Peer Review File

Manuscript Title: Cell-type-resolved mosaicism reveals clonal dynamics of the human forebrain

Reviewer Comments & Author Rebuttals

Reviewer Reports on the Initial Version:

Referees' comments:

Referee #1 (Remarks to the Author):

In the manuscript entitled "Cell-type-resolved somatic mosaicism reveals clonal dynamics of the human forebrain", Chung, Yang, and colleagues conducted heroic work tracking human brain development using somatic mutations as cellular barcodes. The authors collected tissues of the brain and other organs from warm autopsies to study the development pattern of the human brain. For the study, two individuals were investigated, which may need to be more so as to generalize their findings. Using deep whole-genome sequencing followed by validation sequencing, the authors identified mosaic variants (MVs), which were then utilized as cellular barcodes. Single-genome sequencings were also integrated for more profound resolution. Finally, by combining information from genome sequencing and anatomical locations, the authors reconstructed overall patterns of human brain development and reached a few conclusions. It is one of the most profound studies that dissected human brain development using somatic mutations. The study was well conducted, the manuscript reads well, and I believe the topic, in general, will interest the readership of Nature. Below are my comments, which may be constructive for conclusions, helpful to make the manuscript more meaningful, and provide more useful information for future studies.

(1) The authors identified overall ~1,100 MVs from ID01 (n=287 from 35 tissues; 321 total samples) and and ID05 (b=780 from 32 tissues; 147 total samples). ID05 showed ~2.7 times more MVs, although fewer tissues were explored. Is there any good exploration for the variable MV burden?

(2) Full information about the MVs should be shown (i.e., in a Supplementary Table), because the information is valuable as is (for future studies), and also is necessary for validating their findings and conclusions by other groups. Important information includes their genomic positions, wt and variant alleles, variant allele fractions (with wt and mutant allele counts) in each tissue's WGS, amplicon sequencing, and single-genome sequencing.

(3) What are the mutational spectrums (for both SNVs and indels) for all MVs, brain-specific mutations? This information will be essential to understand the "precision" of their mutation calls.

(4) I don't know whether it is possible to answer, but I am wondering about the sensitivity of their approach to the detection of MVs. Extended Data Figs 3c and 3d show that VAFs from WGS are often overestimated for MVs with lower VAFs, presumably due to the detection bias. A deeper analysis using

the data may provide some clues.

(5) The study revealed a few interesting findings, including more lineage-restricted development of hippocampal excitatory neurons, dorsal neocortical origin for a subgroup of DLX1+ inhibitory neurons, and the sequence of L-R, A-P, and D-V axes formation in neurodevelopment. However, these conclusions are primarily qualitative, mainly from clustering the sqrt-t(AF) of many MVs in bulk tissues. Although a dominant fraction of their analyses were conducted from bulk-tissue sequencing (WGS and amplicons) lacking single-cell resolution, because the authors conducted single-genome sequencing as well, I believe that reconstruction of the absolute developmental phylogenies is possible, as shown in Park et al., Nature 2021 and Coorens et al., Nature 2021. The approach will enable to understand the order of acquired embryonic mutations in each lineage, and using the mutation burden as a molecular clock, the authors could reveal the timing of cell fate decision and/or cell migration (for example, when HIP excitatory neurons were branched from other cell types; when L-R axis (or A-P, D-V axes) was determined).

(6) The authors used "brain-specific" variants for lineage tracing. What if we use earlier mutations that were shared by multiple organs? This information may provide clonal dynamics in earlier developmental timing.

(7) Performance of PTA single-genome sequencing. Can it determine the mutational burden in single-nucleus from the PTA data? What is the recall and precision of PTA single-genome sequencing? Mutational signature? Does PTA single-genome sequencing can rescue MVs missed in the bulk tissue sequencing approaches?

(8) How many mosaic mutations do they share for two independent neurons? Does the PTA of single neurons answer the question? These will provide important information about the timing of branching (their most recent common ancestral cell).

(9) Discussion: The study was conducted from the tissues of two donors. The authors should discuss that observation from more individuals will be necessary to generalize their conclusion.

(10) Fig 3g. Are there any other mutations that were shared by DLX1+ nuclei in both prefrontal lobes similar to 2-71776656-C-A? Can the authors narrow down the acquisition timing of these mutations?

Referee #2 (Remarks to the Author):

Chung et al. performs a comprehensive retrospective lineage tracing from two individuals utilizing bulk

deep sequencing and different modalities of single-nuclei sequencing and a joint single nuclei WGS + RNA-seq approach. Tissue biopsies from different brain regions including cortex, hippocampus, basal ganglia, thalamus, cerebellum as well as peripheral organs as an outgroup serves to identify brain mosaic variants.

Using this methodology of single cell and deep sequencing they were able to identify brain region specific mosaic variants which they dub cell-type specific mosaic variant barcode analysis (cMVBA). The enrichment of nuclei based on antibody sorting of DLX1 for cortical inhibitory, TBR1 for cortical excitatory, and COUPTFII for CGE derived neurons were critical in enriching rare populations. These sorts were validated with snRNA-seq.

Main results

From the two individuals they identified 287 and 780 mosaic variants and of these only 85 and 295 were brain specific respectively.

Cortical TBR1+ nuclei share clonality based on (40/43) MVs to basal ganglia DLX1+ compared to hippocampal TBR1+ nuclei (5/1) MVs. Likely points towards local generation of human hippocampal cells during development as seen in Zhong et al (2020).

There are DLX1+/TBR1+ but COUPTFII negative which is consistent with recent findings of progenitors in the cortex giving rise to both lineages from Delgado et al. (2021).

COUPTFII clones are more widely distributed relative to DLX1+/TBR1+ clones.

Joint scRNA+scWGS implies local excitatory lineages and no observation of areal specificity of cortical born inhibitory/excitatory lineages.

Anterior-posterior axis is established before the dorsal ventral-axis based on clonal distribution

Comments

Overall the manuscript is well written and interpretable. This work is very data intensive in terms of the collection and analysis/identification of the MVs. However, the study has limited technical novelty, and the biological insights are largely confirmatory of prior studies. Whether the extent of novelty presented is sufficient to merit publication at Nature is unclear.

The number of MVs is in-line with Bizzotto et al. (2021) SNV number that were observed in brain development and follows a similar bioinformatics pipeline as them as well. For sample ID01 was there a reason a 3 fold reduction/increase relative to ID05 of MVs detected? According to the methods both individuals were females over 70 years old. While there will be variability between individuals this is much more divergence than expected. In Bizzotto et al. (2021) the two individuals profiled in those

studies were 15 and 42 year old females with 219 SNVs and 297 SNVs detected respectively across 250X WGS in peripheral organs as well as snWGS of cortical cells.

It would be helpful if the authors explored the possibility of capturing MVs in snRNA-seq libraries? Specifically, the higher throughput 10x chromium chemistry has previously picked up MVs in the prior studies (Bizzotto et al.). There are pretty straightforward methodologies now to intersect variants with snRNA-seq reads such as vartrix from 10x Genomics, etc.

The snMPAS analysis for MV detection doesn't use SCAN2 (https://www.nature.com/articles/s41588-022-01180-2), which was developed recently to specifically analyze PTA-base data. It would be ideal if further justification could be provided to justify methodological choices.

The overall data presentation could benefit from revised / improved visualization. For example, the current grayscale color gradient featured in allele frequency plots is difficult to appreciate differences. More contrastive color should benefit the data presentation.

Referee #3 (Remarks to the Author):

Summary:
In this paper, Chung et al. extend their prior methodology (introduced in Breuss et al, Nature, 2022) of deep whole-genome sequencing (WGS) followed by deep targeted sequencing and single-cell sequencing to characterize clonal patterns in human brain. The authors profile additional samples from the brain of that prior study plus an additional brain at higher spatial resolutions. Via a new set of analyses, they make the case for 3 biological observations: (1) hippocampus is lineally more distant from cortex and basal ganglia, than cortex and basal ganglia are to each other; (2) there is a dorsal (i.e., cortical) origin for some interneurons, which until recent studies of fetal brain and in vitro cultured progenitor cells, were believed to derive only from the ventral ganglionic eminences; (3) anterior-posterior restriction of the cortex during brain development generally precedes dorsal-ventral restriction. Overall, the paper is expounded clearly, it represents a valiant undertaking with biological findings of interest to many in the fields of somatic mutations and brain development.

However, I am equivocal about publication in Nature due to the following limitations: First, the data supporting the above biological conclusions (2) and (3) is moderate but not as definitive as the authors' findings about left-right axis restriction in their prior paper or their evidence for the above conclusion (1), as detailed in the below specific comments. Second, there is not much technical novelty relative to this group's paper last year in Nature. Third, the most important conclusion of dorsal origins for some interneurons has been indicated by prior fetal brain and in vitro studies, albeit this paper makes the first

direct observation in adult human brain. Fourth, profiling two donors is perhaps sufficient, but not ideal. Understandably, there is an immense cost to profile each individual, and I would not expect profiling of a third brain, but it is important to note that developmental patterns can be highly variable, as evidenced by dramatically different distributions of the lineages from the first cell stages of human development across the early embryo. Therefore, it is possible that the observations in these two brains are a subset of possible clonal patterns.

General comments:
For ID01: the authors should specify in the main text and the methods which samples and data were obtained from the group's prior study (Breuss, et al, Nature, 2022). The methods make it seem like all or nearly all of ID01 is newly profiled.

Identification of brain MVs:
1. It is not clear what quantitative improvement is referred to here: "we further optimized a prior protocol for lower cell number input (100-fold improvement)".
2. The authors should make more clear that the DLX1 and TBR1 nuclei sorting, at least, was developed in the prior Breuss, et al paper.
3. How does the computational pipeline account for the possibility of sequencing or alignment errors that can occur in specific loci, both in the first stage deep WGS and in the second amplicon sequencing stage? This could yield false-positive mutations that seemingly cross-validate between the two methods.
4. It would help to specify more clearly the validation rate between amplicon sequencing and WGS. While the authors likely erred on the side of sensitivity in building the amplicon sequencing panel, providing validation rates would give a measure of the specificity of the WGS pipeline.
5. It would be helpful to specify which tissues were profiled by deep WGS in the text, since subsequently in the section it is stated that about 1/3 of variants were only found in brain. But this fraction is biased by how many of the initial deep WGS samples were from the brain.

Clonal dynamics of cortical inhibitory neurons
1. All the evidence provided for a cortical origin of DLX1+ interneurons depends critically on the purity of the DLX1+ nuclei sorting. However, the authors show in Fig. 1d that DLX1+ neurons contain about 1/4 excitatory neurons, seemingly showing 'contamination' of excitatory neuronal nuclei. While the authors subsequently perform single-cell DNA+RNA sequencing to support their findings, it is not clear to me why this contamination in the bulk sorting does not significantly dampen the strength of all the bulk sequencing data of this section.
2. It is not intuitive why clustering based on allele frequencies of mosaic variants, as opposed to absence/presence of cortical region-specific mosaic variants, is a reliable indicator of lineage origins. Readers would benefit from 1-2 sentences explaining the concept of clustering based on allele frequencies.
3. Can the authors estimate using the bulk (or maybe subsequent single-cell) data what proportion of DLX1+ interneurons are cortical- versus ganglionic eminence-derived?

Dual origins of cortical inhibitory neurons

1. If NeuN+ nuclei were sorted, why were “a few minor non-neuronal cell types” detected “as expected”?
2. This section would benefit from basic statistics on how many single cells were profiled, how many excitatory versus inhibitory, etc.
3. Is it possible that cortical-derived excitatory and inhibitory neurons originate from ventrally-derived progenitor cells that migrate to specific locations and locally give rise to clonally related excitatory and inhibitory neurons? Can the authors’ data support or negate this alternative model (that admittedly is unlikely based on prior literature)?
4. Is the skew towards parvalbumin-positive inhibitory neurons consistent with prior in vitro studies of cortical progenitor-derived interneurons?
5. Did the authors identify any single cells that are inhibitory neurons derived from the ganglionic eminence, based on mosaic variants that these cells have that are entirely distinct from any variants found in cortical-derived neurons? The text states that ganglionic eminence-derived interneurons were identified based on them not having locally-enriched mosaic variants, but this is an exclusion criteria, rather than positively identifying them based on mosaic variants that they specifically carry distinctly from cortical-derived neurons.
6. Per Fig. 4b, was MV 13-69308268-A-G the only mutation detected in both an excitatory and inhibitory interneuron in single-cell profiling? This is important to clarify to assess the strength of the paper’s conclusion regarding a cortical origin for interneurons. While Fig. 4c shows similar allele frequencies in different cortical regions, per above, this could be due to DLX1+ sorting retaining some excitatory neurons in the pool.

Anterior-posterior restriction within a lobe
1. The clustering dendrogram in Fig. 5b seems convincing, but the strength of evidence in Fig. 5d and Figs. 5e-f is not as clear. Specifically, in Fig. 5d, it is difficult to visualize the authors’ observation that allele frequencies (AF) differ more in the AP axis than the DV axis. And in Figs. 5e-f the kernel density estimations on the top and right of each plot do not seem highly distinct. A more direct comparison may be to present an overlap of the delta-DV and delta-AP kernel density estimations.
2. Ext. data Fig. 5 shows UMAP plots based on AFs that show Left versus Right clustering. Can the same be performed for the AP vs DV axis samples?

Discussion:
1. The authors do not distinguish enough between two distinct concepts: lineage restriction that is due to migratory/diffusion barriers and bona-fide lineage commitment in which progenitor cells become epigenetically committed to a specific lineage. In this paper’s retrospective lineage tracing, without directly observing the early progenitors, it is not feasible to distinguish these two phenomena. For example, some early progenitors may relatively restrict to the hippocampus due to developmental anatomic/diffusion barriers without any epigenetic ‘commitment’.
2. The motivation to speculate regarding the specific identity of dorsally-derived interneurons as ‘double bouquet cells’ is not clear. Since the authors have single-cell DNA+RNA sequencing data, could this be assessed directly? If not, why not? And if not, then could the authors feasibly do so via a different method? Without such analyses and attempts, I’m not sure there is added value to hazard a guess as to

the identity of these cells.
3. The paper would benefit from some explanation as to the significance (clinical, physiologic, etc) of their findings that hippocampus is more lineally distinct from cortex and basal ganglia, and that the anterior-posterior restriction precedes dorsal-ventral restriction.

Methods:
1. It is not clear how the 4 different variant callers were integrated. Were variants called by any of the 4 retained, or were only variants called by some subset or all of the callers retained?
2. Bisulfite sequencing of sorted nuclei: “Sorted nuclei DNA on beads” – the meaning of this could be further clarified.

Figures:
1. Fig. 4b: the legend indicates that ‘orange’ is the color for InN1, but that does not match what we see. Perhaps it changed during PDF formatting, or perhaps it is a typo.
2. Figure 4 would benefit from supporting data showing the snRNA-seq analysis and cell type clustering/identification. Since snDNA+RNA-seq is a novel technology, readers will likely want to see supportive evidence regarding its performance. This should also include basic quality metrics (for example, number of genes detected).
3. The schematic in Fig. 4e is confusing due to the numerous colors.
4. Ext data Fig. 2a: were these nuclei also stained with NeuN? The x-axis is labeled ‘488’, but the marker is not specified.

Author Rebuttals to Initial Comments:

Response to Referees for *'Cell-type-resolved somatic mosaicism reveals clonal dynamics of the human forebrain'*

Referee expertise:
Referee #1: somatic evolution
Referee #2: neurodevelopment
Referee #3: genomics, neuroscience

Referee #1 (Remarks to the Author):

In the manuscript entitled "Cell-type-resolved somatic mosaicism reveals clonal dynamics of the human forebrain", Chung, Yang, and colleagues conducted heroic work tracking human brain development using somatic mutations as cellular barcodes. The authors collected tissues of the brain and other organs from warm autopsies to study the development pattern of the human brain. For the study, two individuals were investigated, which may need to be more so as to generalize their findings. Using deep whole-genome sequencing followed by validation sequencing, the authors identified mosaic variants (MVs), which were then utilized as cellular barcodes. Single-genome sequencings were also integrated for more profound resolution. Finally, by combining information from genome sequencing and anatomical locations, the authors reconstructed overall patterns of human brain development and reached a few conclusions. It is one of the most profound studies that dissected human brain development using somatic mutations. The study was well conducted, the manuscript reads well, and I believe the topic, in general, will interest the readership of Nature. Below are my comments, which may be constructive for conclusions, helpful to make the manuscript more meaningful, and provide more useful information for future studies.

R1Q1 The authors identified overall ~1,100 MVs from ID01 (n=287 from 35 tissues; 321 total samples) and ID05 (b=780 from 32 tissues; 147 total samples). ID05 showed ~2.7 times more MVs, although fewer tissues were explored. Is there any good exploration for the variable MV burden?

Response. We appreciate R1 mentioning this question. We think the different sampling strategies in the two cases gave rise to different numbers of MVs. Among 35 tissues of ID01, 10 large lobe samples (see samples annotated as 'LARGE' and '1' in 'Note' and 'WGS performed in Breuss et al' columns, respectively in Supplementary Data 1 ID01) were biopsied in Breuss et al [1], and fixed by formaldehyde. This biopsy method resulted in a lower MV detection rate due to dilution, and a higher false-positive detection rate, which led to many putative MVs dropping out during the validation stage. As our methods evolved, we improved the detection strategy in order to detect more MVs from small-size samples, now labeled as '1' in the 'WGS performed in this study' column of Supplementary Data 1. Even though the methods of MV detection varied slightly between ID01 and ID05, clonal relationships shown were similar, which gives confidence in the conclusions. We now discuss this in the revised manuscript by mentioning, "*Notably, we captured 2.7 times more MVs than ID01 due to the improvement of the employed tissue biopsy*

method."

R1Q2 Full information about the MVs should be shown (i.e., in a Supplementary Table), because the information is valuable as is (for future studies), and also is necessary for validating their findings and conclusions by other groups. Important information includes their genomic positions, wt and variant alleles, variant allele fractions (with wt and mutant allele counts) in each tissue's WGS, amplicon sequencing, and single-genome sequencing.
Response. We apologize for not conveying the complete information about the MVs. In the initial submission, we showed the raw MV information in Supplementary Data 2. This table includes genomic positions, WT and variant alleles, and AFs for both alleles in each tissue from WGS and MPAS. In this revision, we now add a tab showing the information from snMPAS in Supplementary Data 2 as well as other information requested by R1.

R1Q3 What are the mutational spectrums (for both SNVs and indels) for all MVs, brain-specific mutations? This information will be essential to understand the "precision" of their mutation calls.
Response. We appreciate R1's suggestion to check the mutational spectrum. We performed mutational signature analysis using 368 brain-specific SNVs detected in ID01 or ID05. As expected, clock-like mutations such as signatures 1 and 5 were major components, reflecting developmental origins. We now include this data in Extended Data Fig. 4c. Regarding indels, unfortunately, we had only a total of 14 brain-specific indels from the two donors, which was too few to conduct meaningful mutational spectrum analysis. We hope R1 understands this point.

R1Q4 I don't know whether it is possible to answer, but I am wondering about the sensitivity of their approach to the detection of MVs. Extended Data Figs 3c and 3d show that VAFs from WGS are often overestimated for MVs with lower VAFs, presumably due to the detection bias. A deeper analysis using the data may provide some clues.
Response. We agree that AFs from WGS are often overestimated for MVs with lower AFs. It may be because we only took a subset of MV candidates with AF's above the lower binomial confidence intervals (black horizontal error bar) above 0.1% (dashed vertical red line) from WGS (see schematic Fig. R1Q4, and see 'Mosaic SNV/INDEL detection in WGS data' section in Methods) for our follow-up analysis. This means any candidate MVs with lower AF in WGS would not be included in the MPAS quantification. This is the reason why we perform further quantification by MPAS, and use these AFs for inferring clonal relationships. Most of the overrepresented and false-positive MVs from WGS are expected to drop out with the MPAS validation process.

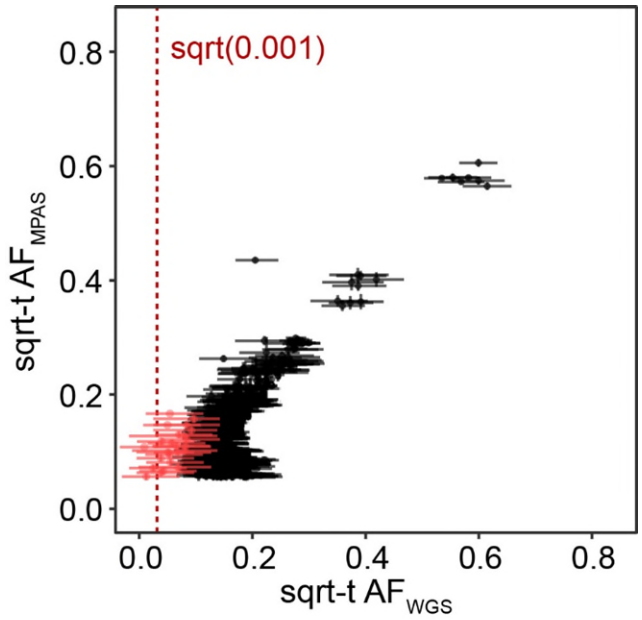

Fig. R1Q4. Schematic correlation plot between sqrt-t AF of individual MVs from WGS and MPAS if MV candidates below the AF threshold were included in the panel design. Dashed red line: AF threshold (lower confidence interval of AF>0.001) for MV calls from WGS. MV calls below the AF threshold were labeled in light red points with exact binomial confidence intervals.

R1Q5 The study revealed a few interesting findings, including more lineage-restricted development of hippocampal excitatory neurons, dorsal neocortical origin for a subgroup of DLX1+ inhibitory neurons, and the sequence of L-R, A-P, and D-V axes formation in neurodevelopment. However, these conclusions are primarily qualitative, mainly from clustering the sqrt-t(AF) of many MVs in bulk tissues. Although a dominant fraction of their analyses were conducted from bulk-tissue sequencing (WGS and amplicons) lacking single-cell resolution, because the authors conducted single-genome sequencing as well, I believe that reconstruction of the absolute developmental phylogenies is possible, as shown in Park et al., Nature 2021 and Coorens et al., Nature 2021. The approach will enable to understand the order of acquired embryonic mutations in each lineage, and using the mutation burden as a molecular clock, the authors could reveal the timing of cell fate decision and/or cell migration (for example, when HIP excitatory neurons were branched from other cell types; when L-R axis (or A-P, D-V axes) was determined).

Response. We appreciate R1's suggestion. Of note, we apologize for the confusion on our single-cell DNA sequencing data in Fig. 4. The data here was generated by a 'targeted amplicon sequencing' followed by PTA amplified DNA from single nuclei called snMPAS, not by single-cell whole genome sequencing. Thus, the sequencing data lacks genome-wide information, which makes it challenging to calculate the molecular clock and estimate the timing of cell fate. Instead, using existing snMPAS data, we made a phylogenic tree by considering alleles on each of the genomic positions as 'pseudo-sequence'. A sequence-based phylogenic tree was then reconstructed to deconvolve the clonal relationship between single cells (Extended Data Fig. 9 and Methods). Based on the bootstrap values reflecting the confidence of the branches, we found 3.5 times more ExN-InN pairs in the same lobe (14 pairs) compared to those in different

lobes (4 pairs) for the closest branches between every 2 samples. This result provides evidence that a significant proportion of human cortical excitatory and inhibitory neurons derive from common cortical progenitor cells. If R1 would prefer, we would be happy to perform single-nucleus WGS, and add this to the manuscript.

R1Q6 The authors used "brain-specific" variants for lineage tracing. What if we use earlier mutations that were shared by multiple organs? This information may provide clonal dynamics in earlier developmental timing.
Response. We appreciate the idea raised by R1. We now investigate AFs of organ-shared variants in two donors, and found evidence of asymmetric clonal branching during early embryonic development, consistent with previous studies [2,3]. We added this observation as Extended Data Fig. 4d-e in the revised manuscript.

R1Q7 Performance of PTA single-genome sequencing. Can it determine the mutational burden in single-nucleus from the PTA data? What is the recall and precision of PTA single-genome sequencing? Mutational signature? Does PTA single-genome sequencing can rescue MVs missed in the bulk tissue sequencing approaches?
R1Q8 How many mosaic mutations do they share for two independent neurons? Does the PTA of single neurons answer the question? These will provide important information about the timing of branching (their most recent common ancestral cell).
Response. We appreciate R1Q7-8 and apologize for the confusion on sequencing method used for PTA-amplified DNA. We think R1 is requesting clarification on WGS from the PTA analysis. To clarify, PTA-analyzed cells were only studied with targeted amplicon sequencing (i.e. snMPAS), which cannot be used to estimate the total mutational burden, mutational signature, rescue rate of MVs missed in bulk tissues, or MRCA. Fortunately, these estimations have already been profiled in the previous PTA publication [4]. Regarding the precision of our PTA data, we were able to estimate the false-negative and false-positive discovery rate of snMPAS, since when we designed our snMPAS panel, we include not only genomic sites for MV candidates but also known homozygous or heterozygous variant sites as true negative or positive controls, respectively (Methods). First, we calculated the chance of failed detection of true-positive variant calls (dropout), using heterozygous variants. Among AF values of the variants known as heterozygous in ID05 bulk and homozygous in control bulk samples, 94.137% (5379/5714) were recovered as heterozygous in snMPAS, showing a false-negative rate of 5.863%. Next, we calculated the chance of false-positive discovery using homozygous genomic sites. The known homozygous genomic positions in bulk samples recovered as homozygous were 98.948% (7149/7225), showing a false-positive rate of 1.052%. We included these rates in the main text and 'Data analysis for MPAS and snMPAS' section of the Methods in the revised manuscript.

R1Q9 The study was conducted from the tissues of two donors. The authors should discuss that observation from more individuals will be necessary to generalize their conclusion.
Response. We now mention that more individuals would be necessary to generalize our conclusions, "*Future studies with additional donors could better generalize our main findings*". We respectfully remind R1 that although clonal divergence can vary [2,5] overall clonal

relationships are likely consistent between subjects. We observed similar clonal relationship patterns from four hemispheres from two individuals, and the bootstrap is statistically consistent that this pattern is unlikely by chance. From our previous experience, the result of MVBA was very consistent across individuals [1]. Moreover, another independent group recently uploaded a manuscript in bioRxiv with similar conclusion on the dual origins of cortical inhibitory neurons using two brains using distinct genetic approaches [6].

R1Q10 Fig 3g. Are there any other mutations that were shared by DLX1+ nuclei in both prefrontal lobes similar to 2-71776656-C-A? Can the authors narrow down the acquisition timing of these mutations?

Response. We appreciate R1's questions. The MV 2−71776656−C−A was a strong example, and there are many other MVs shared between left and right PF DLX1+ nuclear pools, shown in Supplementary Data 6 (11−97326785−G−A, 13−113887705−CCAG−C, 18−56480516−G−A, 4−83599250−T−C, 6−121420086−C−T, 6−125153224−A−C, 6−125153225−G−T, X−54739596−C−G). To answer the second part of the question, six of the MVs (2−71776656−C−A, 4−83599250−T−C, 6−121420086−C−T, 6−125153224−A−C, 6−125153225−G−T, X−54739596−C−G) were present in both neural and non-neural tissues, suggesting they arose during early embryogenesis. The remaining three MVs (11−97326785−G−A, 13−113887705−CCAG−C, 18−56480516−G−A) were restricted to the brain, suggesting later timings of acquisition (Table R1Q10, Supplementary Data 3), although as R1 knows, the precise timing of MV acquisition is not easily determined in human cadavers. If R1 requests, we would be happy to incorporate this supplemental data in the manuscript. However, we avoided emphasizing these MVs or inferring timing of origin since PF samples were only available in ID01.

ID	11-97326785-G-A	13-113887705-CCAG-C	18-56480516-G-A	2-71776656-C-A	4-83599250-T-C	6-121420086-C-T	6-125153224-A-C	6-125153225-G-T	X-54739596-C-G
7614-L-PF-DLX1	0.51	0.65	2.68	1.40	2.33	11.89	9.40	9.54	13.08
7614-R-PF-DLX1	0.39	0.26	3.31	1.23	1.73	12.16	6.43	6.25	6.32
7614-Liver	0.05	0.00	0.06	0.06	0.28	0.41	0.29	0.28	0.16
7614-heart	0.12	0.01	0.06	0.12	0.17	0.60	0.44	0.51	0.18
7614-Kidney-L	0.06	0.00	0.08	0.10	0.37	0.36	0.19	0.24	0.23
7614-Kidney-R	0.11	0.00	0.13	0.28	0.26	0.43	0.36	0.39	0.24

Table R1Q10. The AFs (%) of 9 MVs enriched in DLX1+ nuclei of both prefrontal lobes. AFs with lower confidence intervals above the cut-off (0.001677998) are labeled red. AFs below the cut-off are labeled in black and are considered negative.

Referee #2 (Remarks to the Author):

Chung et al. performs a comprehensive retrospective lineage tracing from two individuals utilizing bulk deep sequencing and different modalities of single-nuclei sequencing and a joint single nuclei WGS + RNA-seq approach. Tissue biopsies from different brain regions including cortex, hippocampus, basal ganglia, thalamus, cerebellum as well as peripheral organs as an outgroup serves to identify brain mosaic variants.

Using this methodology of single cell and deep sequencing they were able to identify brain region specific mosaic variants which they dub cell-type specific mosaic variant barcode analysis (cMVBA). The enrichment of nuclei based on antibody sorting of DLX1 for cortical inhibitory, TBR1 for cortical excitatory, and COUPTFII for CGE derived neurons were critical in enriching rare populations. These sorts were validated with snRNA-seq.

Main results

From the two individuals they identified 287 and 780 mosaic variants and of these only 85 and 295 were brain specific respectively.

Cortical TBR1+ nuclei share clonality based on (40/43) MVs to basal ganglia DLX1+ compared to hippocampal TBR1+ nuclei (5/1) MVs. Likely points towards local generation of human hippocampal cells during development as seen in Zhong et al (2020).
Response. We appreciate R2's kind summary. We want to emphasize the advances in our paper compared with Zhong et al. Our study examines clonal relationships between brain structures using MVs that faithfully reflect in clonality. In contrast, Zhong et al. used pseudo-time analysis based on transcriptomic or epigenomic signatures to estimate developmental processes, which cannot provide direct evidence of clonality. We now added this point in "*Previous studies relied on the …… among human forebrain cell types based on clonality*" in the Discussion of the revised manuscript. More importantly, Zhong et al. did not study clonal relationships between neuronal cell types in basal ganglia or cortex, which are major points of our paper. Lastly, Zhong et al. used samples at fetal stages, where lineages are not yet entirely determined, and thus might not reflect final cell fates. Our study, on the other hand, associated adult brain structures and cell types with embryonic development.

There are DLX1+/TBR1+ but COUPTFII negative which is consistent with recent findings of progenitors in the cortex giving rise to both lineages from Delgado et al. (2021).
Response. We agree there are some consensuses with Delgado et al, but also, we would like to emphasize the difference that Delgado et al used 'fetal' human brain tissue in VZ to differentiate progenitors into cortical cell types, showing dorsal telencephalic progenitor cells have 'potential' to generate inhibitory neurons prospectively. However, they did not demonstrate the cells differentiate into inhibitory neurons and reside in the cortical areas in adult human brains. These questions are addressed in our study in mature neurotypical human brains using MVBA.

COUPTFII clones are more widely distributed relative to DLX1+/TBR1+ clones.

Response. We appreciate R2's summary for COUPTFII+ clones. This finding also demonstrates our novelty distinct from Delgado et al. They suggested that inhibitory neurons derived from the dorsal telencephalon express NR2F2 (encoding COUPTFII) in scRNA-seq, while our COUPTFII+ clones disperse widely across the cortical lobes compared to TBR1+ excitatory neuronal clones residing more locally, suggesting the majority are derived from ventral telencephalon that tangentially migrate. Rather we observed DLX1+ and NR2F2 depleted neuronal clones (InN2) sharing local MVs with ExNs in the same lobe, implying dorsal telencephalic origin. This difference is described at the end of the Discussion in the initial manuscript.

Joint scRNA+scWGS implies local excitatory lineages and no observation of areal specificity of cortical born inhibitory/excitatory lineages. Anterior-posterior axis is established before the dorsal ventral-axis based on clonal distribution.
Response. We apologize that the information on snMPAS was not clear. We did not perform single-nucleus WGS, but conducted panel sequencing (snMPAS) with PTA amplified DNA, genotyping putative MV sites in each single cell. The method we used is described as, "*We thus conducted single-cell simultaneous DNA + RNA sequencing (ResolveOME, see Methods), incorporating primary template-directed amplification (PTA) coupled with single-nuclear MPAS (snMPAS) with full-transcript snRNA-seq, in individual NEUN+ nuclei from right frontal and temporal cortex in ID05*" in the initial manuscript. Since we think the words 'whole genome amplification (WGA)' in Fig. 4 may lead readers to conclude that we performed single-cell whole genome sequencing, we replaced it with PTA and clarified our method in Fig. 4.
Regarding areal specificity of cortically born InN/ExN lineages, we observed many InNs and ExNs containing MVs exclusively in the frontal lobe (labeled as F-only) or temporal lobe (T-only) in Fig. 4b, indicating areal specificity of cortically born inhibitory and excitatory neurons. We added *"We observed numerous excitatory and inhibitory neurons in the same lobe carrying MVs exclusively in the frontal lobe (F-only) or temporal lobe (T-only).*" in the main text.

Comments

R2Q1 Overall the manuscript is well written and interpretable. This work is very data intensive in terms of the collection and analysis/identification of the MVs. However, the study has limited technical novelty, and the biological insights are largely confirmatory of prior studies. Whether the extent of novelty presented is sufficient to merit publication at Nature is unclear.
Response. We thank R2 for comments on our manuscript's clarity. We also thank R2 for the comments on technical novelty. However, from our point of view, we developed and used several novel techniques that made this study possible. These techniques include: 1] methanol-fixed nuclei sorting (MFNS) which enabled high-quality materials for low-abundance cell types. 2] inclusion of deep-learning-based mosaic variant calling software. 3] single-cell DNA+RNA simultaneous sequencing (ResolveOME), which enables large-scale cell-typing and genotyping from the same cell. As mentioned in responses to R1, we now emphasize these technical novelties in the discussion: "*This study also incorporates several technical advances…… patient-archived tissues at the single-cell level*"

We further thank R2's comments on biological insights. As we have emphasized in the revised manuscript, we believe our data provides the first direct evidence for several of our major conclusions and holds significant novelty in meeting the interests of *Nature* readers in various aspects. We responded to this in the above paragraph under each of R2's summaries.

R2Q2 The number of MVs is in-line with Bizzotto et al. (2021) SNV number that were observed in brain development and follows a similar bioinformatics pipeline as them as well. For sample ID01 was there a reason a 3 fold reduction/increase relative to ID05 of MVs detected? According to the methods both individuals were females over 70 years old. While there will be variability between individuals this is much more divergence than expected. In Bizzotto et al. (2021) the two individuals profiled in those studies were 15 and 42 year old females with 219 SNVs and 297 SNVs detected respectively across 250X WGS in peripheral organs as well as snWGS of cortical cells.

Response. We appreciate R2's comments on the variability of the number of MVs. As asked by R1Q1 above, this variability is likely due to the difference in tissue collection strategy between two individuals. Among 35 tissues of ID01, 10 large lobe samples were biopsied in Breuss et al [1] and fixed by formaldehyde. This biopsy method resulted in a lower MV detection rate due to dilution, and a higher false-positive rate, which led to many putative variations dropping out during the validation stage. We only used small (~500 mg) and more spatially diverse biopsied samples in ID05 for WGS to increase the number of MVs useful for lineage relationship reconstruction. DNA samples participating in WGS in the MV discovery phase are now labeled in the 'WGS performed in this study' column of Supplementary Data 1. Despite the variability of the number of MVs, the overall lineage relationship patterns we present are consistent across individuals, making our conclusions convincing.

R2Q3 It would be helpful if the authors explored the possibility of capturing MVs in snRNA-seq libraries? Specifically, the higher throughput 10x chromium chemistry has previously picked up MVs in the prior studies (Bizzotto et al.). There are pretty straightforward methodologies now to intersect variants with snRNA-seq reads such as vartrix from 10x Genomics, etc.

Response. We appreciate R2's suggestion. To implement 10X genomics snRNA-seq in our study, the MVs we detected should be near the 3' end due to the poly-A capture bias. Unfortunately, we were not able to find any MV positioned within 500 bp upstream from the end of 3'UTR. Also, even if we could capture some MVs by snRNA-seq, the total depth for the MVs will be very low (1~4X) according to previous studies, so we expect many would be false positives. Finally, since a recent preprint [6] after our submission used snRNA-seq to show cortical inhibitory neurons sharing variants with excitatory neurons, we believe this complementary experimental design could address R2's concern.

R2Q4 The snMPAS analysis for MV detection doesn't use SCAN2 (https://www.nature.com/articles/s41588-022-01180-2), which was developed recently to specifically analyze PTA-base data. It would be ideal if further justification could be provided to justify methodological choices.

Response. We apologize that it was unclear how the snMPAS experiment was performed. As we mentioned above (see R1Q5), snMPAS was not a single-nucleus WGS, but rather panel

sequencing to genotype PTA-amplified genomic DNA, targeting genomic regions of interest. This strategy enabled focused validation of single-cell genotypes with significantly improved accuracy compared with single-cell WGS due to the higher read depth (5000 vs 300x). Thus, de novo MV discovery using single-cell variant callers like SCAN2 does not apply to snMPAS data.

R2Q5 The overall data presentation could benefit from revised / improved visualization. For example, the current grayscale color gradient featured in allele frequency plots is difficult to appreciate differences. More contrastive color should benefit the data presentation.
Response. We appreciate R2's suggestion. We now improved the contrasts of the heatmaps in Fig. 2,3,5, and Extended Data Fig. 6 to show AF differences more clearly. We kept the monochromatic scale because AFs had only positive values.

Referee #3 (Remarks to the Author):

Summary:
In this paper, Chung et al. extend their prior methodology (introduced in Breuss et al, Nature, 2022) of deep whole-genome sequencing (WGS) followed by deep targeted sequencing and single-cell sequencing to characterize clonal patterns in human brain. The authors profile additional samples from the brain of that prior study plus an additional brain at higher spatial resolutions. Via a new set of analyses, they make the case for 3 biological observations: (1) hippocampus is lineally more distant from cortex and basal ganglia, than cortex and basal ganglia are to each other; (2) there is a dorsal (i.e., cortical) origin for some interneurons, which until recent studies of fetal brain and in vitro cultured progenitor cells, were believed to derive only from the ventral ganglionic eminences; (3) anterior-posterior restriction of the cortex during brain development generally precedes dorsal-ventral restriction. Overall, the paper is expounded clearly, it represents a valiant undertaking with biological findings of interest to many in the fields of somatic mutations and brain development.

However, I am equivocal about publication in Nature due to the following limitations: First, the data supporting the above biological conclusions (2) and (3) is moderate but not as definitive as the authors' findings about left-right axis restriction in their prior paper or their evidence for the above conclusion (1), as detailed in the below specific comments.
Response. We appreciate R3's comments on the definitiveness of conclusions 2 and 3. R3 probably thought conclusions (2) and (3) were not as definitive as the previous finding about L-R restriction due to the possibility of impurity of excitatory neurons in isolated DLX1+ nuclear pools (see below). We now include additional data demonstrating robustness of these analyses by deconvolution and simulations of AF distributions (now Extended Data Fig. 7c-f) to address the R3's concern.

Second, there is not much technical novelty relative to this group's paper last year in Nature.
Response. We thank R3's comments on technical novelty. However, we emphasize several novel techniques developed for this study (ResolveOME, MFNS, AI-based variant calling). Similar to the response to R2Q1, we now provide more emphasis on these technical novelties in the Discussion: "*This study also incorporates several technical advances…… patient-archived tissues at the single-cell level.*"

Third, the most important conclusion of dorsal origins for some interneurons has been indicated by prior fetal brain and in vitro studies, albeit this paper makes the first direct observation in adult human brain.
Response. We thank R3 for recognizing that our study provides the first direct observation in adult human brain. Regarding the previous fetal brain and in vitro studies, they showed the 'potential' of radial glial progenitor cells to differentiate into inhibitory neurons, but did not study adult human brain. Also, as mentioned in the last paragraph of the Discussion, we conclude that dorsally derived inhibitory neurons are distinct from COUPTFII+ neurons in adults, whereas previous in vitro cultured fetal brain studies suggested they may be COUPTFII+ (encoded by NR2F2) [7,8].

Fourth, profiling two donors is perhaps sufficient, but not ideal. Understandably, there is an immense cost to profile each individual, and I would not expect profiling of a third brain, but it is important to note that developmental patterns can be highly variable, as evidenced by dramatically different distributions of the lineages from the first cell stages of human development across the early embryo. Therefore, it is possible that the observations in these two brains are a subset of possible clonal patterns.
Response. R1Q9 also asks this question. We agree that additional subjects will further demonstrate the generalizability of the results. At the same time, we emphasize that we used four hemispheres from two individuals for our conclusion: "…*in four different cortical hemispheres*…" Moreover, an independent preprint with complementary experimental designs also suggests dorsally derived inhibitory neurons in human adult brains through distinct methods and individuals [6]. Planned studies will include additional neurotypical subjects as well as subjects with neurodevelopmental disease.

R3Q1 For ID01: the authors should specify in the main text and the methods which samples and data were obtained from the group's prior study (Breuss, et al, Nature, 2022). The methods make it seem like all or nearly all of ID01 is newly profiled.
Response. Thanks to R3 for suggesting further clarification. We updated the 'Tissue dissection' section in the Methods by adding information on newly dissected samples in this study. We also added information on whether DNA was studied in Breuss et al., in Supplementary Data 1. Finally, MVs used in Breuss et al. are now labeled in Supplementary Data 4.

R3Q2 It is not clear what quantitative improvement is referred to here: "we further optimized a prior protocol for lower cell number input (100-fold improvement)".
Response. We apologize for the unclear description. Evaluation of minor cell types, especially inhibitory neurons, was very limited in previous studies due to the lack of the sorting method that could isolate those cell types with intact DNA. This issue resulted in consumption of human brain tissues samples (an entire lobe) requiring at least 50,000 sorted nuclei to perform 10,000X MPAS sequencing, limiting spatial resolution of MV distribution. However, our current study uses MFNS without formaldehyde fixative, and a modified nuclear isolation protocol, so that we can generate 10,000X MPAS sequencing data with a minimum of 200 nuclei, increasing the efficiency of MV discovery by an estimated 250-fold. We clarified this in the main text: "*we further improved a prior protocol, requiring at least 50,000 nuclei…… comprising three phases*"

R3Q3 The authors should make more clear that the DLX1 and TBR1 nuclei sorting, at least, was developed in the prior Breuss, et al paper.
Response. We apologize for not better clarifying differences from our previous study. Antibodies we used in the current paper were also employed in Breuss et al, but the sorting method was substantially different from the previous paper because we used the MFNS method as mentioned above to isolate multiple cell types and multiple ultra-high-depth genotypes from a single small punch (~200 mg). This high yield enabled a detailed evaluation of MVs from DLX1+, TBR1+, and COUPTFII+ nuclei at the same small punch in parallel and ultimately provided a much higher spatial and cell-type-specific resolution, which was not possible in the

previous paper. We clarified this in the Discussion section: "*This study also incorporates several technical advances in MVBA…… patient-archived tissues at the single-cell level.*"

R3Q4 How does the computational pipeline account for the possibility of sequencing or alignment errors that can occur in specific loci, both in the first stage deep WGS and in the second amplicon sequencing stage? This could yield false-positive mutations that seemingly cross-validate between the two methods.

Response. We appreciate R3's reasonable concern. Since the validation rate of MV candidates from WGS was ~35% in both cadavers (see 'Data analysis for MPAS and snMPAS' in Methods), the false positive rate of the first step of the MV calling pipeline is around 65%. This is a major reason why a validation step is so essential. To account for potential false positives and false negatives from our MPAS validation step, we included homozygous (homo) and heterozygous (het) variants as negative and positive controls in the MPAS panel design, respectively. Based on AF distributions detected by MPAS from homo/het variants, we determined our false-positive and false-negative rates as 5% (Fig. R3Q4-1). The detailed strategy is now further explained in Methods: "*The genotypes of candidate MVs from MPAS were determined by……~5% false discovery rate (FDR) based on the built-in heterozygous and alternative homozygous genomic positions.*" Thus, the false-positive rate for MVs seemingly positive in both WGS and MPAS can be calculated by multiplying the false positive rate in WGS of 65% X the chance of either 95% binomial upper or lower bound of AF passing cut-offs (2.5% X 95% + 97.5% X 5% + 2.5% X 5%) = 4.79%.

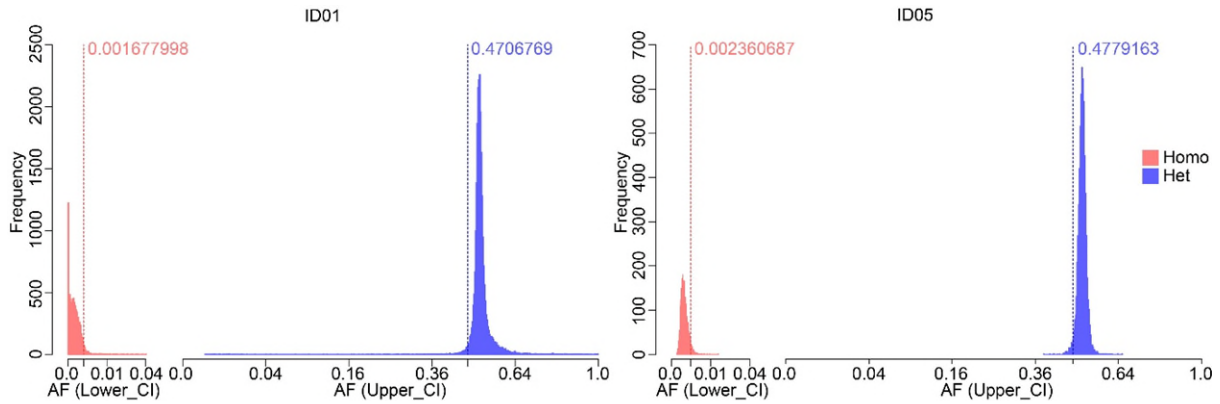

Fig. R3Q4-1 False-positive and negative rate control using homozygous and heterozygous variants. AF distribution of the exact binomial lower bound of MPAS results for ID01 reference homozygous variants (red), and the exact binomial upper bound of heterozygous variants (blue) are shown in ID01 and ID05, respectively. The x-axis is square-rooted transformed AF. The cut-off for the exact binomial lower bound was determined to be 0.001677998 and 0.002360687, and the cut-off for the exact binomial upper bound was determined to be 0.4706769 and 0.4779163, for MPAS detection with a false discovery rate of 5% in ID01 and ID05, respectively.

We also generated Circos plots to determine potential alignment error clustering (Fig. R3Q4-2). MV candidate calls in WGS were widely distributed across genomic positions, excluding localized alignment biases causing false positives.

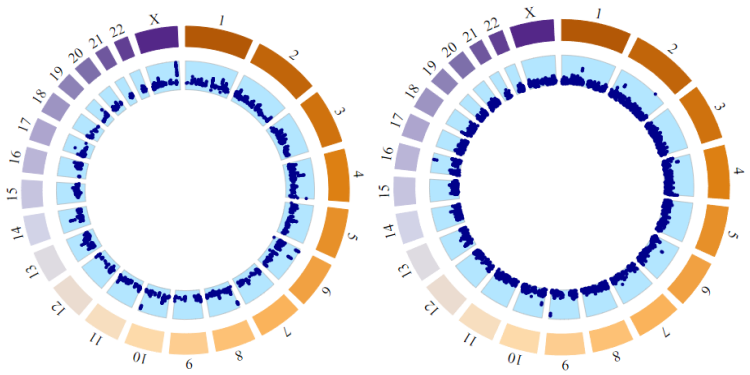

Fig. R3Q4-2 Circos plots for the distributions of MV calls across the genome. Left, ID01; Right, ID05. Blue dots, MV calls in WGS passed MV filters and included in panel designs; x and y-axis of light blue track, genomic position and sqrt-t AF, respectively.

R3Q5 It would help to specify more clearly the validation rate between amplicon sequencing and WGS. While the authors likely erred on the side of sensitivity in building the amplicon sequencing panel, providing validation rates would give a measure of the specificity of the WGS pipeline.
Response. Thanks to R3 for the suggestion. The validation rate of MV candidates was 36.5% (328/898) and 35.5% (780/2195) in ID01 and ID05, respectively. This is now in the 'Data analysis for MPAS and snMPAS' section in Methods.

R3Q6 It would be helpful to specify which tissues were profiled by deep WGS in the text, since subsequently in the section it is stated that about 1/3 of variants were only found in brain. But this fraction is biased by how many of the initial deep WGS samples were from the brain.
Response. Thanks for the suggestion, in line with R2Q2. DNA samples used in WGS in the MV discovery phase are now labeled in the 'WGS performed in this study' and 'WGS performed in Breuss et al' columns of Supplementary Data 1.

R3Q7 All the evidence provided for a cortical origin of DLX1+ interneurons depends critically on the purity of the DLX1+ nuclei sorting. However, the authors show in Fig. 1d that DLX1+ neurons contain about 1/4 excitatory neurons, seemingly showing 'contamination' of excitatory neuronal nuclei. While the authors subsequently perform single-cell DNA+RNA sequencing to support their findings, it is not clear to me why this contamination in the bulk sorting does not significantly dampen the strength of all the bulk sequencing data of this section.
Response. We fully agree with R3, and would like to mention this is the reason we applied ResolveOME to the dataset. To address R3's question, we now further deconvolve 25% of excitatory neuronal proportion within DLX1+ nuclear pools (Fig. 1d), adjust AF values of DLX1+ nuclear pools ('Computational deconvolution for DLX1+ populations' in Methods) and perform similar clustering with COUPTFII and TBR1+ nuclear pools. The results across individuals continue to show similar deconvolved DLX1+ nuclear pools clustered with TBR1+ nuclear pools within the same punches rather than clustering with COUPTFII nuclear pools (Extended Data Fig. 7c and d).

Additionally, we generated pseudo-contaminated nuclear pool data by aggregating 75% of COUPTFII and 25% of TBR1 AF values, clustering with the original COUPTFII and TBR1+ nuclear pools ('Simulated TBR1+ nuclei contamination for COUPTFII+ populations' in Methods). This simulation continues to show strong clustering of COUPTFII + within the pseudo-contaminated nuclear pools (Extended Data Fig. 7e and f). Taken together, this analysis indicates that as great as 25% contamination of excitatory neurons in DLX1+ nuclear pools does not change major results generated by DLX1+ nuclear sorting.

R3Q8 It is not intuitive why clustering based on allele frequencies of mosaic variants, as opposed to absence/presence of cortical region-specific mosaic variants, is a reliable indicator of lineage origins. Readers would benefit from 1-2 sentences explaining the concept of clustering based on allele frequencies.
Response. Thanks for the suggestion. We added this sentence in the main text: "*Just as clustering based on haplotypes allele frequency patterns can assess genetic structures among populations* [9,10]*, clustering of biopsied samples in an individual of MV AFs can be used to infer clonal relationships.*"

R3Q9 Can the authors estimate using the bulk (or maybe subsequent single-cell) data what proportion of DLX1+ interneurons are cortical- versus ganglionic eminence-derived?
Response. We appreciate R3's suggestion. We now coarsely estimated the proportion of dorsally vs ventrally derived DLX1+ inhibitory neurons by the Least Squares method. Specifically, we assumed that COUPTFII+ and TBR1+ nuclei originate exclusively from ventral and dorsal telencephalic sources, respectively. We then computed the optimal proportion of COUPTFII+ and TBR1+ nuclei, minimizing the difference in AFs from the deconvolved DLX1+ nuclei for each lobe (Least square: L(β), Methods). Finally, we determined that the mean proportion of estimated dorsal clones within deconvolved DLX1+ neurons as 58.875% ± 7.887% (with a 95% confidence interval). This aligns with earlier studies employing retroviral labeling in organotypic slice culture of embryonic human forebrain, which reported a proportion of 65% [11]. We added this estimation in Extended Data Fig. 7g. The example performance of L(β) for the left occipital lobe of ID05 was shown in Fig. R3Q9.

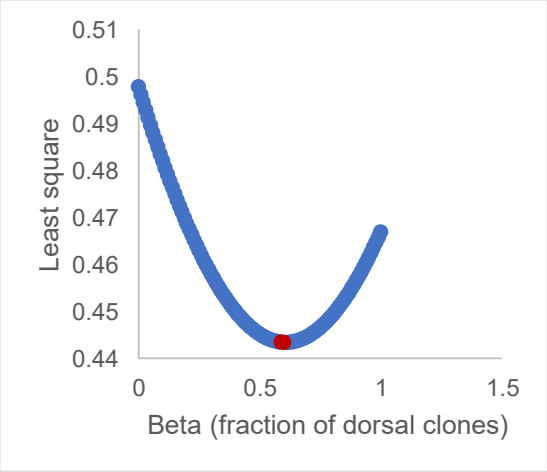

Fig. R3Q9. Example least square function depending on beta. One single global optimal (red) was obtained from the left occipital lobe of ID05.

R3Q10 If NeuN+ nuclei were sorted, why were “a few minor non-neuronal cell types” detected “as expected”?
Response. Thanks for pointing out this. We removed the words “*as expected*” from the main text.

R3Q11 This section would benefit from basic statistics on how many single cells were profiled, how many excitatory versus inhibitory, etc.
Response. In the original text, we mentioned the total number of input NEUN+ nuclei and the number of excitatory and inhibitory neurons used for Fig. 4b. We further clarified these basic statistics in the legend of Fig. 4 in the revised manuscript.

R3Q12 Is it possible that cortical-derived excitatory and inhibitory neurons originate from ventrally-derived progenitor cells that migrate to specific locations and locally give rise to clonally related excitatory and inhibitory neurons? Can the authors’ data support or negate this alternative model (that admittedly is unlikely based on prior literature)?
Response. We appreciate R3’s interesting alternative hypothesis. As R3 mentioned, the tangential migration of excitatory neurons is very unlikely, based on the previous literature. However, since we cannot exclude this rare possibility, we mentioned this scenario in the main text: *“…if we exclude the very unlike scenario … migrate to specific locations together.”*

R3Q13 Is the skew towards parvalbumin-positive inhibitory neurons consistent with prior in vitro studies of cortical progenitor-derived interneurons?
Response. Thanks for mentioning this. Different from ours, Delgado et al proposed that the transcriptomic profile of inhibitory neurons originating from the dorsal telencephalon resembles that of inhibitory neurons derived from the CGE but not MGE, mainly generating PV+ neurons. This discrepancy may be due to the difference in observed development stages, now mentioned in the Discussion, “*Despite our findings, there remain notable disparities…… the need for further investigation of cortical inhibitory neuron diversity.*”

R3Q14 Did the authors identify any single cells that are inhibitory neurons derived from the ganglionic eminence, based on mosaic variants that these cells have that are entirely distinct from any variants found in cortical-derived neurons? The text states that ganglionic eminence-derived interneurons were identified based on them not having locally-enriched mosaic variants, but this is an exclusion criteria, rather than positively identifying them based on mosaic variants that they specifically carry distinctly from cortical-derived neurons.
Response. We apologize for our mistake in terminology. R3 raises the question of how we defined ventrally originated cortical inhibitory neurons because of this paragraph: *“…We also calculated the probability of observing ventrally originated cortical inhibitory neurons carrying seemingly locally enriched MVs by chance using MVs shared in more than two cells in one lobe but none in the other lobe. …*” Actually, our original intention was to calculate the probability of observing just ‘cortical inhibitory neurons’ carrying seemingly locally enriched MVs by chance, using MVs shared in more than two cells in one lobe but none in the other lobe. We now removed the word ‘*ventrally originated*’.

R3Q15 Per Fig. 4b, was MV <13-69308268-A-G> the only mutation detected in both an excitatory and inhibitory interneuron in single-cell profiling? This is important to clarify to assess the strength of the paper's conclusion regarding a cortical origin for interneurons. While Fig. 4c shows similar allele frequencies in different cortical regions, per above, this could be due to DLX1+ sorting retaining some excitatory neurons in the pool.

Response. We apologize for the unclear description in Fig. 4b. There were 13 MVs shared in both excitatory and inhibitory neurons exclusively in the same lobe, and MV 13-69308268-A-G was an example. We clarified this by labeling MVs shared between two cell types exclusively at the same lobe as solid black borders (ExN & InN shared) in new Fig. 4b. Regarding the contamination issue of DLX1+ sorting, we deconvolved AFs of DLX1+ nuclei in Fig. 4c by removing the excitatory neuronal AF component as mentioned in the response of R3Q9 and Methods. The result below shows that deconvolved DLX1+ pools have very similar AF patterns like Fig. 4c.

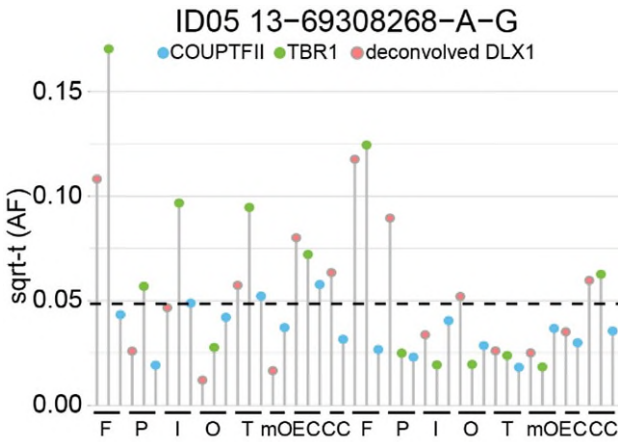

Fig. R3Q15. Lolliplot for ID05 13-69308268-A-G

R3Q16 The clustering dendrogram in Fig. 5b seems convincing, but the strength of evidence in Fig. 5d and Figs. 5e-f is not as clear. Specifically, in Fig. 5d, it is difficult to visualize the authors' observation that allele frequencies (AF) differ more in the AP axis than the DV axis. And in Figs. 5e-f the kernel density estimations on the top and right of each plot do not seem highly distinct. A more direct comparison may be to present an overlap of the delta-DV and delta-AP kernel density estimations.

Response. We appreciate R3's suggestion. We increased the contrast of the color indicating sqrt-t AF to visualize the AFs more clearly in Fig. 5d. We also overlapped the contour plots of DV and AP kernel density estimation and found the distinct density distribution. The updated figure is now in Fig. 5e and f.

R3Q17 Ext. data Fig. 5 shows UMAP plots based on AFs that show Left versus Right clustering. Can the same be performed for the AP vs DV axis samples?

Response. We appreciate this suggestion. We added a UMAP plot in new Extended Data Fig. 10 and found that the samples were clustered by the A-P axis rather than the D-V axis consistent with the result in Fig. 5.

R3Q18 The authors do not distinguish enough between two distinct concepts: lineage restriction that is due to migratory/diffusion barriers and bona-fide lineage commitment in which progenitor cells become epigenetically committed to a specific lineage. In this paper's retrospective lineage tracing, without directly observing the early progenitors, it is not feasible to distinguish these two phenomena. For example, some early progenitors may relatively restrict to the hippocampus due to developmental anatomic/diffusion barriers without any epigenetic 'commitment'.
Response. Thanks for pointing out this equivocal expression of 'commitment'. We changed 'commitment' to 'restriction' to avoid confusion.

R3Q19 The motivation to speculate regarding the specific identity of dorsally-derived interneurons as 'double bouquet cells' is not clear. Since the authors have single-cell DNA+RNA sequencing data, could this be assessed directly? If not, why not? And if not, then could the authors feasibly do so via a different method? Without such analyses and attempts, I'm not sure there is added value to hazard a guess as to the identity of these cells.
Response. We agree with the R3's opinion. We now removed the entire paragraph describing double bouquet cells from the main text.

R3Q20 The paper would benefit from some explanation as to the significance (clinical, physiologic, etc) of their findings that hippocampus is more lineally distinct from cortex and basal ganglia, and that the anterior-posterior restriction precedes dorsal-ventral restriction.
Response. We appreciate R3's kind suggestion. We added more clinical and physiological implications on the lineage restriction of the hippocampus and AP vs DV restriction in the discussion section: "*From the clinical perspective, this restriction may explain…… insights into brain dysplasias.*" and "*The cell-type-resolved spatial clonal relationship within single cortical lobes was…… insights for refining surgical strategies.*"

R3Q21 It is not clear how the 4 different variant callers were integrated. Were variants called by any of the 4 retained, or were only variants called by some subset or all of the callers retained?
Response. We apologize that we were unclear in the description of our variant calling methods. Four different calling methods were further clarified by numbering as follows in the Methods section: "*Mosaic single nucleotide variants/mosaic small…… were collected for sample-specific variants.*" Detailed computational pipelines and parameter files are provided at GitHub.

R3Q22 Bisulfite sequencing of sorted nuclei: "Sorted nuclei DNA on beads" – the meaning of this could be further clarified.
Response. We apologize that we were unclear in the use of "sorted nuclei DNA on beads". Typically, the DNA purification step uses magnetic bead selection followed by elution. However, since long and intact genomic DNA can be strongly attached to beads, it is hard to elute DNA by the solvent, leading to significant loss. Not enough DNA is extracted for downstream experiments if the starting material is limited. Thus, we skipped the magnet separation step during elution and added low-TE resuspension containing DNA attached to beads to the Lightning Conversion Reagent in the Pico Methyl-Seq™ Library Prep Kit. We now clarified this in the Methods: "*The low-TE resuspension including DNA and beads were processed by Pico*

Methyl-Seq Library Prep Kit (Zymo Research, D5455) to generate bisulfite sequencing libraries. Samples were sequenced at PE150 on the Illumina NovaSeq 6000 platform." Also, we moved the 'Low-input DNA extraction from sorted nuclei' section right before the 'Bisulfite sequencing of sorted nuclei for cell type of origin' section, so that the real workflow can be matched with the text flow.

R3Q23 Fig. 4b: the legend indicates that 'orange' is the color for InN1, but that does not match what we see. Perhaps it changed during PDF formatting, or perhaps it is a typo.
Response. We appreciate R3's suggestion. We now replaced 'orange' with 'light red' for the color description of InN1.

R3Q24 Figure 4 would benefit from supporting data showing the snRNA-seq analysis and cell type clustering/identification. Since snDNA+RNA-seq is a novel technology, readers will likely want to see supportive evidence regarding its performance. This should also include basic quality metrics (for example, number of genes detected).
Response. We appreciate R3's suggestion. We added information about the number of genes and read counts for each nucleus in Supplementary Data 10.

R3Q25 The schematic in Fig. 4e is confusing due to the numerous colors.
Response. We now reduced the colors used in Fig. 4e.

R3Q26 Ext data Fig. 2a: were these nuclei also stained with NeuN? The x-axis is labeled '488', but the marker is not specified.
Response. We apologize for not clarifying the x-axis in Extended Data Fig. 2a-c. We did not stain the NeuN-488 antibody in this sorting. Rather this channel is used to exclude nuclei with autofluorescence signals. We clarified this in the figure legend.

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3 Ju, Y. S. *et al.* Somatic mutations reveal asymmetric cellular dynamics in the early human embryo. *Nature* **543**, 714-718, doi:10.1038/nature21703 (2017).
4 Luquette, L. J. *et al.* Single-cell genome sequencing of human neurons identifies somatic point mutation and indel enrichment in regulatory elements. *Nat Genet* **54**, 1564-1571, doi:10.1038/s41588-022-01180-2 (2022).
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Reviewer Reports on the First Revision:

Referees' comments:

Referee #1 (Remarks to the Author):

The authors addressed most of my comments and now the manuscript is clarified and reads well. I am sorry that there is no single-nucleotide-PTA-WGS. If single-cell resolution data was possible, more interesting analyses would be possible.

Referee #2 (Remarks to the Author):

The authors have addressed my comments and recommendation and I am supportive of the publication.

Referee #3 (Remarks to the Author):

Summary:
Overall, the authors addressed my critiques, with the exception of a few minor comments below. The most important additions were their addressing my concern (by simulations) about contamination in their DLX1+ sorting, and their clarification of their improved sorting protocol that represents a technical novelty. I support publication of this paper in Nature.

Specific comments:

Response to R3Q1. I think it is important to also mention in the main text that a subset of the samples were from a prior study. Otherwise it gives the impression that 100% of the samples are new.

Response to R3Q4. This approach does not account for locus-specific false positive events that happen both in WGS and in MPAS, due to sequence/region-specific artifacts in the human genome. The homo and het variants are distinct loci, and while they provide a general measure of false positives, their false positive rate may underestimate a subset of loci with higher false positive rates. The circos plot showing that mosaic variants are widely distributed across the genome doesn't address the possibility that a specific small subset of loci may be false positives due to local alignment or other sequencing errors that occur both in WGS and MPAS. Is there any other technical reason that would make MPAS differentially susceptible to locus-specific false positives than WGS that would mitigate this issue and enable MPAS to be a more independent validation method? If not, then I think somewhere in the discussion, these false positive estimates should be qualified, and the possibility of cross-method false positives should be acknowledged.

Response to R3Q24. The columns in the new Supplementary Table are not defined. The authors should define each column whose meaning is not intuitive, in this and all the other supplementary tables.

Author Rebuttals to First Revision:

<Second round>

Referees' comments:

Referee #1 (Remarks to the Author):

The authors addressed most of my comments and now the manuscript is clarified and reads well. I am sorry that there is no single-nucleotide-PTA-WGS. If single-cell resolution data was possible, more interesting analyses would be possible.
We appreciate R1's positive response. We agree that analysis of snPTA and snWGS could identify even more interesting evidence of human-specific features. We will definitely take this approach into consideration for our future studies.

Referee #2 (Remarks to the Author):

The authors have addressed my comments and recommendation and I am supportive of the publication.
We appreciate R2's positive response.

Referee #3 (Remarks to the Author):

Summary:
Overall, the authors addressed my critiques, with the exception of a few minor comments below. The most important additions were their addressing my concern (by simulations) about contamination in their DLX1+ sorting, and their clarification of their improved sorting protocol that represents a technical novelty. I support publication of this paper in Nature.
We appreciate R3's positive response.

Specific comments:

Response to R3Q1. I think it is important to also mention in the main text that a subset of the samples were from a prior study. Otherwise it gives the impression that 100% of the samples are new.
We agree R3's suggestion. We now mentioned a subset of samples derived from a previous study in the main text.

Response to R3Q4. This approach does not account for locus-specific false positive events that happen both in WGS and in MPAS, due to sequence/region-specific artifacts in the human genome. The homo and het variants are distinct loci, and while they provide a general measure of false positives, their false positive rate may underestimate a subset of loci with higher false positive rates. The circos plot showing that mosaic variants are widely distributed across the genome doesn't address the possibility that a specific small subset of loci may be false positives due to local alignment or other sequencing errors that occur both in WGS and MPAS. Is there any other technical reason that would make MPAS differentially susceptible to locus-specific

false positives than WGS that would mitigate this issue and enable MPAS to be a more independent validation method? If not, then I think somewhere in the discussion, these false positive estimates should be qualified, and the possibility of cross-method false positives should be acknowledged.
We agree with R3's suggestion. We added text to the Discussion acknowledging our pipeline cannot identify double false-positive mutations. Although we estimate double false-positives account for less than 5 percent of all mutations, we also mention that incorporating additional independent validation assays may help to further increase accuracy in the Discussion.

Response to R3Q24. The columns in the new Supplementary Table are not defined. The authors should define each column whose meaning is not intuitive, in this and all the other supplementary tables.
We now added a legend providing column information to Supplementary Table 10.