FOXP1 orchestrates neurogenesis in human cortical basal radial glial cells

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Summary

During cortical development, human basal radial glial cells (bRGCs) are highly capable of sustained self-renewal and neurogenesis. Selective pressures on this cell type may have contributed to the evolution of the human neocortex, leading to an increase in cortical size. bRGCs have enriched expression for Forkhead Box P1 (FOXP1), a transcription factor implicated in neurodevelopmental disorders such as autism spectrum disorder. However, the cell type-specific roles of FOXP1 in bRGCs during cortical development remain unexplored. Here, we examine the requirement for FOXP1 gene expression regulation underlying the production of bRGCs using human brain organoids. We examine a developmental time point when FOXP1 expression is highest in the cortical progenitors, and the bRGCs, in particular, begin to actively produce neurons. With the loss of FOXP1, we show a reduction in the number of bRGCs, as well as reduced proliferation and differentiation of the remaining bRGCs, all of which lead to reduced numbers of excitatory cortical neurons over time. Using single-nuclei RNA sequencing and cell trajectory analysis, we uncover a role for FOXP1 in directing cortical progenitor proliferation and differentiation by regulating key signaling pathways related to neurogenesis and neurodevelopmental disorders. Together, these results demonstrate that FOXP1 regulates human-specific features in early cortical development.
Introduction

The neocortex consists of diverse cell types that are produced in a highly species-specific manner under strict spatiotemporal control throughout development. Compared to lissencephalic species, the gyrencephalic human neocortex is endowed with an expanded outer subventricular zone (oSVZ) that is occupied by the basal progenitors such as the basal radial glial cells (bRGCs) (Dennis and Eichler, 2016; Fan et al., 2020; Otani et al., 2016). The human bRGCs are known for their exceptional capacity for self-renewal, and neurogenesis. bRGCs are known to produce neurons through both direct neurogenesis, a production of neurons via intermediate progenitors, and indirect neurogenesis, a production of neurons directly from bRGCs. Both of these mechanisms contribute to the prolonged birth of excitatory neurons (ENs), which underlie human cortical expansion (Lui et al., 2011; Pollen et al., 2015). Gene expression in these cell types during early development is tightly regulated, as any abnormal changes at this stage may have irreversible consequences for brain development (Parikshak et al., 2015; State and Sestan, 2012; Xu et al., 2018). However, the genetic and molecular components involved in the formation of early cortical neural cells that give rise to the neocortex have not yet been fully defined.

A crucial regulator of the molecular mechanisms underlying cortical development is the transcription factor (TF) Forkhead Box P1 (FOXP1). FOXP1 has been linked to neurodevelopmental disorders, such as autism spectrum disorder (ASD) and intellectual disability (ID) (Co et al., 2020; Lozano et al., 2021). Previous studies have shown that either knockdown (KD) or knockout (KO) of FOXP1 leads to varying phenotypes reflecting abnormal neurogenesis in the cortex (Braccioli et al., 2017; Li et al., 2015; Pearson et al., 2020). However, no study has examined the role of FOXP1 in basal progenitors in relation to cortical development (Vaid and Huttner, 2020). A recent study detected the expression of FOXP1 in the ventricular zone (VZ) of the developing human cortex at gestation week (GW) 14, the last week of fetal development that FOXP1 expression remains high in the cortical progenitors (Pearson et al., 2020). The same study showed that FOXP1 was detected in as many as 70% of human bRGCs, whereas FOXP1-positive (FOXP1+) bRGCs were not observed in the mouse cortex at an equivalent developmental stage. Currently, there is very little known about the cell type-specific roles of FOXP1 in the cortex. Single-cell or nuclei RNA-sequencing (scRNA-seq and snRNA-seq, respectively) studies of other FOXP1+ cell types in the brain, such as the spiny projection neurons of the striatum, revealed that FOXP1 regulates distinct gene expression programs within each spiny projection neuron subtype (Anderson et
al., 2020). These findings show that FOXP1 has cell-type specific contributions to the development of the striatum and suggest that a similar role may occur in the cortex as well.

In our study, we investigate several unanswered questions regarding the cell type specific contribution of FOXP1 to human cortical development, specifically in regard to bRGCs. First, we wanted to determine whether FOXP1 regulates gene expression programs that are important for the development of bRGCs. Second, we wanted to examine if FOXP1 regulates the proliferation and differentiation of bRGCs. Third and last, we sought to identify changes in the expression of corticogenesis and NDD-relevant genes with loss of FOXP1 in a cell type-dependent manner. In this way, we could determine how a lack of FOXP1 leads to NDD-relevant features in the developing human cortex. To capture the FOXP1+ bRGCs that cannot be studied using mouse models, we utilized 3-D human brain organoids in combination with single-nuclei RNA sequencing (snRNA-seq) (Figure 1A and 1B). We manipulated FOXP1 expression using CRISPR/Cas9 and evaluated how the loss of FOXP1 affects the development of bRGCs and ENs within the brain organoids. Using this system, we found that the loss of FOXP1 negatively affects both bRGC production and the differentiation of ENs. snRNA-seq enabled us to determine the differentially regulated genes associated with neurogenesis and NDDs in bRGCs with loss of FOXP1.

Results

Generation of human brain organoids with loss of FOXP1

Disruption of the FOXP1 locus results in a collection of cognitive and neurodevelopmental symptoms known as FOXP1 syndrome (Lozano et al., 2021; Siper et al., 2017). The individuals diagnosed with this disorder have loss of function mutations such as deletions, frameshifts or de novo point mutations in one copy of FOXP1 (Siper et al., 2017; Urreizti et al., 2018). To study the essential function of FOXP1 in a manner that reflects human brain development, we applied two different CRISPR-mediated gene KO strategies at the stem cell stage of human brain organoids. The first strategy involved a complete gene deletion (KO-1) yielding no FOXP1 mRNA and protein. The second strategy employed a reporter knock-in into one allele and a double stranded break and frameshift in another allele (KO-2) yielding a non-functional FOXP1 protein (Figure 1C). We then differentiated these edited stem cells into cerebral brain organoids (Figure 1B and Figure S1A-E), which are a model system known to reliably recapitulate many characteristics of human cortical neural progenitors and neurons (Kelava et
al., 2022; Kelava and Lancaster, 2016; Lancaster et al., 2013). FOXP1 mRNA and protein were absent from the KO-1 organoids, whereas the FOXP1 protein was gone but FOXP1 mRNA persisted in KO-2 organoids as expected (Figure 1D, 1E, and S2A-C).

FOXP1 is enriched in cortical progenitors early in the second trimester (Pearson et al., 2020). To capture FOXP1 expression reflective of the early second trimester fetal brain, we performed immunostaining on the organoids using RGC and EN markers at day (D) 25, 40, 60, and 100 after inducing organoid formation (Figure S1A). We found that at D25, the organoids primarily contained SOX2+ progenitors with a thin layer of TUJ1+ neurons, a pattern analogous to a pre-plate stage (Figure S1A). By D40, TUJ1+, CTIP2+, and CUX1+ staining indicated the presence of deep-layer neurons situated adjacent to the progenitors in an organization reminiscent of a cortical plate (CP) (Figure S1B–E). At D60, the CP was more developed, and FOXP1 expression was greater in the CP cells than at D40. By D100 (month 3), FOXP1 expression is primarily in CP cells, overlapping with the TUJ1+ ENs, with little expression within the SOX2+ RGCs (Figure S1A and B). This pattern of expression is similar to FOXP1 expression in the late second trimester fetal brain (Pearson et al., 2020). Among the time points we examined, D40 appeared to be most analogous to early second trimester fetal brain development. At D40, the cortical-like structures in the brain organoids expressed proteins abundant in the dorsal telencephalic lineage cells in a pattern specific to a developing mammalian neocortex, such as FOXG1 in the progenitors and ENs, TBR2 in the intermediate progenitor cells (IPCs) lining the VZ, and CTIP2, and CUX1 in the ENs adjacent to the VZ (Figure S1C–E). To capture the beginning of neurogenesis when FOXP1 expression is abundant in the cortical progenitors, we performed the majority of our experiments at D40 ± 1-2 days (Mora-Bermúdez et al., 2016; Otani et al., 2016).

**Identification of bRGC subtypes using single-cell transcriptomics**

To study the cell type-specific functions of FOXP1 during cortical development, we performed snRNA-seq on wildtype (WT) and FOXP1 KO organoids (Figure 2A). After ensuring quality control (see Methods and Figure S2D and S2E), we obtained 151,336 nuclei across 9 samples, with an average of 30,310 reads per nucleus (Table S1); this was sufficient to resolve the major cell types in the human brain organoids at this stage. FOXP1 mRNA was absent from the KO-1 organoids, whereas FOXP1 mRNA persisted in KO-2 organoids as expected (Figure S2A-C). We observed FOXP1 expression in 79.35% of the bRGCs (Figure S2C). Based on
our snRNA-seq clustering and annotation (Kanton et al., 2019; Nowakowski et al., 2017), the brain organoids exhibited cell types typically represented in the dorsal telencephalic lineage (Figure S3A-C), which is consistent with other brain organoid studies (Camp et al., 2015; Mora-Bermúdez et al., 2016; Otani et al., 2016). We identified two bRGC clusters (clusters 17 and 25) (Figure 2A-B) based on the annotation (Figure S3C). We further confirmed the cell type identity of these two clusters by performing a Fisher’s exact test against a list of bRGC-enriched genes derived from a previous study (Heng et al., 2017) (Figure 2C). These results are in line with recent single-cell studies showing multiple subtypes of bRGCs in the developing human cortex (Zhong et al., 2018). To focus on cortical cell types that express FOXP1, we removed non-dorsal telencephalic EN clusters from our analysis (See Methods). Additionally, as our KO-1 and KO-2 models showed similar transcriptional profiles, we merged them into one new dataset (KO) for the rest of our analyses (Figure S4A and B).

**FOXP1 deletion leads to changes in the developmental trajectory of bRGCs**

Next, we sought to examine the developmental trajectories of the major cortical cell types reflected in the gene expression sequences and how the progression would be different between the WT and FOXP1 KO organoids. We performed pseudotemporal ordering of the cells using Monocle 3 (Cao et al., 2019). We designated the root cells—assigned “0” in both the pseudotime uniform manifold approximation and projection (UMAP) (Figure 2D) and the density plot (Figure 2E)—as those triple-positive for SOX2, PAX6, and HES5 transcripts. The apical RGCs (aRGCs), which gives rise to all progenitors and neurons in the cortex, are known to express these TFs in the earliest stage of cortical development (Bella et al., 2021). The end point, designated as “1,” is the point at which no SOX2+PAX+HES5+ expression occurs and the greatest amount of transcriptional changes from the root cells is present. In the pseudotime UMAP, the relative changes in gene expression from the root cells are reflected in the color scale from one cluster to another (Figure 2E). bRGC clusters 17 and 25 showed the most striking color changes in the KO compared to the WT (Figure 2D, in dotted line). Furthermore, when we plotted these changes using density plots, which show the number of cells in pseudotemporal order in a bar graph format, the bRGCS showed reduced gene expression changes from the root cells with the loss of FOXP1. We also observed a more skewed distribution of ENs in the KO (Figure 2E), which represents abundant dysregulation of gene expression programs and impaired neuronal differentiation. Additionally, we observed
significantly decreased levels of some of the bRGC-enriched genes (i.e., PTPRZ1, TNC, HOPX1, LIFR, and FAM107A) (Heng et al., 2017; Pollen et al., 2015) in the KOs (Figure S3D). These genes are known to be associated with proliferation and maturation of bRGCs (Pollen et al., 2015).

Examination of bRGCs in KO1 and KO2 separately showed similar pattern of gene expression changes in the density plot (Figure S4B). We also saw similar patterns of gene expression changes when we examined bRGC subclusters #17 and #25 using both relative cell density or absolute cell numbers (Figure S4C). Other cell types, such as aRGCs and IPCs, showed subtle changes in the FOXP1 KO compared to WT (Figure 2D and 2E). Together, these results suggest impaired differentiation of bRGCs with loss of FOXP1.

**FOXP1 deletion leads to decreased proliferation and differentiation of bRGCs**

We next wanted to assess how deletion of FOXP1 would affect the proliferation and differentiation of bRGCs at the protein level. First, we examined the number of bRGCs in both WT and FOXP1 KO. bRGCs are morphologically characterized by the loss of apical contacts and low expression of TBR2, an IPC marker (Andrews et al., 2020; Betizeau et al., 2013; Kalebic et al., 2019; Ostrem et al., 2014; Pollen et al., 2015; Vaid and Huttner, 2020). We sparsely transduced organoids with an adenovirus-expressing GFP (AD-EGFP) to identify basally located cells without apical contacts (Figure 3A). We then co-stained the transduced organoids with an antibody to TBR2, an IPC marker, to more confidently label the bRGCs, since bRGCs have a relatively low expression of TBR2 compared to IPCs (Andrews et al., 2020; Pollen et al., 2015; Vaid and Huttner, 2020) (Figure 3B). We found fewer basally located EGFP+TBR2- cells without apical contacts in the KO (Figure 3C).

To determine whether the decreased number of bRGCs was due to the reduced proliferative capacity of bRGCs, we also performed BrdU assays by pulsing the organoids at D40 with 100uM BrdU for 2 hours and harvesting the samples 24 hours later (Figure 3D). We examined the organoids by immunostaining with a bRGC-enriched marker HOPX and the relative position of HOPX+ nuclei compared to adjacent cells (Figure 3E-G). We observed fewer number of proliferating HOPX+BrdU+ bRGCs in the KO (Figure 3H). We supplemented this analysis by using another bRGC-enriched marker, PTPRZ, and found fewer PTPRZ+TBR2- bRGCs in the KO (Figure S5A-B). In addition to cellular proliferation, we also wanted to examine differentiation of bRGCs. Therefore, we counted TBR2+ cells located asymmetrically to HOPX+ cells, which represented neurogenic IPCs that underwent indirect neurogenesis, and observed fewer of these cells in the KO (Figure 3I). When we examined
HOPX+ bRGCs located asymmetrically to CTIP2+ENs, which represent direct neurogenesis from bRGCs to ENs, we did not find significant differences between the genotypes (Figure S5C–E). Together, our data show that loss of FOXP1 may reduce the self-renewal capacity of bRGCs, and also negatively affect indirect neurogenesis from bRGCs to IPCs, but not direct neurogenesis from bRGC to ENs. We did not observe changes in the number of neurons or the number of EN cells that exited the cell cycle to become neurons at this stage (Figure S6C, F and G).

We then assessed how a reduction in bRGCs during the early phase of neurogenesis might affect later neurogenesis. We cultured the organoids for ~3 months (100 days) and examined the number of neurons produced. In month 3, the progenitor pool becomes much smaller, as the majority of progenitors have differentiated into neurons (Figure S1A-B). In later cortical development stages, from mid-corticogenesis to early postnatal stages, FOXP1 is primarily expressed in post-mitotic ENs in the CP and overlaps significantly with expression of SATB2, which is an abundant marker of ENs in cortical layers 2–5 (Li et al., 2015; Usui et al., 2017). We found that FOXP1 expression in our WT organoids at D100 also strongly overlaps with SATB2+ ENs (Figure S7A). When we examined whether loss of FOXP1 would impact these SATB2+ ENs (Figure S7B), we observed that there were significantly fewer SATB2+ ENs with the loss of FOXP1 (Figure S7C). The proportion of IPCs compared to the number of ENs was not statistically significantly different in this later stage of brain organoid development (Figure S7-F). These results indicate that impaired neurogenesis in early cortical development with the loss of FOXP1 ultimately results in fewer neurons overall in later cortical development in this human model system.

**FOXP1 regulates NDD-associated genes**

We wanted to determine which gene expression changes were potentially responsible for the shift in the developmental trajectory of bRGCs that we observed in the pseudotime analysis. Loss of FOXP1 led to a substantial number of differentially expressed genes (DEGs) in each cell type with progenitors having the greatest number of DEGs among the four major cell types (Figure 4A and Tables S2 and S3). Among the DEGs, we were specifically interested in neurogenesis and NDD-relevant genes that are dysregulated with the loss of FOXP1 in bRGCs. Our previous study indicated that FOXP1 regulates genes that are associated with different types of NDDs such as ASD, ID and Fragile X Mental Retardation Protein (FMRP) target genes (Araujo...
et al., 2015). Therefore, we carried out a disease-gene enrichment analysis that examined the overlap between our DEGs and ASD-, ID-, and FMRP-associated genes (see Methods) in a cell type-specific manner. The FOXP1 DEGs showed the greatest overlap with ASD genes in the bRGCs compared to the other cell types (Figure 4B). This indicates that with the loss of FOXP1, genetic vulnerability to ASD is most prevalent in the cell type that is linked to human cortical expansion in the early stage of corticogenesis.

When we examined individual genes, we found many gene expression regulators, such as TFs (e.g., MEF2C, ASXL3, and ZBTB20), chromatin remodelers (e.g., CHD2, ANDP, SMARCA2 and ANKRD11), and RNA-binding proteins (e.g., TNRC6B) among the genes altered in the DEGs (Figure 4B and Table S3). For example, the gene encoding MEF2C, a TF involved in promoting neuronal differentiation and function (Cho et al., 2011; Li et al., 2008), is downregulated in bRGCs. Another gene, ANKRD11, which encodes a chromatin regulator that controls transcription through histone acetylation and is implicated in cortical development, is downregulated in bRGCs. In a previous study, knockdown of ANKRD11 in both developing mouse cortical precursors at E12.5 and human ESC-derived cortical neural precursors led to decreased proliferation and differentiation (Gallagher et al., 2015).

Catenins (i.e., CTNNBP2; CTNND2), extracellular matrix (i.e., LAMA1), and cell adhesion molecules (i.e., NRXN3) were other notable gene categories regulated by FOXP1 in the bRGCs (Figure 4B and Table S3). Catenins often interact with the extracellular matrix or cell adhesion molecules in the modulation of synapse structure formation, acquisition of growth factors, functioning of cell-signaling pathways, or synaptic communication with other cells (Bienz, 2005; Kim et al., 2008). For example, CTNND2, a gene encoding for δ-Catenin that is involved in WNT signaling through its interaction with N-cadherin as well as neurite outgrowth and function (Bareiss et al., 2010; Nopparat et al., 2015; Schaffer et al., 2018; Zhang et al., 2018), is upregulated in the bRGC and EN clusters. The enrichment of CTNND2 in fetal bRGCs is consistent with a published scRNA-seq of human fetal bRGCs (Pollen et al., 2015). These cell type-specific expression patterns indicate that FOXP1 may regulate the timing and amount of excitatory cortical projection neuronal differentiation from bRGCs by controlling the amount of CTNND2 expression.

In order to examine how FOXP1 regulates gene expression in different subtypes of bRGCs, we examined DEGs that are up- and down-regulated by FOXP1 in either the bRGC cluster 17 or 25 (Figure S8A and Table S4). The gene ontology analysis for each subcluster of bRGCs showed that neurogenesis and projection neuron
formation-related terms are commonly dysregulated in both directions with loss of FOXP1 (Figure S8B), which indicates that FOXP1 regulates multiple aspects of projection neuronal differentiation. To provide insights into the direct targets of FOXP1 in bRGCs, we overlapped the DEGs from each bRGC cluster and the DEGs from a previously published chromatin immunoprecipitation sequencing (ChIP-seq) dataset (Araujo et al., 2015) from our lab (Figure S8C). Doing so yielded genes associated with diverse neurogenesis-related functions such as gene expression regulators, catenin/ECM/cell adhesion molecules, and projection neuronal formation in both clusters. The majority of the genes pertaining to those same broadly categorized functions were different between the two subclusters of bRGCs, which may indicate that FOXP1 regulates neurogenesis in the bRGCs through different mechanisms in each bRGC subtype. Additionally, our analysis showed that FOXP1 may directly regulate high confidence ASD genes (i.e., WWOX, NEO1, NRXN3, ANKRD11, MEIS2, NFIA, CHD3, ST8SIA2, NLGN1, CDH8, and MAP4) early in the differentiation of bRGCs.

Discussion

The developing human neocortex harbors highly heterogeneous cell types that are generated from seemingly homogenous progenitors under strict spatiotemporal control. bRGCs specifically play a vital role in the development of the human cortex. The dynamic changes that occur in bRGCs are initiated by regulatory elements, such as TFs. Here, we examined the dynamic gene orchestration governed by FOXP1 in bRGCs. We used human cerebral organoids to model the production of bRGCs in a developing human cortical model. Using snRNA-seq, we distinguished the subtypes of bRGCs based on their gene expression signatures. We highlighted the altered developmental trajectory of bRGCs with the loss of FOXP1, as well as several neurogenesis- and NDD-related genes and signaling pathways. By immunostaining using cell type-specific markers, EGFP labeling, and BrdU assays, we were able to observe bRGC production, the reduction of bRGC to IPC and EN differentiation, and the reduced production of ENs in FOXP1 KOs. In addition to the regulation of neurogenesis by FOXP1 in bRGCs, another potential mechanism contributing to the reduction in neuronal numbers could be through indirect neurogenesis mediated via IPCs. Based on immunostaining for SOX2+RGCs, TBR2+IPCs and CTIP2+ENs, we observed premature transition from IPCs to ENs with loss of FOXP1 (Figure S6A-C). This early depletion of progenitors may have led to the observed reduction in neurons. While this shift in the developmental
trajectory of IPCs was not reflected in the snRNA-seq pseudotime analysis, it would be important to know how FOXP1 regulates other basal progenitor cell types with human-specific modifications.

FOXP1 is a high-confidence ASD gene (Satterstrom et al., 2020). We found that FOXP1-regulated DEGs have a stronger association with ASD genes in bRGCs than other cell types. This indicates that, with a loss of FOXP1, genetic vulnerability to ASD is prevalent in a cell type linked to human cortical expansion in early corticogenesis. In addition to genes involved in proliferation and differentiation mentioned above, we also discovered DEGs related to neuronal functions, such as synaptic transmission and intrinsic excitability, in the bRGCs (Figure 4B and Figure S8C). These genes encode for glutamate receptors (e.g., GRIND2B, and GRID2), and potassium (e.g., KCNB1) and chloride (e.g., GABRB3) ion channels. Previous studies have reported regulation of neuronal function in adult mouse hippocampal CA1 neurons and striatal spiny projection neurons by FOXP1 (Araujo et al., 2015; Bacon et al., 2015). Besides neuronal firing, neuronal function-related genes such as neurotransmitter receptors and ion channels are known to play important roles for proliferation and differentiation of progenitors (Chou et al., 2021; Jansson et al., 2013; Xing and Huttner, 2020; Young et al., 2011). Our results suggest that FOXP1 primes neurogenic progenitor cells to proliferate as well as to differentiate into functional neurons.

Using currently available 3-D brain organoid protocols, we successfully recapitulated many important characteristics of the developing cortex, such as the initial formation of cortical basal progenitors, followed by the differentiation of excitatory neurons into a layered fashion reminiscent of an early cortical plate that is comprised of deep layer neurons. Mouse models with complete Foxp1 KO are embryonic lethal due to a heart defect (Wang et al., 2004). However, FOXP1 KO brain organoids are free of those organismal complications and can be used to elucidate the essential contributions of FOXP1 to brain development effectively. However, while 3-D brain organoids generate distinct ventricular-like zones and a primordial cortical plate, they do not develop an elaborate six-layered cortical plate that contains mature excitatory neurons, in part due to the absence of inputs from other parts of the organoid. Therefore, it is challenging to study the alteration of the organization of excitatory neurons residing in the cortical plate. Moreover, due to a lack of fully mature organized lamination and circuitry, studying how human FOXP1 affects radial migration as the cortical plate expands remains to be elucidated.
In summary, our study has successfully shown that FOXP1 contributes to human-specific elements of the developing cortex such as basal radial glial cells and governs a multitude of NDD-relevant neurogenesis pathways. These data provide opportunities for further exploration of the human-relevant gene expression driven by FOXP1 during brain development. In addition, validation of the key downstream target genes we identified can facilitate the development of therapeutics to treat FOXP1 syndrome and other forms of NDD.

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Author contributions

S.E.P. and G.K. conceptualized the study. S.E.P. performed all the experiments and analyzed all the data. A.K. analyzed snRNA-seq datasets and wrote the method section of snRNA-seq analysis. S.E.P. and G.K. wrote the manuscript.

Declaration of interests

The authors declare no competing interests.
FIGURE LEGENDS

Figure 1. Generation of FOXP1 KO cerebral organoids from stem cells.

(A) Schematic diagram showing the experimental design. (B) Cerebral organoid protocol illustrating typical morphology observed at each time point during differentiation from embryoid body formation to maturation. Scale bar = 600uM (C) The two CRISPR-Cas9 strategies used in generating FOXP1 KOs. (D) Representative immunostaining results showing FOXP1 expression in SOX2+ and TUJ1+ cells in WT and absence of FOXP1 expression in the KOs at day 40 (D40) in-vitro. Scale bar = 100uM (E) Representative Western blot results with quantification from n=4.

Figure 2. snRNA-seq and pseudotime analysis of WT and FOXP1 KO organoids.

(A) Representation of diverse cell types in UMAP space. WT, KO-1 and KO-2 datasets are integrated and shown in the same UMAP. (B) A UMAP highlighting bRGC clusters 17 and 25. (C) Enrichment analysis of the bRGC genes (Heng et al., 2017) based on Fisher’s exact test. (D) UMAPs with a scaled color scheme as a function of gene expression changes from the root cells (SOX2+PAX6+HES5+). Changes in the basal progenitors are noted in a dotted line. WT (left) and the combined FOXP1 KO-1 and KO-2 datasets (right) are shown. (E) Density bar plots showing the number of pseudobulked cells (aRGC, bRGC, IPC and EN) in the relative pseudotime scale. The root (‘0’) is same as in the above UMAP.

Figure 3. Loss of FOXP1 results in decreased number of bRGCs.

(A) Schematic showing the design of the ‘Ad-EGFP’ virus infection experiment. (B) Representative images of immunostaining performed on the ‘Ad-EGFP’ infected organoids at week 6 showing detection of bRGCs by morphology including loss of ventricular contact. (C) Quantification of progenitors that are without apical attachment and are TBR2-. (D) Schematic showing the design of the BrdU assays. Incubation with 100uM BrdU for 2 hours was followed by media change, 24-hour incubation and harvesting the organoids for fixation. (E) Representative images of immunostaining performed on BrdU treated organoids at week 6. (F) Schematic diagrams showing bRGC divisions. (G) Magnified images showing overlap between different markers. (H) Quantification of HOPX+ dividing bRGCs. (I) Quantification of HOPX+ basal progenitors going through neurogenesis via IPCs. For all quantifications, n=3-8 organoids per sample per genotype were used. In each
organoid, 3-8 cortical structures with clear lamination patterns were examined. Data are represented in bar graphs as mean ± STD with individual data as dots; n.s. means p > 0.05, *p < 0.05, p** < 0.01, and ***p <0.001, Kruskal-Wallis ANOVA test with Dunn’s multiple comparisons test as a post hoc was used. Scale bar = 100uM.

**Figure 4. Differential expression analysis of bRGC subtypes.**

(A) Number of DEGs in each cell type. Significant DEGs are defined as log2(FC) ≥ 0.25, FDR ≤ 0.05, percent expression > 0.10. (B) Dot plots showing the enrichment of NDD-relevant DEGs in aRGC, bRGC, IPC and EN cell types. ASD = high confidence ASD genes with score 1-3 from SFARI, ID=intellectual disability relevant genes, and FMRP=Fragile X relevant genes. On the bottom are lists of ASD-associated genes enriched in bRGCs. OR=Odd’s ratio.

**Materials availability**

Transgenic iPSC cell lines generated in this study can be provided upon request.

**Data and code availability**

The single-nuclei RNA-sequencing data reported in this paper can be accessed at NCBI GEO (https://www.ncbi.nlm.nih.gov/geo/query/acc.cgi?acc=GSE195510) with the reviewer token= mrchykuyjtmmbip. Code that was used to perform data pre-processing, clustering and differential gene expression analysis is available at GitHub repository (https://github.com/konopkalab/organoidseq).

**METHODS DETAILS**

**Stem cell culture**

Stem cell work described in this manuscript has been conducted under the oversight of the UT Southwestern Stem Cell Research Oversight (SCRO) Committee. UT Southwestern uses the International Society for Stem Cell Research (ISSCR) "Guidelines for Stem Cell Research and Clinical Translation" and NIH "National Institutes of Health Guidelines for Human Stem Cell Research" when establishing stem cell research standards. A male human iPSC line (WTC-11, cat #GM25256) was provided by Bruce R. Conklin (The Gladstone
Institutes and UCSF). The cell lines were verified to have normal karyotype based on G-banding technique, free of mycoplasma contamination, and were kept under passage number 10 for differentiation. Proliferation markers (OCT4, SOX2, KLF4, and NANOG) were checked by qPCR or immunostaining. The cell lines were cultivated in mTeSRTM1 medium (cat #85870, Stemcell Technologies) using feeder-free culture protocols in six-well plates (cat #4936, Corning) that were coated with growth factor-reduced Matrigel matrix hESC-qualified (cat #354277, BD Biosciences). Cells were passaged 1:4 every 3-4 days, using Gentle Cell Dissociation Medium (cat #07174, Stemcell Technologies) and ROCK inhibitor was added at a final concentration of 10uM (cat #50-863-6, Fisher Scientific) for the first 16-20 hours of passage. The cells were maintained with daily medium change without ROCK inhibitor.

**CRISPR-Cas9-edited iPSC line generation**

The FOXP1-KO cell lines using strategy 1 (KO-1) were generated by targeting the 5’ and 3’ ends of FOXP1 with a pair of sgRNAs. For 5’ prime UTR targeting, the following set of sgRNAs were used: 5’-CACCGACAAACTTTCGGGTTCCCGC-3’ and 5’- AAACGCGGGAACCCGAAAGTTTGTC-3’. For 3’ prime UTR targeting: 5’-CACCGCATCTTACAAGACGGACTCT-3’ and 5’- AAACAGAGTCCGTCTTGTAAGATGC-3’.

We identified putative 5’ and 3’ ends as ±1000bp of the first and last exons of FOXP1 isoform 1 (OMIM: 605515). To mitigate potential off-target effects of gene editing, sgRNA candidates were analyzed using the online CRISPR Design tool developed by the Zhang laboratory (http://crispr.mit.edu/) and the sgRNA sequences with fewest off-target sites in the human genome were selected for use. Each sgRNA was inserted into ‘pSpCas9(BB)-2A-GFP (PX458),’ which was obtained from Addgene (Plasmid #48138). Cells were incubated with ROCK inhibitor for 1-3 hours and then electroporated with the pair of sgRNAs. 48-72 hours post electroporation, cells were subjected to fluorescence-activated cell sorting (FACS) for GFP+ signals and replated as single clones for the subsequent 10-12 days. The second FOXP1 KO (KO-2) cell lines were generated using the CRISPR Paint strategy (Schmid-Burgk et al., 2016). Two sgRNAs were designed to target exon 11 or 14. For exon 11 targeting, the following set of sgRNAs are used: 5’ CACCGGTCCATTGGTAGAGGCATGT and 5’-AAACACATGCCTCTACCAATGGACc-3’. For exon 14 targeting: the following set of gRNAs are used: 5’-CACCGGTAAGTATTGATCCCCACCA -3’ and 5’- AAACTGGTGGGGATCAATACTTACc -3’. sgRNA, selector
and GFP donor DNA were electroporated in a 1:1:3 molar ratio using a 4D Lonza electroporator (cat # V4XP-3024, Lonza).

Cells were subjected to puromycin selection (0.3-0.5μg/ml) following the method from a published study (Steyer et al., 2018). To verify deletion of FOXP1, positive clones from both strategies were confirmed via Sanger sequencing, qPCR, immunostaining, and western blot.

**Brain organoid generation**

Brain organoid generation was performed using a modification of a commercially available cerebral organoid kit (cat # 08571, Stem Cell Technologies) together with the method published from a published study (Lancaster et al., 2013). After day 40, we used the maturation medium following Lancaster et al., 2013. Brain organoids were kept on an orbital shaker (cat # BT4500, Benchmark) starting on D11 at 79 rpm. Brain organoids were analyzed at 40 days in-vitro (DIV) for proper cortical lamination using the following markers: SOX2, PAX6, and HOPX for NSC and RG, TBR2 for IPC, CTIP2 and TBR1 for pre-plate or deep layer neurons, TUJ1 for immature neurons, and FOXG1 for dorsal telencephalic neural cells.

**RT-qPCR**

Organoid RNA was extracted following the protocol supplied with RNeasy Total RNA Kit (cat #533179, Qiagen) and TRIzol reagent (cat # 15596018, Thermo Fisher Scientific). The extracted RNA was reverse transcribed following the protocol supplied with SSIII First-Strand Super Mix for RT-PCR (cat # 18080-400, Invitrogen Life Technologies). Quantitative real-time PCR (qPCR) was carried out using the CFX384 Real-Time System (Bio-Rad). Reactions were run in triplicate or quadruplicates and expression of each gene was normalized to the geometric mean of 18s and β-actin as housekeeping genes and WT values to generate ΔΔCT. The primer sequences of each gene can be provided upon request.

**Western blot**

Western blotting was performed as previously described in a published study from our laboratory (Usui et al., 2017). Individual organoids were lysed in radioimmunoprecipitation assay (RIPA) buffer containing protease inhibitors. Protein concentrations were determined through a Bradford assay (Bio-Rad Laboratories).
30-50 μg of proteins for each of the genotypes were run on an SDS-PAGE gel for two hours at RT and transferred to an immune-Blot PVDF Membrane (Bio-Rad Laboratories) for 16 hours at 4°C. Blots were imaged using an Odyssey Infrared Imaging System (LI-COR Biosciences). GAPDH (cat #MAB374, Milipore, 1:1000 dilution), FOXP1 (cat #2005S, Cell Signaling, 1:1000 dilution), and FOXP1 (Spiteri et al., 2007)(1:1000 dilution) antibodies were used.

**Immunocytochemistry (ICC) staining**

The organoid samples were fixed with 4% paraformaldehyde at 4°C overnight on a shaker. The next day, the organoids were washed 3 times for 5 minutes with PBS and transferred to 30% sucrose for another overnight incubation at 4°C. Then, the samples were carefully embedded in Tissue-Tek CRYO-OCT Compound (cat #14373-65, Thermo Fisher Scientific), which slowly solidified on dry ice. The frozen organoid samples were sectioned at 20 μM thickness with a cryostat and warmed to room temperature. Some antibodies were subjected to antigen retrieval using citrate buffer (10 mM tri-sodium citrate, 0.05% Tween-20, pH 6) for 10 min at 95 °C. Free aldehydes were quenched with 0.3 M glycine in TBS for 1 h at room temperature. This was followed by blocking for 1 hr at RT in 1% (w/v) bovine serum albumin (BSA) and 5% (v/v) normal donkey serum (NDS) in TBST (150 mM NaCl, 10 mM Tris pH 8, 0.05% Tween 20). The primary antibodies were diluted in the blocking buffer and incubated for two nights at 4°C. Coverslips were then washed five times in TBS and incubated overnight at 4°C with the relevant fluorescent-conjugated secondary antibody. Sections were washed 3 times, stained for DAPI for 5 minutes and then washed twice more. Finally, coverslips were mounted using ProLong Diamond Antifade Mountant (cat #P35970, Thermo Fisher Scientific). Analysis of cell morphology and differentiation was performed across three separate batches of differentiation experiments, using at least 3 samples per experiment. Only sections from the middle of the organoids were used. WT and KO samples were sectioned and mounted onto the same slide so that the immunostaining condition were the same across different genotypes. Only DAPI+ cells were analyzed. Antibodies used were: FOXP1 (cat #2005S, Cell Signaling, 1:1000 dilution), FOXP1 (Spiteri et al., 2007)(1:1000 dilution), FOXG1 (cat #18259, Abcam, 1:500 dilution), SOX2 (ab3045S, Abcam, Cell Signaling, 1:500 dilution), SOX2 (cat #sc17320, Satan Cruz, 1:500 dilution), PAX6 (cat # HPA030775, Sigma Aldrich, 1:1000 dilution), EOMES (cat # AF6166SP, Fisher Scientific, 1:500 dilution), EOMES (cat #ab23345, Abcam, 1:500 dilution), HOPX (cat #11419-1-AP, Proteintech, 1:100 dilution), TBR1
(cat #ab31940, Abcam, 1:100 dilution), CTIP2 (cat # ab18465, Abcam, 1:500 dilution), PTPRZ1 (cat #HPA015103, Sigma Aldrich, 1:500), BrdU (cat # MA5-11285, Thermo Fisher, 1:500 dilution), BrdU (cat #ab6326, Abcam, 1:1000 dilution), GFP (cat #600-101-215, Rockland, 1:1000 dilution), GFP (cat #GFP-1010, Aves, 1:1000 dilution), pVimentin (cat # 50-459-01, MBL international, 1:1000 dilution), and SATB2 (cat #51502, Abcam, 1:500 dilution).

**Microscope imaging and analysis**

Images were generated using a Zeiss confocal laser scanning microscope (LSM880). 6 z-stack images for samples with 16uM thickness were collected using a 20x lens within a 1024x1024 pixel field of view across all images and averaged per section. All the results were quantified either manually using ImageJ (http://imagej.nih.gov/ij) or automatically using CellProfiler (http://cellprofiler.org). Differences between genotypes or conditions were assessed using either the combination of t-test and Kolmogorov-Smirnov test or a one-way ANOVA with multiple comparisons and Brown-Forsythe and Welch ANOVA tests. GraphPad Prism software was used to perform statistical tests and obtain p-values. Sample size for each experiment is indicated in the figure legends.

**Analyses of cortical neural cell distribution**

Analyses of the number of different cell types - RGCs, IPCs, ENs or any combination of their overlaps - were performed following previously described methods (Lancaster et al., 2013; Qian et al., 2016) with some modifications. The modifications are as follows: for staining, we used the middle 8-10 sections of each organoid at 20 uM thickness. Since the size and shape of the cortical structures varied within the same organoid as well as across different ones, we took images of all the cortical structures whose shape did not overlap or fuse with another cortical structure and were positive for dorsal telencephalic markers unbiasedly. In addition to the cell type specific markers, well-differentiated organoids show individual cortical structures with a clear presence of a ventricular space, a VZ that is full of radially organized cells, and a dense CP that is separated from the VZ by a cell-sparse zone. We drew a rectangular ROI (477 x 238 microns) from the basal surface to the apical surface and quantified the number of cells either manually using FIJI or automatically using CellProfiler for each image. ROI drawing and quantifications were performed in a bias free manner and the same quantification method was
used across the genotypes in every experiment throughout the data generation. For each experiment, we used n=3-8 WT, KO-1, and KO-2 organoids that were generated from the isogenic iPSC lines.

**AD-EGFP infection**

AD-EGFP (cat#106, vector lab) was added to media at 1:2,000 and incubated for 24 hours. The virus was added at or around D40 (±2-3 days), incubated for 24 hours, and organoids were collected 72 hours later. The organoids were then stained with anti-GFP (cat #AB011, Evrogen, 1:1000 dilution) for better visualization of GFP infected cells.

**BrdU labeling proliferation and differentiation assays**

Cell proliferation and differentiation rates were determined by labeling with 5-bromo-2-deoxyuridine (BrdU, cat # B5002, Sigma-Aldrich). The organoids were pulsed with a single dose of 100uM BrdU for 2 hours, washed 3 times with PBS and either harvested right away or chased in the organoid medium without BrdU for 24 hours. Anti-BrdU (cat# ab6326, Abcam or cat# MA5-11285, Thermo Fisher Scientific, 1:400 dilution) was used in conjunction with anti-EOMES (cat #50-4877-42 Thermo Fisher Scientific or cat# AF6166SP Thermo Fisher Scientific, 1:400 dilution) or anti-CTIP2 (cat #ab18465, Abcam, 1:400 dilution) to examine proliferating cells in active S-phase or differentiating cells that had just finished the last division to become neurons, respectively.

**Cell cycle exit analysis**

In conjunction with BrdU, which marks cells going through S-phase, anti-Ki67 (cat #ab15580, Abcam) was also used to examine cells that are in all stages of the cell cycle (S, G1, M, and G2). The difference between BrdU⁺ and Ki67⁺ cells (BrdU⁺Ki67⁻) was used to examine proliferating or differentiating G-M cell population, which reflected the number of cells exiting the cell cycle.

**Nuclei isolation and library generation**

Nuclei isolation was performed following published methods (Ayhan et al., 2021; Habib et al., 2017). snRNA-seq was performed using a 10x Genomics Chromium system following published methods (Ayhan et al., 2021).
Cortical regions of the organoids were micro-dissected using the following method (Camp et al., 2015). The organoids used in these experiments were n=3 for each genotype: iPSC-derived WT, FOXP1 KO-1 and FOXP1 KO-2. 10,000 nuclei per sample per genotype were targeted for the experiment. Droplet-based snRNA-seq libraries were prepared using the Chromium Single Cell 3’ v3 kit (cat #120237 10x Genomics) according to the manufacturer’s protocol and were sequenced using an Illumina Nova-Seq 6000 and NEXT-seq 500 for a total of over 3.5 billion reads.

**Sequence alignment and counting**

Raw sequencing data were acquired from the North Texas Genome Center at the University of Texas at Arlington and McDermott Sequencing Core at UT Southwestern in the form of binary base call (BCL) files. Raw BCL files were then demultiplexed with 10x Genomics i7 indices (used during library preparation) using Illumina’s bcl2fastq v2.19.1 and ‘cellranger mkfastq’ from 10x Genomics CellRanger v3.0.2 tools. Extracted paired-end reads (28 bp long R1 – 16 bp 10x cell barcode and 12 bp UMI sequence information, 124 bp long R2 - transcript sequence information from cDNA fragment) were first checked for read quality using FASTQC v0.11.5 (FastQC, Babraham Bioinformatics, URL: [https://www.bioinformatics.babraham.ac.uk/projects/fastqc](https://www.bioinformatics.babraham.ac.uk/projects/fastqc)) Extracted paired-end reads were then aligned to the reference human genome (GRCh38.p12) from University of California Santa Cruz (UCSC) genome browser and reference human annotation (Gencode v28) and counted using ‘cellranger count’ from 10x Genomics CellRanger v3.0.2 tools. Since the nuclear transcriptome contained unspliced transcripts, reads mapping to a pre-mRNA reference file were counted. The resulting raw UMI count matrix contains genes as rows and nuclei as columns and was further used for downstream analysis.

**Clustering and cell-type annotation**

Raw and combined UMI counts for a total of 151,336 nuclei (62,899 nuclei for WT, 58,466 nuclei for KO and 29,971 nuclei for KO2) were used for clustering using the Seurat R analysis pipeline (from 10X Genomics CellRanger v3.0.1 tools). For each genotype, nuclei with more than 15,000 molecules (number of UMIs per nucleus) and nuclei with more than 10% mitochondrial content were filtered out to discard potential doublets and unhealthy cells. Also, genes with no expression in any nucleus and genes from chromosomes X, Y and M were removed. Seurat objects with filtered datasets for each genotype (59,041 nuclei for WT, 53,230 nuclei for KO
and 28,438 nuclei for KO2) were then normalized using ‘sctransform’ and scored for cell-cycle genes following Seurat guidelines. Individual sctransformed and cell-cycle scored Seurat objects were then integrated using the reciprocal PCA (RPCA) approach and clustered using the original Louvain algorithm. Clusters were visualized with Uniform Manifold Approximation and Projection (UMAP) (Becth et al., 2019; Kulkarni et al., 2019) in two dimensions. A resolution of 1.2 was selected based on clustering stability using ‘clustree’ R package (Zappia and Oshlack, 2018). Clusters were further annotated into a broad category of cell types using the gene markers enriched (FDR ≤ 0.05 and log2 (fold change) ≥ 0.25) in every cluster identified using ‘FindAllMarkers’ and performing Fisher-exact based enrichment against cell classes (Kanton et al., 2019; Nowakowski et al., 2017). Further, only nuclei identified as from the dorsal telencephalic lineage (DTL) were retained by sub-setting the Seurat object. 13 clusters (out of 36) of non-telencephalic or unknown identity were removed. Raw counts for a total of 116,607 DTL nuclei (50,533 nuclei for WT, 40,028 nuclei for KO and 26,046 nuclei for KO2) were then integrated using RPCA, followed by identifying clusters for DTL lineage nuclei. Clustered nuclei were visualized using UMAP. A resolution of 1.6 was selected based on clustering stability using the ‘clustree’ R package (Zappia and Oshlack, 2018). Clusters were further annotated into cell-types using the gene markers enriched in every cluster identified using ‘FindAllMarkers’ and performing Fisher-exact based enrichment against cell classes (Kanton et al., 2019; Nowakowski et al., 2017). The identification of bRGC clusters was determined by a Fisher-exact based enrichment analysis of cluster of RG types with genes annotated as markers of bRGCs (Heng et al., 2017). Clusters that were identified as non-dorsal telencephalic cortical cell types and inhibitory interneurons based on our annotation method were removed from the analysis. We also further confirmed the removal of these cell types using the marker genes derived from a published study (Kanton et al., 2019). Out of 140,709 total number of nuclei, 24,102 nuclei and 7,191 nuclei that were annotated as non-dorsal telencephalic cortical cell types and inhibitory neurons (IN), respectively, were discarded from the final dataset that was used for downstream analysis.

**Pseudobulk differential expression analysis**

Clusters were grouped into broad categories such as RG, IPC and EN based on cell-type markers and enrichment against the reference dataset using Fisher’s exact test. For differential gene analysis, cells corresponding to WT or KO genotypes were grouped within each broad cellular category. Genes with altered
expression in KO were then identified using a Wilcoxon test from Seurat v3 (Butler et al., 2018) FDR ≤ 0.05 and log2(fold change) ≥ 0.25). The functional annotation of differentially expressed genes was performed using the ToppGene Suite (Chen et al., 2009) with a background of 8023 genes. The background genes are genes that are expressed in either WT or KO across all cell-types. Gene ontology categories with Benjamini-Hochberg FDR ≤ 0.05 were summarized using REVIGO (Supek et al., 2011).

Pseudotime trajectory analysis

The filtered Seurat object for DTL nuclei without IN neurons described above was first split into genotype-specific subsets for WT and KO and then converted into Monocle compatible objects using ‘as.cell_data_set’ command. Genotype-specific subsets were then pre-processed (cluster_cells, learn_graph) using the standard Monocle pipeline. Nuclei with the greatest SOX2, PAX6 and HES5 expression were then selected as a root population for performing pseudotime trajectory analysis (order_cells). UMAP plots colored by scaled pseudotime values were then generated accompanied by density plots and histograms corresponding to broad cell-types (RG, IPC, EN).

ASD gene enrichment analysis

The enrichment of pseudobulk differentially expressed genes and ASD-relevant genes was performed using the Fisher-exact test. Disease-relevant gene sets were used from a previous study (Berto et al., 2021). Fisher exact tests were performed in R with the following parameters: alternative = “greater”, conf.level = 0.85. Bubble dot plots were generated using odds ratio (OR) and Benjamini–Hochberg-adjusted P values (FDR).

Gene overlap analysis

Scaled Venn diagrams were made using https://www.biovenn.nl/. Gene overlap analysis was performed using Fisher’s exact test. DEGs from ChIP-seq experiments were obtained from a previous publication (Araujo et al., 2015).

Quantification and Statistical Analysis
For snRNA-seq transcriptomic data, non-parametric Wilcoxon rank-sum tests were used for differential gene analysis. The methods for differential gene expression using Seurat v3 are detailed in the ‘Pseudobulk differential expression analysis’ section. The results of differential gene expression analyses are listed in Table S2 and S3 and subsets of these comparisons are included in Figures 2, 4, and S8.

For statistical analysis of IHC quantification, individual organoids were treated as biological replicates. Organoid samples were randomly taken from the culture for experiments and analyses. The sample sizes were designed to account for the variability between organoids within the same batch of differentiation and meet current standards in human brain organoid-related studies. Data analyses comparing WT and FOXP1 KO organoids were performed blindly or using an automated quantification method that was applied similarly across the different genotypes. Data are presented as mean ± S.E.M., or mean ± S.D., unless otherwise indicated in the Figure Legends. Statistical analyses were performed using Prism software. The appropriate statistical tests for each experiment are stated in Figure Legends. Statistical significance was defined by p value or adjusted p-values < 0.05.

To calculate enrichment of overlapping datasets, Fisher’s exact test was used. A Benjamini-Hochberg adjusted p-value was applied as a multiple comparisons adjustment. The results of these tests are shown in Figures 2, 4, S3, and S8.

For gene ontology enrichments, a one-sided hypergeometric test was used to test overrepresentation of functional categories. A Benjamini-Hochberg adjusted p-value was applied as a multiple comparisons adjustment, and the results are shown in Figure S8 and Table S4.

**ADDITIONAL RESOURCES**

The data can also be accessed through an interactive R shiny application at the Github repository: https://github.com/konopkalab/organoidseq.
SUPPLEMENTAL INFORMATION

Figure S1 (Related to Figure 1). Brain organoid development over time.

(A) Organoid immunostaining at D25, D40, D60 and D100 showing expression of SOX2, TUJ1 and FOXP1. (B) Region of interest (ROI) selected from panel A showing FOXP1 expression. (C) Overlap between cell type markers and FOXP1. FOXG1 marks dorsal telencephalic lineage cells, TBR2 marks IPCs, and CTIP2 marks ENs. (D) Magnified images from panel C showing the overlap among cell type markers. (E) Organoids at D40 showing expression of TBR2 and another EN marker, CUX1. Scale bar = 100uM for panels A, C and E, 50uM for panel Fig B

Figure S2. snRNA-seq quality control and FOXP1 expression in the CRISPR edited organoids.

(A) FOXP1 expression level in each of the pseudo-bulked cell types, RGCs, IPCs and ENs per genotype. (B) Genome track files at the FOXP1 gene showing the pileup of the sequencing reads in this region of the genome for each genotype. (C) Percentage of FOXP1⁺ cells in individual clusters. (D) UMI count, number of detected genes and percentage mitochondria gene in each cluster. (E) UMI count, number of detected genes, and percentage mitochondrial genes in each genotype.

Figure S3. Representation of the diverse cell types in profiled in the organoids.

(A) Feature plots showing distribution of cells across different genotypes. (B) Violin plots showing gene expression of markers representing the dorsal telencephalic lineage versus other brain regions. OR=Odd’s ratio. (C) Dot plot showing gene expression correlation between our brain organoid dataset and a human fetal cortex scRNA-seq dataset from the second trimester of gestation (Nowakowski et al., 2017). Dotted lines in blue indicate bRGC cell clusters. (D) Violin plots showing the expression level of bRGC-marker genes (Pollen et al., 2015) that are differentially regulated between WT and KO in each bRGC subcluster. Y-axis represents log10(CPM) values.

Figure S4 (Related to STAR Methods). Similar gene expression pattern of KO-1 and KO-2.
(A) Correlation of DEGs between KO-1 and KO-2 organoids in all cells as well as each of the pseudo-bulked cell types, aRGC, bRGC, IPC and ENs. (B) Density plots showing the number of pseudobulked cells (aRGC, bRGC, IPC and EN) of KO-1 and KO-2 organoids separately.

Figure S5 (Related to Figure 3). bRGC-driven direct neurogenesis, quantification of PTPRZ+ bRGCS.

(A) Immunostaining showing PAX6, TBR2, and the bRGC marker PTPRZ in week 6 organoids. Scale bar = 50uM (B) Quantification of bRGCS using PTPRZ+TBR2- bRGCS. (C) Schematic showing the experimental design of the BrdU treatment at week 6. (D) Immunostaining showing CTIP2, HOPX, BrdU and DAPI in week 6 organoids. Scale bar = 50uM (E) Quantification of neurogenic bRGCS, which are CTIP2+ cells located next to HOPX+ cells. These cells may represent neurons born directly from bRGC to EN. For all quantifications, n=3-8 organoids per sample per genotype was used. In each organoid, 3-9 cortical structures with clear lamination patterns were examined. Data are represented in bar graphs as mean ± STD with individual data as dots; n.s. = not statistically significant, *p < 0.05, Kruskal-Wallis ANOVA test with Dunn’s multiple comparisons test as a post hoc.

Figure S6 (Related to Figure 3). Deletion of FOXP1 results in increased numbers and protracted differentiation of IPCs.

(A) Schematic showing changes observed in layering with the loss of FOXP1 (top) and examples of regions of interest (ROI) selected from left panel (bottom). SOX2 expression marks RGCs, TBR2 marks IPCs and CTIP2 marks ENs. Scale bar = 100uM for the upper images and 50uM for the ROI images on the bottom. (B) A summary diagram of the lamination changes between WT and KO organoids at D40. (C) Quantification of RGCs, IPCs and ENs. (D) Quantification of SOX2+ RGCs that express IPC marker TBR2. (E) Quantification of CTIP2+ ENs that express IPC marker TBR2. (F) Representative images of BrdU-treated brain organoids sectioned and stained for Ki67, BrdU, CTIP2 expression. Scale bar = 50uM (G) The difference between BrdU and Ki67 staining showing cell cycle exit rate in the CTIP2+ postmitotic neurons. For all quantifications, n=3-8 organoids per sample per genotype was used. In each organoid, 3-9 cortical structures with clear lamination patterns were examined. Data are represented in bar graphs as mean ± STD with individual data as dots; n.s. means p > 0.05, *p < 0.05, **p < 0.01, and ****p<0.0001. Mixed-effects analysis multiple comparisons test with Tukey’s multiple
comparisons test as a post hoc was used for panel C. Kruskal-Wallis ANOVA test with Dunn’s multiple comparisons test as a post hoc was used for supplementary figure 6 D and E.

**Figure S7 (Related to Figure 3). Impaired late neurogenesis in 3 month (D100) organoids.**

(A) Immunostaining images of D100 organoids showing expression of SOX2 for RGCs, SATB2 for ENs, and FOXP1 in a representative WT organoid. Scale bar = 300uM. (B) Immunostaining showing IPCs (TBR2+) and ENs (SATB2+) at D100. Scale bar = 300uM for panel B on the left and 100uM for ROI selected images on the right. (C) - (F) Immunostaining results showing percentage of RGCs, IPCs and ENs at D100 normalized to all DAPI+ cells. For all quantifications, n=3-8 organoids per sample per genotype was used. In each organoid, 3-9 cortical structures with clear lamination patterns were examined. Data are represented in bar graphs as mean ± STD with individual data as dots; n.s. means p > 0.05, ***p <0.001, and ****p <0.0001 Kruskal-Wallis ANOVA test with Dunn’s multiple comparisons test as a post hoc was used.

**Figure S8 (Related to Figure 4). Differential expression analysis of bRGC subtypes.**

(A) Number of DEGs identified in bRGC clusters. (log2(FC) ≥ 0.25, FDR ≤ 0.05, percent expression > 0.10) (B) Summarized biological process GO terms for upregulated and downregulated DEGs for each bRGC cluster. Significantly changed gene categories are defined as Benjamini-Hochberg FDR ≤ 0.05 (dashed line in red). (C) Scaled Venn diagram showing the overlap between the DEGs in bRGCs and a previously published chromatin immunoprecipitation assay with sequencing (ChIP-seq) dataset from our laboratory (Araujo et al., 2015). Fisher’s exact test was performed to calculate statistical significance.

**Figure S9 (Related to STAR Methods). Representative karyotype analysis for iPSCs G-banding technique showing normal chromosomes of metaphase cells across all three genotypes.**

**Supplementary Tables**

**Table S1:** Sequencing read numbers, number of nuclei sequenced, number of expressed genes, and number of UMIs. Related to STAR Methods

**Table S2:** List of DEGs per major cell type. Related to Figure 4 and S8
Table S3: List of DEGs from bRGC subtypes. Related to Figure S8

Table S4: Gene ontology biological processes for bRGC subtypes. Related to Figure S8
REFERENCES

Anderson, A.G., Kulkarni, A., Harper, M., and Konopka, G. (2020). Single-Cell Analysis of Foxp1-Driven Mechanisms Essential for Striatal Development. Cell reports 30, 3051-3066.e3057. 10.1016/j.celrep.2020.02.030 PMID - 32130906.

Andrews, M.G., Subramanian, L., and Kriegstein, A.R. (2020). mTOR signaling regulates the morphology and migration of outer radial glia in developing human cortex. eLife 9, e58737. 10.7554/elife.58737 PMID - 32876565.

Araujo, D.J., Anderson, A.G., Berto, S., Runnels, W., Harper, M., Ammanuel, S., Rieger, M.A., Huang, H.-C., Rajkovich, K., Loerwald, K.W., et al. (2015). FoxP1 orchestration of ASD-relevant signaling pathways in the striatum. Genes & Development 29, 2081-2096. 10.1101/gad.267989.115 PMID - 26494785.

Ayhan, F., Kulkarni, A., Berto, S., Sivaprakasam, K., Douglas, C., Lega, B.C., and Konopka, G. (2021). Resolving cellular and molecular diversity along the hippocampal anterior-to-posterior axis in humans. Neuron 109, 2091-2105.e2096. 10.1016/j.neuron.2021.05.003.

Bacon, C., Schneider, M., Magueresse, C.L., Froehlich, H., Sticht, C., Gluch, C., Monyer, H., and Rappold, G.A. (2015). Brain-specific Foxp1 deletion impairs neuronal development and causes autistic-like behaviour. Molecular Psychiatry 20, 632-639. 10.1038/mp.2014.116 PMID - 25266127.

Bareiss, S., Kim, K., and Lu, Q. (2010). Delta-catennin/NPRAP: A new member of the glycogen synthase kinase-3beta signaling complex that promotes beta-catennin turnover in neurons. J Neurosci Res 88, 2350-2363. 10.1002/jnr.22414.

Becht, E., McInnes, L., Healy, J., Dutertre, C.-A., Kwok, I.W.H., Ng, L.G., Ginhoux, F., and Newell, E.W. (2019). Dimensionality reduction for visualizing single-cell data using UMAP. Nature Biotechnology 37, 38-44. 10.1038/nbt.4314.

Bella, D.J.D., Habibi, E., Stickels, R.R., Scalia, G., Brown, J., Yadollahpour, P., Yang, S.M., Abbate, C., Biancalani, T., Macosko, E.Z., et al. (2021). Molecular logic of cellular diversification in the mouse cerebral cortex. Nature 595, 554-559. 10.1038/s41586-021-03670-5 PMID - 34163074.

Berto, S., Fontenot, M.R., Seger, S., Ayhan, F., Caglayan, E., Kulkarni, A., Douglas, C., Tamminga, C.A., Lega, B.C., and Konopka, G. (2021). Gene expression correlates of the oscillatory signatures supporting human episodic memory encoding. Nature neuroscience 24, 554-564. 10.1038/s41593-021-00803-x PMID - 33686299.

Betizeau, M., Cortay, V., Patti, D., Pfister, S., Gautier, E., Bellemin-Ménard, A., Afanassieff, M., Huissoud, C., Douglas, Rodney J., Kennedy, H., and Dehay, C. (2013). Precursor Diversity and Complexity of Lineage Relationships in the Outer Subventricular Zone of the Primate. Neuron 80, 442-457. 10.1016/j.neuron.2013.09.032 PMID - 24139044.

Bienz, M. (2005). beta-Catenin: a pivot between cell adhesion and Wnt signalling. Curr Biol 15, R64-67. 10.1016/j.cub.2004.12.058.

Braccioli, L., Vervoort, S.J., Adolfs, Y., Heijnen, C.J., Basak, O., Pasterkamp, R.J., Nijboer, C.H., and Coffer, P.J. (2017). FOXP1 Promotes Embryonic Neural Stem Cell Differentiation by Repressing Jagged1 Expression. Stem Cell Reports 9, 1530 - 1545. 10.1016/j.stemcr.2017.10.012.

Butler, A., Hoffman, P., Smibert, P., Papalexi, E., and Satija, R. (2018). Integrating single-cell transcriptomic data across different conditions, technologies, and species. Nature Biotechnology 36, 411-420. 10.1038/nbt.4096.
Camp, J.G., Badsha, F., Florio, M., Kanton, S., Gerber, T., Wilsch-Bräuninger, M., Lewitus, E., Sykes, A., Hevers, W., Lancaster, M., et al. (2015). Human cerebral organoids recapitulate gene expression programs of fetal neocortex development. Proceedings of the National Academy of Sciences 112, 15672-15677. 10.1073/pnas.1520760112 PMID - 26644564.

Cao, J., Spielmann, M., Qiu, X., Huang, X., Ibrahim, D.M., Hill, A.J., Zhang, F., Mundlos, S., Christiansen, L., Steemers, F.J., et al. (2019). The single cell transcriptional landscape of mammalian organogenesis. Nature 566, 496-502. 10.1038/s41586-019-0969-x PMID - 30787437.

Chen, J., Bardes, E.E., Aronow, B.J., and Jegga, A.G. (2009). ToppGene Suite for gene list enrichment analysis and candidate gene prioritization. Nucleic Acids Res 37, W305-W311. 10.1093/nar/gkp427.

Cho, E.G., Zaremba, J.D., McKercher, S.R., Talantova, M., Tu, S., Masliah, E., Chan, S.F., Nakanishi, N., Terskikh, A., and Lipton, S.A. (2011). MEF2C enhances dopaminergic neuron differentiation of human embryonic stem cells in a parkinsonian rat model. PLoS One 6, e24027. 10.1371/journal.pone.0024027.

Chou, S.M., Li, K.X., Huang, M.Y., Chen, C., Lin King, Y.H., Li, G.G., Zhou, W., Teo, C.F., Jan, Y.N., Jan, L.Y., and Yang, S.B. (2021). Kv1.1 channels regulate early postnatal neurogenesis in mouse hippocampus via the TrkB signaling pathway. Elife 10. 10.7554/eLife.58779.

Co, M., Anderson, A.G., and Konopka, G. (2020). FOXP transcription factors in vertebrate brain development, function, and disorders. Wiley Interdisciplinary Reviews: Developmental Biology 9, e375. 10.1002/wdev.375 PMID - 31999079.

Dennis, M.Y., and Eichler, E.E. (2016). Human adaptation and evolution by segmental duplication. Current Opinion in Genetics & Development 41, 44-52. 10.1016/j.gde.2016.08.001 PMID - 27584858.

Fan, X., Fu, Y., Zhou, X., Sun, L., Yang, M., Wang, M., Chen, R., Wu, Q., Yong, J., Dong, J., et al. (2020). Single-cell transcriptome analysis reveals cell lineage specification in temporal-spatial patterns in human cortical development. Science Advances 6, eaaz2978. 10.1126/sciadv.aaz2978 PMID - 32923614.

Gallagher, D., Voronova, A., Zander, Mark A., Cancino, Gonzalo I., Bramall, A., Krause, Matthew P., Abad, C., Tekin, M., Neilsen, Paul M., Callen, David F., et al. (2015). Ankrd11 Is a Chromatin Regulator Involved in Autism that Is Essential for Neural Development. Developmental Cell 32, 31-42. 10.1016/j.devcel.2014.11.031 PMID - 25556659.

Habib, N., Avraham-David, I., Basu, A., Burks, T., Shekhar, K., Hofree, M., Choudhury, S.R., Aguet, F., Gelfand, E., Ardlie, K., et al. (2017). Massively parallel single-nucleus RNA-seq with DroNc-seq. Nature Methods 14, 955-958. 10.1038/nmeth.4407.

Heng, X., Guo, Q., Leung, A.W., and Li, J.Y. (2017). Analogous mechanism regulating formation of neocortical basal radial glia and cerebellar Bergmann glia. Elife 6. 10.7554/eLife.23253.

Jansson, L.C., Louhivuori, L., Wigren, H.K., Nordström, T., Louhivuori, V., Castrén, M.L., and Åkerman, K.E. (2013). Effect of glutamate receptor antagonists on migrating neural progenitor cells. Eur J Neurosci 37, 1369-1382. 10.1111/ejn.12152.

Kalebic, N., Gilardi, C., Stepien, B., Wilsch-Brauning, M., Long, K.R., Namba, T., Florio, M., Langen, B., Lombardot, B., Shevchenko, A., et al. (2019). Neocortical Expansion Due to Increased Proliferation of Basal Progenitors Is Linked to Changes in Their Morphology. Cell Stem Cell 24, 535-550 e539. 10.1016/j.stem.2019.02.017.

Kanton, S., Boyle, M.J., He, Z., Santel, M., Weigert, A., Sanchis-Calleja, F., Guijarro, P., Sidow, L., Fleck, J.S., Han, D., et al. (2019). Organoid single-cell genomic atlas uncovers human-specific features of brain development. Nature 574, 418-422. 10.1038/s41586-019-1654-9.
Kelava, I., Chiaradia, I., Pellegrini, L., Kalinka, A.T., and Lancaster, M.A. (2022). Androgens increase excitatory neurogenic potential in human brain organoids. Nature 602, 112-116. 10.1038/s41586-021-04330-4.

Kelava, I., and Lancaster, M.A. (2016). Dishing out mini-brains: Current progress and future prospects in brain organoid research. Developmental Biology 420, 199-209. 10.1016/j.ydbio.2016.06.037 PMID - 27402594.

Kim, H., Han, J.R., Park, J., Oh, M., James, S.E., Chang, S., Lu, Q., Lee, K.Y., Ki, H., Song, W.J., and Kim, K. (2008). Delta-catenin-induced dendritic morphogenesis. An essential role of p190RhoGEF interaction through Akt1-mediated phosphorylation. J Biol Chem 283, 977-987. 10.1074/jbc.M707158200.

Kulkarni, A., Anderson, A.G., Merullo, D.P., and Konopka, G. (2019). Beyond bulk: a review of single cell transcriptomics methodologies and applications. Curr Opin Biotechnol 58, 129-136. 10.1016/j.copbio.2019.03.001.

Lancaster, M.A., Renner, M., Martin, C.-A., Wenzel, D., Bicknell, L.S., Hurles, M.E., Homfray, T., Penninger, J.M., Jackson, A.P., and Knoblich, J.A. (2013). Cerebral organoids model human brain development and microcephaly. Nature 501, 373-379. 10.1038/nature12517.

Lancaster, M.A., Renner, M., Martin, C.-A., Wenzel, D., Bicknell, L.S., Hurles, M.E., Homfray, T., Penninger, J.M., Jackson, A.P., and Knoblich, J.A. (2013). Cerebral organoids model human brain development and microcephaly. Nature 501, 373-379. 10.1038/nature12517.

Li, H., Radford Jonathan, C., Ragusa Michael, J., Shea Katherine, L., McKercher Scott, R., Zaremba Jeffrey, D., Soussou, W., Nie, Z., Kang, Y.-J., Nakanishi, N., et al. (2008). Transcription factor MEF2C influences neural stem/progenitor cell differentiation and maturation in vivo. Proceedings of the National Academy of Sciences 105, 9397-9402. 10.1073/pnas.0802876105.

Li, X., Xiao, J., Fröhlich, H., Tu, X., Li, L., Xu, Y., Cao, H., Qu, J., Rappold, G.A., and Chen, J.-G. (2015). Foxp1 regulates cortical radial migration and neuronal morphogenesis in developing cerebral cortex. PloS one 10, e0127671-e0127671. 10.1371/journal.pone.0127671.

Lozano, R., Gbekie, C., Siper, P.M., Srivastava, S., Saland, J.M., Sethuram, S., Tang, L., Drapeau, E., Frank, Y., Buxbaum, J.D., and Kolevzon, A. (2021). FOXP1 syndrome: a review of the literature and practice parameters for medical assessment and monitoring. Journal of Neurodevelopmental Disorders 13, 18. 10.1186/s11689-021-09358-1 PMID - 33892622.

Lui, Jan H., Hansen, David V., and Kriegstein, Arnold R. (2011). Development and Evolution of the Human Neocortex. Cell 146, 18-36. 10.1016/j.cell.2011.06.030 PMID - 21729779.

Mora-Bermúdez, F., Badsha, F., Kanton, S., Camp, J.G., Vernot, B., Köhler, K., Voigt, B., Okita, K., Maricic, T., He, Z., et al. (2016). Differences and similarities between human and chimpanzee neural progenitors during cerebral cortex development. eLife 5, e18683. 10.7554/elife.18683 PMID - 27669147.

Nopparat, J., Zhang, J., Lu, J.P., Chen, Y.H., Zheng, D., Neufer, P.D., Fan, J.M., Hong, H., Boykin, C., and Lu, Q. (2015). β-Catenin, a Wnt/β-catenin modulator, reveals inducible mutagenesis promoting cancer cell survival adaptation and metabolic reprogramming. Oncogene 34, 1542-1552. 10.1038/onc.2014.89.

Nowakowski, T.J., Bhaduri, A., Pollen, A.A., Alvarado, B., Mostajo-Radji, M.A., Lullo, E.D., Haeussler, M., Sandoval-Espinoza, C., Liu, S.J., Velmeshev, D., et al. (2017). Spatiotemporal gene expression trajectories reveal developmental hierarchies of the human cortex. Science 358, 1318 - 1323. 10.1126/science.aap8809.

Ostrem, B.E., Lui, J.H., Gertz, C.C., and Kriegstein, A.R. (2014). Control of outer radial glial stem cell mitosis in the human brain. Cell Rep 8, 656-664. 10.1016/j.celrep.2014.06.058.

Otani, T., Marchetto, M.C., Gage, F.H., Simons, B.D., and Livesey, F.J. (2016). 2D and 3D Stem Cell Models of Primate Cortical Development Identify Species-Specific Differences in Progenitor Behavior Contributing to Brain Size. Cell Stem Cell 18, 467-480. 10.1016/j.stem.2016.03.003 PMID - 27049876.

Parikshak, N.N., Gandal, M.J., and Geschwind, D.H. (2015). Systems biology and gene networks in neurodevelopmental and neurodegenerative disorders. Nat Rev Genet 16, 441-458. 10.1038/nrg3934.
Pearson, C.A., Moore, D.M., Tucker, H.O., Dekker, J.D., Hu, H., Miquelajuregui, A., and Novitch, B.G. (2020). Foxp1 Regulates Neural Stem Cell Self-Renewal and Bias Toward Deep Layer Cortical Fates. Cell Reports 30, 1964-1981.e11963. 10.1016/j.celrep.2020.01.034 PMID - 32049024.

Pollen, A.A., Nowakowski, T.J., Chen, J., Retallack, H., Sandoval-Espinosa, C., Nicholas, C.R., Shuga, J., Liu, S.J., Oldham, M.C., Diaz, A., et al. (2015). Molecular identity of human outer radial glia during cortical development. Cell 163, 55-67. 10.1016/j.cell.2015.09.004.

Qian, X., Nguyen, Ha N., Song, Mingxi M., Hadiono, C., Ogden, Sarah C., Hammack, C., Yao, B., Hamersky, Gregory R., Jacob, F., Zhong, C., et al. (2016). Brain-Region-Specific Organoids Using Mini-bioreactors for Modeling ZIKV Exposure. Cell 165, 1238-1254. 10.1016/j.cell.2016.04.032 PMID - 27118425.

Satterstrom, F.K., Kosmicki, J.A., Wang, J., Breen, M.S., Rubeis, S.D., An, J.-Y., Peng, M., Collins, R., Grove, J., Klei, L., et al. (2020). Large-Scale Exome Sequencing Study Implicates Both Developmental and Functional Changes in the Neurobiology of Autism. Cell 180, 568-584.e523. 10.1016/j.cell.2019.12.036 PMID - 31981491.

Schaffer, A.E., Breuss, M.W., Caglayan, A.O., Al-Sanaa, N., Al-Abdulwahed, H.Y., Kaymakçalan, H., Yilmaz, C., Zaki, M.S., Rosti, R.O., Copeland, B., et al. (2018). Bi-allelic loss of human CTNNA2, encoding αN-catenin, leads to ARP2/3 over-activity and disordered cortical neuronal migration. Nature genetics 50, 1093-1101. 10.1038/s41588-018-0166-0 PMID - 30013181.

Schmid-Burgk, J.L., Höning, K., Ebert, T.S., and Hornung, V. (2016). CRISPaint allows modular base-specific gene tagging using a ligase-4-dependent mechanism. Nat Commun 7, 12338. 10.1038/ncomms12338.

Siper, P.M., Rubeis, S.D., Trelles, M.d.P., Durkin, A., Marino, D.D., Muratet, F., Frank, Y., Lozano, R., Eichler, E.E., Kelly, M., et al. (2017). Prospective investigation of FOXP1 syndrome. Molecular Autism 8, 57. 10.1186/s13229-017-0172-6 PMID - 29090079.

Spiteri, E., Konopka, G., Coppola, G., Bomar, J., Oldham, M., Ou, J., Vernes, S.C., Fisher, S.E., Ren, B., and Geschwind, D.H. (2007). Identification of the transcriptional targets of FOXP2, a gene linked to speech and language, in developing human brain. Am J Hum Genet 81, 1144-1157. 10.1086/522237.

State, M.W., and Sestan, N. (2012). Neuroscience. The emerging biology of autism spectrum disorders. Science 337, 1301-1303. 10.1126/science.1224989.

Steyer, B., Bu, Q., Cory, E., Jiang, K., Duong, S., Sinha, D., Steltzer, S., Gamm, D., Chang, Q., and Saha, K. (2018). Scarless Genome Editing of Human Pluripotent Stem Cells via Transient Puromycin Selection. Stem cell reports 10, 642-654. 10.1016/j.stemcr.2017.12.004.

Supek, F., Bošnjak, M., Škunca, N., and Šmuc, T. (2011). REVIGO Summarizes and Visualizes Long Lists of Gene Ontology Terms. PLOS ONE 6, e21800. 10.1371/journal.pone.0021800.

Urreizti, R., Damanti, S., Esteve, C., Franco-Valls, H., Castilla-Vallmanya, L., Tonda, R., Cormand, B., Vilageliu, L., Opitz, J.M., Neri, G., et al. (2018). A De Novo FOXP1 Truncating Mutation in a Patient Originally Diagnosed as C Syndrome. Scientific Reports 8, 694. 10.1038/s41598-017-19109-9.

Usui, N., Co, M., Harper, M., Rieger, M.A., Dougherty, J.D., and Konopka, G. (2017). Sumoylation of FOXP2 Regulates Motor Function and Vocal Communication Through Purkinje Cell Development. Biological Psychiatry 81, 220-230. 10.1016/j.biopsych.2016.02.008 PMID - 27009683.

Vaid, S., and Huttner, W.B. (2020). Transcriptional Regulators and Human-Specific/Primate-Specific Genes in Neocortical Neurogenesis. International Journal of Molecular Sciences 21, 4614. 10.3390/ijms21134614 PMID - 32610533.
Wang, B., Weidenfeld, J., Lu, M.M., Maika, S., Kuziel, W.A., Morrisey, E.E., and Tucker, P.W. (2004). Foxp1 regulates cardiac outflow tract, endocardial cushion morphogenesis and myocyte proliferation and maturation. Development 131, 4477-4487. 10.1242/dev.01287.

Xing, L., and Huttner, W.B. (2020). Neurotransmitters as Modulators of Neural Progenitor Cell Proliferation During Mammalian Neocortex Development. Front Cell Dev Biol 8, 391. 10.3389/fcell.2020.00391.

Xu, G., Strathearn, L., Liu, B., and Bao, W. (2018). Prevalence of Autism Spectrum Disorder Among US Children and Adolescents, 2014-2016. Jama 319, 81-82. 10.1001/jama.2017.17812.

Young, A., Machacek, D.W., Dhara, S.K., Macleish, P.R., Benveniste, M., Dodla, M.C., Sturkie, C.D., and Stice, S.L. (2011). Ion channels and ionotropic receptors in human embryonic stem cell derived neural progenitors. Neuroscience 192, 793-805. 10.1016/j.neuroscience.2011.04.039.

Zappia, L., and Oshlack, A. (2018). Clustering trees: a visualization for evaluating clusterings at multiple resolutions. Gigascience 7. 10.1093/gigascience/giy083.

Zhang, P., Schaefer-Klein, J., Cheville, J.C., Vasmatzis, G., and Kovtun, I.V. (2018). Frequently rearranged and overexpressed δ-catenin is responsible for low sensitivity of prostate cancer cells to androgen receptor and β-catenin antagonists. Oncotarget 9, 24428-24442. 10.18632/oncotarget.25319.

Zhong, S., Zhang, S., Fan, X., Wu, Q., Yan, L., Dong, J., Zhang, H., Li, L., Sun, L., Pan, N., et al. (2018). A single-cell RNA-seq survey of the developmental landscape of the human prefrontal cortex. Nature 555, 524-528. 10.1038/nature25980.
Figure 1. Generation of FOXP1 KO cerebral organoids from stem cells. (A) Schematic diagram showing the experimental design. (B) Cerebral organoid protocol illustrating typical morphology observed at each time point during differentiation from embryoid body formation to maturation. Scale bar = 600 uM (C) The two CRISPR-Cas9 strategies used in generating FOXP1 KOs. (D) Representative immunostaining results showing FOXP1 expression in SOX2+ and TUJ1+ cells in WT and absence of FOXP1 expression in the KOs at day 40 (D40) in vitro. Scale bar = 100 uM (E) Representative Western blot results with quantification from n=4.
Figure 2. snRNA-seq and pseudotime analysis of WT and FOXP1 KO organoids. (A) Representation of diverse cell types in UMAP space. WT, KO-1 and KO-2 datasets are integrated and shown in the same UMAP. (B) A UMAP highlighting bRGC clusters 17 and 25. (C) Enrichment analysis of the bRGC genes (Heng, Guo et al. 2017) based on Fisher’s exact test. (D) UMAPs with a scaled color scheme as a function of gene expression changes from the root cells (SOX2+PAX6+HES5+). Changes in the basal progenitors are noted in a dotted line. WT (left) and the combined FOXP1 KO-1 and KO-2 datasets (right) are shown. (E) Density bar plots showing the number of pseudobulked cells (aRGC, bRGC, IPC and EN) in the relative pseudotime scale. The root (‘0’) is same as in the above UMAP.
Figure 3. Loss of FOXP1 results in decreased number of bRGCs. (A) Schematic showing the design of the ‘Ad-EGFP’ virus infection experiment. (B) Representative images of immunostaining performed on the ‘Ad-EGFP’ infected organoids at week 6 showing detection of bRGCs by morphology including loss of ventricular contact. (C) Quantification of progenitors that are without apical attachment and are TBR2-. (D) Schematic showing the design of the BrdU assays. Incubation with 100uM BrdU for 2 hours was followed by media change, 24-hour incubation and harvesting the organoids for fixation. (E) Representative images of immunostaining performed on BrdU treated organoids at week 6. (F) Schematic diagrams showing bRGC divisions. (G) Magnified images showing overlap between different markers. (H) Quantification of HOPX+ dividing bRGCs. (I) Quantification of HOPX+ basal progenitors going through neurogenesis via IPCs. For all quantifications, n=3-8 organoids per sample per genotype were used. In each organoid, 3-8 cortical structures with clear lamination patterns were examined. Data are represented in bar graphs as mean ± STD with individual data as dots; n.s. means p > 0.05, *p < 0.05, **p < 0.01, and ***p <0.001, Kruskal-Wallis ANOVA test with Dunn’s multiple comparisons test as a post hoc was used. Scale bar = 100uM.
Figure 4. Differential expression analysis. (A) Number of DEGs in each cell type. Significant DEGs are defined as log2(FC) ≥ 0.25, FDR ≤ 0.05, percent expression > 0.10. (B) Dot plots showing the enrichment of NDD-relevant DEGs in aRGC, bRGC, IPC and EN cell types. ASD = high confidence ASD genes with score 1-3 from SFARI, ID=intellectual disability relevant genes, and FMRP=Fragile X relevant genes. On the bottom are lists of ASD-associated genes enriched in bRGCs. OR=Odd’s ratio.
Figure S1. Brain organoid development over time. (A) Organoid immunostaining at D25, D40, D60 and D100 showing expression of SOX2, TUJ1 and FOXP1. (B) Region of interest (ROI) selected from panel A showing FOXP1 expression. (C) Overlap between cell type markers and FOXP1. FOXG1 marks dorsal telencephalic lineage cells, TBR2 marks IPCs, and CTIP2 marks ENs. (D) Magnified images from panel C showing the overlap among cell type markers. (E) Organoids at D40 showing expression of TBR2 and another EN marker, CUX1. Scale bar = 100uM for panels A, C and E, 50uM for panel B.
Figure S2

(A) FOXP1 expression level in each of the pseudo-bulked cell types, RGCs, IPCs and ENs per genotype. (B) Genome track files at the FOXP1 gene showing the pileup of the sequencing reads in this region of the genome for each genotype. (C) Percentage of FOXP1+ cells in individual clusters. (D) UMI count, number of detected genes and percentage mitochondria gene in each cluster. (E) UMI count, number of detected genes, and percentage mitochondrial genes in each genotype.

Figure S2. snRNA-seq quality control and FOXP1 expression in the CRISPR edited organoids. (A) FOXP1 expression level in each of the pseudo-bulked cell types, RGCs, IPCs and ENs per genotype. (B) Genome track files at the FOXP1 gene showing the pileup of the sequencing reads in this region of the genome for each genotype. (C) Percentage of FOXP1+ cells in individual clusters. (D) UMI count, number of detected genes and percentage mitochondria gene in each cluster. (E) UMI count, number of detected genes, and percentage mitochondrial genes in each genotype.
Figure S3

A

WT
KO-1
KO-2
Merged

B

Dorsal telencephalic

MAP2
FOXG1
NEURON
BCL11B
EOMES
GAD1
GBX2
PCP4
EBF1
OTX2

Non-dorsal telencephalic

C

D

bRG_17
bRG_25

PTPRZ1
LIFR
TNC
HOPX
Figure S3. Representation of the diverse cell types in profiled in the organoids. (A) Feature plots showing distribution of cells across different genotypes. (B) Violin plots showing gene expression of markers representing the dorsal telencephalic lineage versus other brain regions. OR=Odd’s ratio. (C) Dot plot showing gene expression correlation between our brain organoid dataset and a human fetal cortex scRNA-seq dataset from the second trimester of gestation (Nowakowski et al., 2017). Dotted lines in blue indicate bRGC cell clusters. (D) Violin plots showing the expression level of bRGC-marker genes (Pollen, Nowakowski et al. 2015) that are differentially regulated between WT and KO in each bRGC subcluster. Y-axis represents log10(CPM) values.
Figure S4

(A) Correlation of DEGs between KO-1 and KO-2 organoids in all cells as well as each of the pseudo-bulked cell types, aRGC, bRGC, IPC and ENs. (B) Density plots showing the number of pseudobulked cells (aRGC, bRGC, IPC and EN) of KO-1 and KO-2 organoids separately.
Figure S5. bRGC-driven direct neurogenesis, quantification of PTPRZ+ bRGCs. (A) Immunostaining showing PAX6, TBR2, and the bRGC marker PTPRZ in week 6 organoids. Scale bar = 50uM (B) Quantification of bRGCs using PTPRZ+TBR2- bRGCs. (C) Schematic showing the experimental design of the BrdU treatment at week 6. (D) Immunostaining showing CTIP2, HOPX, BrdU and DAPI in week 6 organoids. Scale bar = 50uM (E) Quantification of neurogenic bRGCs, which are CTIP2+ cells located next to HOPX+ cells. These cells may represent neurons born directly from bRGC to EN. For all quantifications, n=3-8 organoids per sample per genotype was used. In each organoid, 3-9 cortical structures with clear lamination patterns were examined. Data are represented in bar graphs as mean ± STD with individual data as dots; n.s. = not statistically significant, *p < 0.05, Kruskal-Wallis ANOVA test with Dunn’s multiple comparisons test as a post hoc was used.
Figure S6

A

WT  |  KO-1  |  KO-2

B

at D40

WT  |  FOXP1 - KO

C

RGC-IPC-EN ratios

D

% TBR2+ RGCs

E

% TBR2+ ENs

F

DAPI  |  K67  |  BrdU  |  CTIP2

WT  |  KO-1  |  KO-2

G

EN cell cycle exit rate

% K67+BrdU+CTIP2+CTIP2+BrdU+
Figure S6. Deletion of FOXP1 results in increased numbers and protracted differentiation of IPCs. (A) Representative images showing changes observed in layering with the loss of FOXP1 (top) and examples of regions of interest (ROI) selected from left panel (bottom). SOX2 expression marks RGCs, TBR2 marks IPCs and CTIP2 marks ENs. Scale bar = 100uM for the upper images and 50uM for the ROI images on the bottom. (B) A summary diagram of the lamination changes between WT and KO organoids at D40. (C) Quantification of RGCs, IPCs and ENs. (D) Quantification of SOX2+ RGCs that express IPC marker TBR2. (E) Quantification of CTIP2+ ENs that express IPC marker TBR2. (F) Representative images of BrdU-treated brain organoids sectioned and stained for Ki67, BrdU, CTIP2 expression. BrdU experiment was conducted in the same way as Gladstone institute iPSC-derived brain organoids. Scale bar = 50uM (G) The difference between BrdU and Ki67 staining showing cell cycle exit rate in the CTIP2+ postmitotic neurons. For all quantifications, n=3-8 organoids per sample per genotype was used. In each organoid, 3-9 cortical structures with clear lamination patterns were examined. Data are represented in bar graphs as mean ± STD with individual data as dots; n.s. means p > 0.05, *p < 0.05, **p < 0.01, and ****p<0.0001. Mixed-effects analysis multiple comparisons test with Tukey’s multiple comparisons test as a post hoc was used for panel C. Kruskal-Wallis ANOVA test with Dunn’s multiple comparisons test as a post hoc was used for supplementary figure 6D and E.
**Figure S7**

(A) Immunostaining images of D100 organoids showing expression of SOX2 for RGCs, SATB2 for ENs, and FOXP1 in a representative WT organoid.  

(B) Immunostaining showing IPCs (TBR2+) and ENs (SATB2+) at D100. Scale bar = 300uM for panel B on the left and 100uM for ROI selected images on the right.  

(C) – (F) Immunostaining results showing percentage of RGCs, ENs and IPCs at D100 normalized to all DAPI+ cells. For all quantifications, n=3-8 organoids per sample per genotype was used. In each organoid, 3-9 cortical structures with clear lamination patterns were examined. Data are represented in bar graphs as mean ± STD with individual data as dots; n.s. means p > 0.05, **p <0.001, and ****p <0.0001 Kruskal-Wallis ANOVA test with Dunn’s multiple comparisons test as a post hoc was used. Scale bar = 100uM for Supp Fig 7A. Scale bar = 300uM for Supp Fig 7B on the left and 100uM for ROI selected images on the right.

**Figure S7. Impaired late neurogenesis in 3 month (D100) organoids.** (A) Immunostaining images of D100 organoids showing expression of SOX2 for RGCs, SATB2 for ENs, and FOXP1 in a representative WT organoid. (B) Immunostaining showing IPCs (TBR2+) and ENs (SATB2+) at D100. Scale bar = 300uM for panel B on the left and 100uM for ROI selected images on the right. (C) – (F) Immunostaining results showing percentage of RGCs, ENs and IPCs at D100 normalized to all DAPI+ cells. For all quantifications, n=3-8 organoids per sample per genotype was used. In each organoid, 3-9 cortical structures with clear lamination patterns were examined. Data are represented in bar graphs as mean ± STD with individual data as dots; n.s. means p > 0.05, **p <0.001, and ****p <0.0001 Kruskal-Wallis ANOVA test with Dunn’s multiple comparisons test as a post hoc was used. Scale bar = 100uM for Supp Fig 7A. Scale bar = 300uM for Supp Fig 7B on the left and 100uM for ROI selected images on the right.
Figure S8. **Differential expression analysis of bRGC subtypes.** (A) Number of DEGs identified in bRGC clusters. \((\log_{2}(FC) \geq 0.25, \text{FDR} \leq 0.05, \text{percent expression} > 0.10)\) (B) Summarized biological process GO terms for upregulated and downregulated DEGs for each bRGC cluster. Significantly changed gene categories are defined as Benjamini-Hochberg FDR \(\leq 0.05\) (dashed line in red). (C) Scaled Venn diagram showing the overlap between the DEGs in bRGCs and a previously published chromatin immunoprecipitation assay with sequencing (ChIP-seq) dataset from our laboratory (Araujo et al., 2015). Fisher’s exact test was performed to calculate statistical significance.
Figure S9

IPSC WT

IPSC KO-1

IPSC KO-2

Figure S9. Representative karyotype analysis for iPSCs G-banding technique showing normal chromosomes of metaphase cells across all three genotypes.
| Sample name | Genotype        | Total Reads (NextSeq) | Total Reads (NovaSeq) | Total Nuclei | Mean Reads per Nucleus | Median Genes per Nucleus | Median UMI per Nucleus |
|-------------|-----------------|-----------------------|-----------------------|--------------|------------------------|--------------------------|------------------------|
| WT_1        | WT              | 54,992,799            | 308,137,509           | 13,853       | 26,213                 | 1,986                    | 3,046                  |
| WT_2        | WT              | 55,999,689            | 329,936,982           | 31,477       | 12,260                 | 1,420                    | 2,002                  |
| WT_3        | WT              | 59,056,503            | 332,920,320           | 17,569       | 22,310                 | 1,902                    | 2,846                  |
| KO-1_1      | P1 gene body deletion | -                      | 391,215,611           | 13,066       | 29,941                 | 1,870                    | 2,837                  |
| KO-1_2      | -               | -                     | 376,442,968           | 4,725        | 79,670                 | 1,746                    | 2,705                  |
| KO-1_3      | -               | -                     | 414,955,518           | 12,180       | 34,068                 | 1,875                    | 2,880                  |
| KO-2_1      | FOXP1 KO Puro KI | 52,016,272            | 300,208,358           | 14,096       | 24,987                 | 1,830                    | 2,835                  |
| KO-2_2      | FOXP1 KO Puro KI | 56,731,346            | 358,688,357           | 30,304       | 13,708                 | 1,528                    | 2,208                  |
| KO-2_3      | FOXP1 KO Puro KI | 60,792,883            | 356,049,243           | 14,066       | 29,634                 | 2,251                    | 3,805                  |
| TOTAL       |                 | 3,508,144,358         | 151,336               |              |                        |                          |                        |

Table S1. Sequencing read numbers, number of nuclei sequenced, number of expressed genes, and number of UMI.