trimester (13–19 post-conceptional weeks), providing a relatively focused developmental window. Although there is a ~6-week range between the earliest and latest samples, this is a narrower interval compared to many existing studies that examine developmental trajectories across broader time spans, including comparisons between fetal, postnatal, and adult tissue. Thus, we believe our dataset captures a critical and relatively comparable window of neurodevelopment. Additionally, we would like to highlight a recent paper published in Nature by Marderstein et al., (2024) that performed RNA velocity using CellRank on a cohort of 20 fetal brains (5 disomic and 15 trisomic) spanning an even broader gestational range (11–19 PCW). This study, despite the wider developmental interval, still identified alterations in erythroid lineage formation that were biologically meaningful.

**Reviewer Comment:** Line 239-241: There is no differentiation of cell types in the proteomics analysis, and evidence such as statistical charts or tables for proteomics differences is lacking.

**Author Response:** We have now included a new analysis and figure (Supplementary Fig. 3a, b) that depicts the cell type-specific expression pattern of differentially abundant proteins from the proteomics analysis, which allows us to visualize their likely cell subtype origin. We have also included a new Supplementary Table 5 with proteomics data and statistics. We have improved the integration and proteomics and snRNA-seq analyses in the Results, which strengthens the overall conclusions.

**Reviewer Comment:** Line 260: The logical relationship between ATR—chronic replication stress—increased gene dosage is not clear, and the markers for the existence of chronic replication stress are not provided.

**Author Response:** We have completely restructured the Results section in response to other constructive comments from the Reviewer, and we have removed this reference to ATR and chronic replication stress. The Results now focus more clearly on Gene Ontology-based processes, in order to provide a clearer structure for readers.

**Reviewer Comment:** Line 281-283: The snRNA-seq profiled the transcriptome of TEs. The evidence for the causal relationship between Hsa21 triplication and transposon alteration is mainly correlative. The specific mechanism by which Hsa21 triplication disrupts epigenetic modifications to cause transposon alteration is not clearly described.

**Author Response:** We have made several revisions to the manuscript to address this constructive comment. First, we have improved the justification for specifically examining the

expression of TEs in the prenatal human DS brain. Second, we do not intend to suggest a causal relationship, as that would require extensive functional experiments. We have revised our conclusions to be more consistent with the data we provide, and we have expanded upon the Discussion to include a potential mechanistic model linking LINE1 expression and Trisomy 21, that can be tested in future experiments.

**Reviewer Comment:** Line 294: The limitations of using only S9.6 antibody immunostaining to confirm transposon differences are pointed out, including potential cross-reactions and affected quantitative accuracy. Suggestions are provided to improve the quantification of transposon levels by combining nucleic acid hybridization with quantitative PCR and to use sequencing methods and functional validation in cell/animal models.

**Author Response:** We appreciate the Reviewer's point regarding the S9.6 antibody. To bettersupport our conclusions, we have now performed immunostaining with a widely used LINE1 antibody and found an increased LINE1 ORF1 signal in DS NPCs (Fig. 4f, g). This is direct supporting evidence for our conclusions. qPCR would not be an appropriate method of confirmation, as our findings note an interesting cell lineage specificity to transposable element activation (Fig. 4e). Functional validation in human cell models is beyond the scope of this manuscript; such work, for instance in Down Syndrome iPSC-derived neural progenitor cells, would require 12+ months of work at least. This will be the focus of a follow-up paper, and such future experiments are expanded upon in the Discussion.

**Reviewer Comment:** Line 302: The specific selection criteria for the 500 genes used in the analysis are not clearly described, and it is not certain whether these genes can comprehensively and accurately cover all important aspects related to DS.

**Author Response:** We have expanded upon the justification for the selection of the MERFISH gene panel in the Results. The gene list was curated based on review of existing literature of the Ts65Dn mouse line, focusing on specific molecular processes that have been suggested as common pathways for dysregulation; this has been clarified in the text. We have also amended the Discussion to acknowledge the limitation that MERFISH is a targeted in situ approach and thus is subject to bias in the selection of gene targets. However, we also highlight approaches to leverage targeted MERFISH datasets to accurately impute the entire transcriptome at spatial resolution, thus increasing the potential Resource utility of the dataset.

**Reviewer Comment:** Line 343: It is questioned whether 6mo mice are sufficient to reflect aging signals. The study should increase the description of how gene expression changes translate into cell function changes and affect cell phenotypes and overall brain function during neurodevelopment and aging.

**Author Response:** Based on the Reviewer's feedback, we have reframed the manuscript to remove references to "aging", as 6mo mice may not sufficiently reflect aging. Instead, we emphasize the utility of describing two timepoints in the Ts65Dn mouse model (developmental and mature brain).

**Reviewer Comment:** Line 389-391: The relationship between signal transduction changes and the inflammatory environment is oversimplified. The study should consider other factors that

may affect the inflammatory response and explore in-depth how these signal transduction changes interact with other factors to affect the formation and development of the inflammatory environment. The use of non-spatial sequencing for cell communication analysis in the mouse model is questioned.

**Author Response:** We have removed the CellChat analysis for both the human and mouse datasets, as suggested above, and we have clarified the Results to avoid over-speculation regarding the inflammatory environment in DS.

**Reviewer Comment:** Line 395, 401: I assume these are mis-references of figures.

**Author Response:** Thank you. These figure references have been corrected.

**Reviewer Comment (Summary):** While this study attempts to advance our understanding of Down syndrome neurobiology through multi-omics approaches, several fundamental limitations cast doubt on the broader applicability and reliability of its findings. The extremely limited sample size, coupled with significant technical constraints in both human and mouse studies, raises serious concerns about the generalizability of the results. The lack of functional validation for key molecular findings, absence of protein-level confirmation, and heavy reliance on correlative observations without clear mechanistic insights significantly weakens the study's conclusions. The methodological gaps in controlling for post-mortem artifacts, limited temporal coverage, and insufficient cross-species validation suggest that many of the reported molecular signatures may reflect technical artifacts or specimen-specific variations rather than true biological phenomena. Furthermore, the absence of thorough documentation and incomplete statistical rigor compromises the reproducibility of these findings. While the study introduces potentially interesting hypotheses about altered lineage commitment and transposable element dysregulation, substantial additional work with larger cohorts, rigorous functional validation, and more comprehensive controls will be necessary before these findings can meaningfully contribute to our understanding of DS neurodevelopment or inform therapeutic strategies. This work ultimately highlights the significant challenges and methodological improvements needed in the field rather than providing definitive insights into DS pathobiology.

**Author Response:** We thank the reviewer for the detailed critique. As previously mentioned, we acknowledge the challenges inherent in working with limited human DS and euploid brain tissue. We have worked within this constraint to generate as much meaningful data as possible from a narrow developmental window. We recognize that there is potential for sample-to-sample variability, and as such, we have taken care to explicitly state this limitation in the revised version of the manuscript and softened the results/conclusions to reflect the discovery-focused nature of our findings. To address concerns regarding robustness and reproducibility of our findings, we implemented a LOSO approach, as mentioned above, for our microenvironment tool. This approach was designed during our revision period to test whether our findings were driven by any individual sample, in both human and mouse samples. Our results indicate that the findings are quite robust across samples. Additionally, our Github repository (https://github.com/annaminyifeng/Molecular-Cartography-of-DS-Brain) contains the custom code for the microenvironment analysis and all relevant codes for the snRNA-seq, Slide-seq, and MERFISH datasets to increase reproducibility of our work. We also recognize the importance of validating molecular signatures at the protein level and have expanded our dataset to include protein-level validations for key findings, including TP53, LINE1-ORF1, and ROBO1. Lastly, while we agree that additional validation in larger cohorts is needed, our

exploratory analysis offers one of the first comprehensive multi-omic views of DS neurodevelopment during a critical and understudied window.

**Reviewer Comment:** Codes should be additionally reviewed by specialists.

**Author Response:** All custom code has been provided via GitHub (https://github.com/annaminyifeng/Molecular-Cartography-of-DS-Brain). To facilitate transparency and reproducibility, we have also included a small test dataset (P0 Ts65Dn corpus callosum) within the GitHub. The code is organized into clearly labelled folders corresponded to each type of analysis performed.

**REVIEWER 2:**

**Reviewer Comment:** Overview and Summary:
Down syndrome (DS) presents the dual challenges of abnormal neurodevelopment, and accelerated age- and Alzheimer's disease-related neurodegeneration. How these aberrant processes interact to result in impaired intellect, hyperactive immune responses, and defects in other critical systems in DS is only partially understood. To define neurodevelopmental abnormalities, and map the molecular landscape of trisomy-related changes during perinatal development with cellular resolution, the set of studies by Feng et al used cross-species, integrated single-nucleus RNAseq, spatial transcriptomics and proteomics approaches, with a focused analysis of the maintenance, differentiation and proliferation of neural precursor cells (NPCs). These studies were largely performed using prenatal human DS and euploid brains, with additional support from data generated using Ts65Dn DS model mice.

Alterations in core cellular processes in DS NPCs
SnRNAseq performed with DS and euploid human brain (second trimester) identified five subtypes of NPCs (outer radial glia, oRG; ventricular radial glia, vRG; cycling progenitors, CP; intermediate progenitors fated to an excitatory lineage, IP ExN; and IP fated to an inhibitory lineage, IP InN). These subpopulations of NPCs were spatially defined to the ventricular zone using slide-seqV2.

Significant down-regulation of genes regulating transcription, translation and proteosomal proteolysis was observed in oRG, IP, and newborn excitatory neurons (NB ExN) from DS brain, including genes encoding DNA and RNA polymerases, ribosomal subunits and translation initiation factors. Components of other core cellular processes, such as tRNA and mRNA maturation factors, were also found down-regulated in DS NPCs. Several genetic components of DNA repair machinery and DNA damage checkpoint control were found to be down-regulated in DS NPCs in the current study, consistent with previous findings that elevated oxidative stress and inefficient DNA repair processes contribute to the manifestation of DS. Consistent with nuclear disruption and senescence, LMNB1 was decreased in DS. Genes regulating maintenance of NPC multipotency were also disrupted in DS brain.

Microenvironmental organization
Spatial transcriptomics with slide-seqV2 was used to examine identity changes in the cellular neighborhood proximal to NPCs. In DS brains, a sparsity of oRG was reported in the vicinity of IP cells in the ventricular zone. Since oRG cells differentiate into IP cells, the finding raises the possibility of a defective expansion of the stem cell niche in DS.

The organization of the microenvironment depends on ligand-receptor signaling during neurodevelopment. To investigate potential alterations in cell-cell communication in DS, the authors inferred incoming and outgoing signaling tone by comparing ligand and cognate receptor expression across cell types. In human DS brain, stronger incoming and outgoing signals from inhibitory neurons in cortex and striatum were inferred, whereas stronger signals from excitatory neurons were inferred in euploid brain. The authors used network analysis to infer that BMP and WNT signaling is increased in several cell types in DS brain, including WNT5B signaling from cortical neurons, and BMP7 from astrocytes, to NPCs. On the other hand, decreased SLIT-ROBO, signaling was inferred in DS NPCs, and decreases in ROBO1, SLIT1, were additionally supported by slide-seqV2. Considering the established role of SLIT-ROBO signaling in neuronal migration in the developing brain, this decrease may contribute to architectural disorganization in DS.

Disrupted lineage progression in DS
By comparing the isoform ratios of spliced and unspliced pools of RNA within progenitor cells, lineage progression and rate of differentiation can be predicted (RNA velocity analysis). The authors used the technique to predict that most cell types in DS brain follow a similar pattern of differentiation to controls, but identify exceptions in NPCs, newborn superficial excitatory neurons, and inhibitory neurons in cortex and striatum. Notably, in DS, NPCs were predicted, based on splicing pattern, to undergo decelerated differentiation, while newborn excitatory neurons were predicted to show early termination of differentiation, and/or entrance into senescence; several driver genes of maturation, migration and synaptogenesis were depleted in these cells in DS, including SLIT-ROBO. Taken together, these data predict a slower differentiation of NPCs, and early terminal differentiation of newborn excitatory neurons, in DS.

Proteomics of the prenatal DS brain
The authors performed proteomics by MS with 4 DS and 3 control brains, age-matched with the transcriptomics experiments (13-19 weeks post-conception). Differential protein expression analysis found that of almost 900 significantly altered proteins, 2/3 are up-regulated, and 1/3 down-regulated in DS versus control. In DS, phagocytosis, proteolysis, inflammation and innate and adaptive immune responses were enriched ontology terms; proteins involved in mRNA processing, synapse assembly and cortical migration were down-regulated, consistent with RNA data presented earlier in the manuscript. Some proteins involved in DNA damage and repair were up-regulated in DS (e.g. ATR, CHEK1, CDK2, MCM3-7, RAD9A/17 and ORC), while others were concurrently downregulated (e.g. p53, RAD51C, HMGA1), suggesting an impaired ability to respond effectively to DNA damage. Neuroimmune dysregulation was supported by proteomics data indicating increased expression of innate and adaptive immune response (including interferon regulatory transcription factor IRF3).

Pronounced de-repression of transposable elements in DS
RNA and protein data from the current study, and others, indicate a down-regulation of chromatin-associated proteins and epigenetic modifiers like methyltransferases. Since such changes have been linked to de-repression of transposable elements, including DNA transposons and retrotransposons, the authors tested a hypothesis that NPCs in DS have aberrantly activated TE expression. Significant enrichment of long interspersed nuclear elements (LINEs) was found in NPCs, newborn and mature excitatory neurons, and vascular cells; this was further evidenced by increased immunostaining for RNA-DNA hybrids, which result from retrotransposon processing and can trigger cellular stress responses.

Mouse: Spatial transcriptomics in the neonatal and mature Ts65Dn forebrain
Coronal sections of P0 and 6 month-old Ts65Dn and euploid mice were processed for

MERFISH (n=3 per genotype). A panel of 500 probes was curated to examine proliferation, differentiation, inflammation and immunity, and findings were in accord with changes described herein for human tissue. Ligand-receptor analyses predicted increased signaling by astrocytes, OPCs and excitatory neurons via CX3CL1 to CX3CR1 receptors on microglia in perinatal DS; this elevation persists at 6 months of age, where increased CCL8-CCR3 pro-inflammatory signaling is predicted. Altered WNT, SHH and NOTCH signaling was inferred by MERFISH in NPCs, and systems previously identified as aberrant in human NPCs (translation and transcription regulation, DNA repair and cell cycle checkpoint regulation, self-renewal), were also disrupted in Ts65Dn mice. At 6 months, trisomic NSCs showed elevated transcription of interferon-related signaling and neuroinflammatory responses involving CCL8-CCR2 and CX3CL1-CX3CR1 signaling axes.

Conclusion:
The paper represents an informative and important contribution to understanding the developing DS brain. As such, will be of interest to investigators in DS and, more generally, to those in neurodevelopment. The key limitation, as acknowledged, is that while a large collection of interesting observations with possible inferences is presented, it is only when the suggested molecular pathways and mechanisms have been further investigated will the salience of findings be defined. This is not a criticism but it would be useful to further expand in the discussion as to suggested priorities for future mechanistic studies. The manuscript would also benefit from consideration of the several considerations/ suggestions, below.

**Author Response:** Thank you for your suggestions. We have significantly revised the Discussion to include future directions based on the hypotheses generated by this Resource.

**Reviewer Comment:** The authors should consider this small change in title for the paper: “Molecular Cartography of the Brain Developing Human Down Syndrome Brain and the Developing and Mature Ts65Dn Mouse Model Brain”.

**Author Response:** Thank you for the suggestion. We respectfully find the recommended title, “Molecular Cartography of the Brain Developing Human Down Syndrome Brain and the Developing and Mature Ts65Dn Mouse Model Brain” to be a bit lengthy. We instead propose the title, “Molecular Cartography of the Human Down Syndrome and Trisomic Mouse Brain”.

**Reviewer Comment:** The findings obtained in the mouse model are broadly consistent with those made in human tissue, and indicate disruptions in core cellular processes like translation, transcription, chromatin dynamics, DNA repair, differentiation, renewal, and migration in NPCs.The authors predicted decelerated differentiation of NPCs, and premature terminal differentiation in newborn excitatory neurons in human DS. Since the mouse model enables fine temporal studies of NPC lineage progression that are implausible in human, these predictions could be tested empirically in the days/weeks following birth. The cross-species validation of general effects provided here is welcome, but leveraging the mouse model to test these predictions about lineage progression, which are hypothesized from RNA velocity analyses, would have raised the impact of this section of the study.

**Author Response:** Thank you for this insight. We have revised the manuscript to include clearer cross species comparisons at the end of the Results section. In particular, we have used published snRNA-seq of the Ts65Dn mouse model to test for the activation of transposable elements in the trisomic mouse brain. No developmental sn(sc)RNA-seq dataset of the

developing Ts65Dn mouse model is currently available, thus limiting our ability to perform RNA velocity from the Ts65Dn mouse.

**Reviewer Comment:** On line 247, the word “assembly” appears twice, and the sentence requires further small adjustment to be complete.

**Author Response:** Thank you. We have revised this line to correct this sentence.

**Reviewer Comment:** The proteomics data from brain lysate partially validates key findings inferred from transcriptomics methods, though lacking single-cell resolution. We appreciate that single-cell proteomics may be out of the scope of the study, but a protein-based, cell-type specific validation (i.e. IHC) of key findings would bolster them, e.g. to confirm or reject predictions about premature terminal differentiation of newborn excitatory neurons with markers of mature synapses, and to validate the predictions about enriched and depleted incoming and outgoing pro-inflammatory signals in glia and neurons.

**Author Response:** Thank you for this suggestion. In the revised manuscript, we have included a new figure (Supplementary Fig. 3a, b) showing the cell type-specific expression of key proteins in the human DS fetal brain that demonstrated differential abundance by proteomics. While this is not the same as single cell proteomics, this adds to the single-cell resolution of the findings. We have also validated several of our findings by immunofluorescence of ROBO1, and LINE1-ORF, and TP53, now in Fig. 2d, Fig.4f, and Supplementary Fig. 2e.

**Reviewer Comment:** Since chromatin organization defects are described (TE activation, up- and down-regulation of DNA damage repair genes/proteins), what is the status of trisomic chromatin-associated factors such as Usp16, Hmgn1 and Dyrk1a in these cells?

**Author Response:** We observed a general upregulation of USP16 and DYRK1A across most cell types in the snRNA-seq analysis. HMGN1 was generally downregulated in DS compared to euploid across most cell types, with the exceptions of OPCs, OLs, and astrocyte progenitors. These genes are now referenced in the Results. To facilitate further exploration of these and other genes of interest, we have developed a dedicated website for easy visualization of data.

**Reviewer Comment:** In the 6 month-old mouse brain, were transposable elements activated by trisomy, as in neonatal brain? By 6 months, are there other cell types in which transposable elements have been activated?

**Author Response:** As stated above, we have now leveraged previously published snRNA-seq of the Ts65Dn mouse model to test for the activation of transposable elements in the trisomic mouse brain. We have added this analysis to a new section at the end of the Results comparing the mouse and human data. We do identify changes in cell type-specific transposable element

expression in the 6mo Ts65Dn brain, but not at all similar to the profound lineage-specific effects in the human prenatal brain.

**Reviewer Comment:** Since TE activation is observed in NPCs, what is the status of cytosolic DNA sensing pathways, e.g. cGAS-STING, in proteomic and transcriptomic datasets? This knowledge may be useful in defining correlates of senescence in this setting.

**Author Response:** Interestingly, we did not observe evidence of cGAS/STING activation, either transcriptionally or by proteomics. We have included this in the Discussion, highlighting potential mechanisms of TE activation and unresolved questions for future study.

**Reviewer Comment:** I was struck by the wide differences in gene expression for HSA21 genes, as depicted in S Figure 1. The authors may want to further address what appears to a quite variable pattern demonstrating increases and decreases across cell types. I note in particular that APP gene expression was rarely increased in neurons, an in most cases decreased, a finding at odds with much earlier data in the brains of mouse models. Perhaps the authors could consult with other sources of data in humans and respond.

**Author Response:** We appreciate the reviewer's comments and have revised the figure legend for Supplementary Figure 1 (now Supplementary Figure 2a) to clarify that the z-score heatmap was used to visualize cell type-specific transcriptional patterns of genes within the DSCR without direct comparison between DS and euploid conditions. This figure reflects the relative gene expression across different cell types, highlighting variability in the expression of chromosome 21 genes across cell types, rather than making direct comparisons between groups. Indeed, APP expression is higher in almost every cell type population in DS compared to euploid.

**Reviewer Comment:** Given the large amount of data, it would help to more frequently reference the source of observations – e.g. when citing the results of proteomic studies, etc. As

an example, on page 11 in two paragraphs we read of changes in a number of proteins. Specifically calling out the locus of these data will help.

**Author Response:** Thank you for this suggestion to improve clarity. We have revised the text, both in the Results and Discussion, to reference the source of observations.

**Reviewer Comment:** In the legend to Figure 1, the panels for neural progenitors and excitatory neurons are misstated.

**Author Response:** Thank you. This has been corrected.

**Reviewer Comment:** On pages 16 and 17, the authors reference S Figure 5, but I have no such figure. I believe they mean to reference S Figure 4 panels h through k.

**Author Response:** Thank you. This has been corrected.

**REVIEWER 3**

**Reviewer Comment:** I co-reviewed this manuscript with one of the reviewers who provided the listed reports. This is part of the Nature Communications initiative to facilitate training in peer review and to provide appropriate recognition for Early Career Researchers who co-review manuscripts.

**Author Response:** Thank you.

**Reviewer Comment:** I co-reviewed this manuscript with one of the reviewers who provided the listed reports. This is part of the Nature Communications initiative to facilitate training in peer review and to provide appropriate recognition for Early Career Researchers who co-review manuscripts.

**Author Response:** Thank you.

**REVIEWER COMMENTS**

**Reviewer #1 (Remarks to the Author):**
**Reviewer Comment:** The study has been significantly enhanced through a series of comprehensive revisions, which have significantly strengthened the study through additional validations (e.g., TP53, ROBO1, LINE1-ORF1 immunofluorescence), enhanced analyses (e.g., LOSO, SenMayo GSEA, SoloTE for transposable elements), and improved clarity. The publicly accessible data (GEO: GSE280175, GSE280170, GSE280177; MassIVE: MSV000096108) and code (GitHub: https://github.com/KalishLab/Molecular-Cartography-of-DS-Brain) further enhance its value as a resource for Down syndrome (DS) research. However, to ensure the robustness and interpretability of key findings, I have a few follow-up questions addressing unresolved or partially addressed points from my original comments. These questions aim to refine the manuscript and maximize its scientific impact.

Senescence Conclusions (Comment 2, Results p. 10, Fig. 2i, Supplementary Table 2): The addition of the SenMayo gene set analysis (Fig. 2i) and LMNB1 immunofluorescence validation (Supplementary Fig. 2f, g) provides valuable evidence for a senescence-like state in DS neural progenitor cells (NPCs), and the softened conclusions are appreciated. However, the original comment noted that Figure 3b does not show the expression of other canonical senescence genes (e.g., CDKN2A, CDKN1A), which are critical for robustly supporting senescence claims given its complex phenotype. Could you provide the expression data for CDKN2A, CDKN1A, or other senescence markers in NPCs (e.g., oRG, vRG, IP) from Supplementary Table 2, including log2 fold change and FDR values? If these genes were not detected or analyzed, please explain why (e.g., low expression levels, technical limitations) and discuss how this impacts the senescence interpretation.

**Author Response:**
We appreciate the reviewer's thoughtful feedback and the opportunity to clarify our interpretation regarding senescence in DS NPCs. We have updated our differential gene expression (DGE) to include biological replicates as a covariate to address potential inter-sample variability and strengthen our DGE analysis. This adjustment improves the sensitivity to condition-specific transcriptional changes. Differentially expressed genes (DEGs) were identified using a threshold of $|\log_2 FC| > 0$ and FDR ≤ 0.05. As noted by the reviewer, canonical senescence markers such as *CDKN2A* and *CKDKN1A* are not reported in Supplemental Table 2. This omission reflects their low expression levels, which fell below the threshold set by our MAST DGE analysis pipeline. Specifically, our analysis set a min.pct parameter of 0.05, requiring that genes be expressed in >5% of cells within a cluster to be included in the DE output. Consequently, low-abundance transcripts, including certain cyclin-dependent kinase inhibitors, were not detected. However, raw expression values for these genes are publicly available via our interactive portal.

We acknowledge the absence of robust *CDKN2A* and *CDKN1A* signal limits the strength of a definitive senescence phenotype. Importantly, our data reveal downregulation of key chromatin regulators implicated in senescence, including histone deacetylases (*HDAC1/2*), polycomb-group proteins (*EZH2*, *EED*), and chromatin modifiers such as *KAT2A*, *AURKB*, and *VRK1*. These findings align with the results published by Meharena et al. (PMID 34995493).
We interpret these observations not as evidence of complete senescence but rather as indicative of a primed or transitional state. We have modified the Results and Discussion accordingly to clarify our interpretation of the data. Specifically, we focus on the chromatin regulatory changes in NPCs and make reference to the alignment with Meherena et al., without making overt claims about senescence. We propose that features observed here represent an

early-stage program, the full manifestation of which may depend on additional temporal and cellular contexts. We also emphasize the need for longitudinal sampling across neurodevelopmental and neurodegenerative timepoints to delineate the onset and trajectory of senescence in DS. We believe the changes in the Results and Discussion now accurately reflect our data, without over-stating any claims regarding senescence.

Key chromatin regulators:

| avg_log2FC | p_val_adj | gene | celltype | FDR | sig | p_val |
|---|---|---|---|---|---|---|
| -0.14726 | 1 | HDAC1 | Cycling_progenitors | 0.003147 | TRUE | 0.002547 |
| -0.52281 | 1.16E-05 | HDAC1 | IP | 4.98E-10 | TRUE | 3.17E-10 |
| 0.082578 | 2.82E-27 | HDAC1 | oRG | 1.73E-31 | TRUE | 7.70E-32 |
| -0.10187 | 1 | HDAC1 | vRG | 0.000546 | TRUE | 0.000303 |
| -0.20959 | 0.000156 | HDAC2 | Cycling_progenitors | 1.81E-08 | TRUE | 4.26E-09 |
| -0.18838 | 1.18E-26 | HDAC2 | IP | 3.05E-30 | TRUE | 3.24E-31 |
| -0.22986 | 3.89E-44 | HDAC2 | oRG | 6.36E-48 | TRUE | 1.06E-48 |
| -0.11535 | 0.181677 | HDAC2 | vRG | 1.53E-05 | TRUE | 4.96E-06 |
| -0.1271 | 0.009842 | EZH2 | Cycling_progenitors | 7.45E-07 | TRUE | 2.69E-07 |
| -0.05816 | 2.19E-16 | EZH2 | IP | 2.81E-20 | TRUE | 5.97E-21 |
| -0.14955 | 2.53E-45 | EZH2 | oRG | 4.49E-49 | TRUE | 6.91E-50 |
| 0.027133 | 0.001344 | EZH2 | vRG | 2.27E-07 | TRUE | 3.67E-08 |
| -0.47061 | 1 | EED | Cycling_progenitors | 0.000854 | TRUE | 0.00063 |
| -0.09649 | 9.32E-10 | EED | IP | 6.17E-14 | TRUE | 2.55E-14 |
| -0.46538 | 2.90E-11 | EED | oRG | 9.69E-16 | TRUE | 7.93E-16 |
| -0.20758 | 1 | EED | vRG | 0.000726 | TRUE | 0.000419 |
| -0.25473 | 0.03376 | AURKB | Cycling_progenitors | 2.25E-06 | TRUE | 9.22E-07 |
| -0.46988 | 0.403994 | AURKB | IP | 1.23E-05 | TRUE | 1.10E-05 |
| -0.58395 | 0.000706 | AURKB | oRG | 2.02E-08 | TRUE | 1.93E-08 |
| -0.19517 | 0.196507 | VRK1 | Cycling_progenitors | 1.10E-05 | TRUE | 5.37E-06 |
| -0.51066 | 5.04E-08 | VRK1 | IP | 2.76E-12 | TRUE | 1.38E-12 |
| -0.33621 | 5.69E-18 | VRK1 | oRG | 2.37E-22 | TRUE | 1.55E-22 |
| -0.59352 | 0.019804 | VRK1 | vRG | 2.29E-06 | TRUE | 5.41E-07 |

**Reviewer Comment:** Transposable Element De-repression Mechanism (Comment 6, Discussion p. 29-30, Supplementary Tables 2, 5, 6): The LINE1-ORF1 validation (Fig. 4f, g) and discussion of potential mechanisms (e.g., chromatin accessibility changes, VPS37A downregulation) strengthen the transposable element (TE) de-repression findings. However, the mechanism by which Hsa21 triplication drives TE de-repression remains general, particularly regarding which Hsa21 genes disrupt epigenetic modifications. Supplementary Table 2 shows altered expression of Hsa21 genes like DYRK1A and HMGN1 in DS NPCs, but their role in TE regulation is not discussed. Could you specify which Hsa21 genes (e.g., DYRK1A, HMGN1) might regulate epigenetic modifications (e.g., DNA methylation, histone acetylation) to drive LINE1 de-repression, and provide preliminary data (e.g., correlation analysis between DYRK1A/HMGN1 and LINE1 expression from Supplementary Tables 2 and 6)? If such data are unavailable, please propose a more specific testable hypothesis in the Discussion to guide future studies.

**Author Response:**

Thank you for this suggestion. We have expanded the Discussion to propose the hypothesis that overexpression of specific Hsa21 epigenetic regulators, such as *N6AMT1*, *DNMT3L*, and

*MIS18A*, may disrupt chromatin states, leading to aberrant TE activation in DS. This is based on prior literature on the role of these molecules in regulating heterochromatin and/or TEs. We are hesitant to provide more specific claims about singular candidates (i.e. via correlative analysis), as specific gain/loss of function experiments would be necessary to convincingly link a set of Hsa21 candidates to TE activation. Further, these molecules may act in concert or have cascading effects that cannot be appreciated by a single transcriptional snapshot.

**Reviewer Comment:** Functional Impact of Gene Expression Changes in Ts65Dn Mice (Comment 9, Results p. 24-25, Discussion p. 29, Supplementary Table 8): Re-framing the 6-month Ts65Dn timepoint as a “mature” rather than “aging” brain clarifies the study’s scope, and the detailed differential gene expression (DEG) data (Fig. 6a-c, Supplementary Table 8) provide insights into dysregulated pathways (e.g., WNT, SHH, NOTCH). However, the original comment requested more description of how these DEGs affect cell functon(e.g., NPC proliferation, migration, gliogenesis) and brain functon(e.g., cortical development, cognition). The Discussion briefly mentions reduced NSC proliferation (citing Chakrabarti et al., 2007), but does not elaborate on how DEGs like Mki67, Dcx (downregulated in P0 NPCs), or Gfap, Aqp4 (upregulated at 6 months) contribute to DS phenotypes. Could you expand on the potential functional impacts of these DEGs on NPC behavior and brain development, supported by relevant Ts65Dn phenotype studies (e.g., Chakrabarti et al., 2007; Stagni et al., 2022)? If this is beyond the current scope, please discuss the potential links between these DEGs and DS neurodevelopmental deficits in the Discussion.

**Author Response:**
We appreciate the reviewer’s insightful comment regarding the need for more discussion on how differentially expressed genes (DEGs) may affect NPC and brain function in Ts65Dn mice. In response, we have expanded the Discussion section to address these points more directly. Specifically, we now elaborate on how downregulation of *Mki67* and *Dcx* in P0 NPCs may reflect impaired NPC proliferation and migration—core processes implicated in early brain development deficits in DS. At the 6-month time point, we highlight how upregulation of astrocytic genes such as *Gfap* and *Aqp4* is consistent with an emerging reactive glial phenotype. These transcriptional changes parallel previously reported phenotypes of astrocyte proliferation and hypertrophy in the Ts65Dn hippocampus at later stages (e.g., Stagni et al., 2022), supporting the model of progressive astrogliosis and potential neuroinflammation in DS.

**Reviewer Comment:** Control of Inter-sample Variability and Post-mortem Artifacts (Summary Comment, Methods p. 33-34, Discussion p. 30, Supplementary Table 1): The LOSO analysis, stringent quality control (e.g., mitochondrial gene filtering, SCTransform normalization, Harmony batch correction), and data/code availability significantly enhance result robustness. However, the original comment raised concerns about post-mortem artifacts (e.g., RNA degradation, cell state alterations) in human prenatal brain samples, which could affect gene expression and TE analyses. The Discussion acknowledges potential inconsistencies in post-mortem tissue sectioning but does not detail specific controls for post-mortem intervals (PMI) or RNA integrity. Supplementary Table 1 lacks PMI or preservation condition details. Could you clarify the measures taken to control post-mortem artifacts (e.g., PMI range, RNA integrity number, or storage conditions)? Additionally, while the GitHub code is accessible, has it been reviewed by external specialists to ensure reproducibility, or do you plan to facilitate such a review (e.g., via bioRxiv community feedback)? These clarifications would strengthen confidence in the dataset’s reliability.

Addressing these points will further refine the manuscript and solidify its contribution to

understanding DS neurodevelopment.

**Author Response:**
Thank you for the suggestions. We have expanded our Methods to clarify the measures taken to control post-mortem artifacts, which includes the postmortem interval and storage conditions. To ensure reproducibility of our microenvironment code, we have also uploaded the microenvironment code to Code Ocean, which enables executable, version-controlled environments. Code Ocean is the custom code service supported by Nature Communications. Our capsule has passed peer-review and will be published alongside the manuscript upon acceptance. This code reproduces our results from Fig 6d.

**Reviewer #2 (Remarks to the Author):**

I found the revisions satisfactory. As stated before, I believe it makes a significant contribution to research in DS. I would suggest fixing a few minor typographical errors:

**Reviewer Comment:** Line 84: modify “single nucleus” to “single-nucleus”

**Author Response:** Thank you, this has been corrected.

**Reviewer Comment:** Lines 55-56: modify “high resolution” to “high-resolution”

**Author Response:** Thank you, this has been corrected.

**Reviewer Comment:** Lines 995-995: modify “an Vanquish” to “a Vanquish”

**Author Response:** Thank you, this has been corrected.

**Reviewer Comment:** Lines 578-579: modify “reveals” to “reveal” to reflect that the subject “dysregulation” is singular

**Author Response:** Thank you, this has been corrected.

**Reviewer Comment:** Reviewer #3 (Remarks to the Author):

I co-reviewed this manuscript with one of the reviewers who provided the listed reports. This is part of the Nature Communications initiative to facilitate training in peer review and to provide appropriate recognition for Early Career Researchers who co-review manuscripts.

**Author Response:** Thank you for the suggestions.