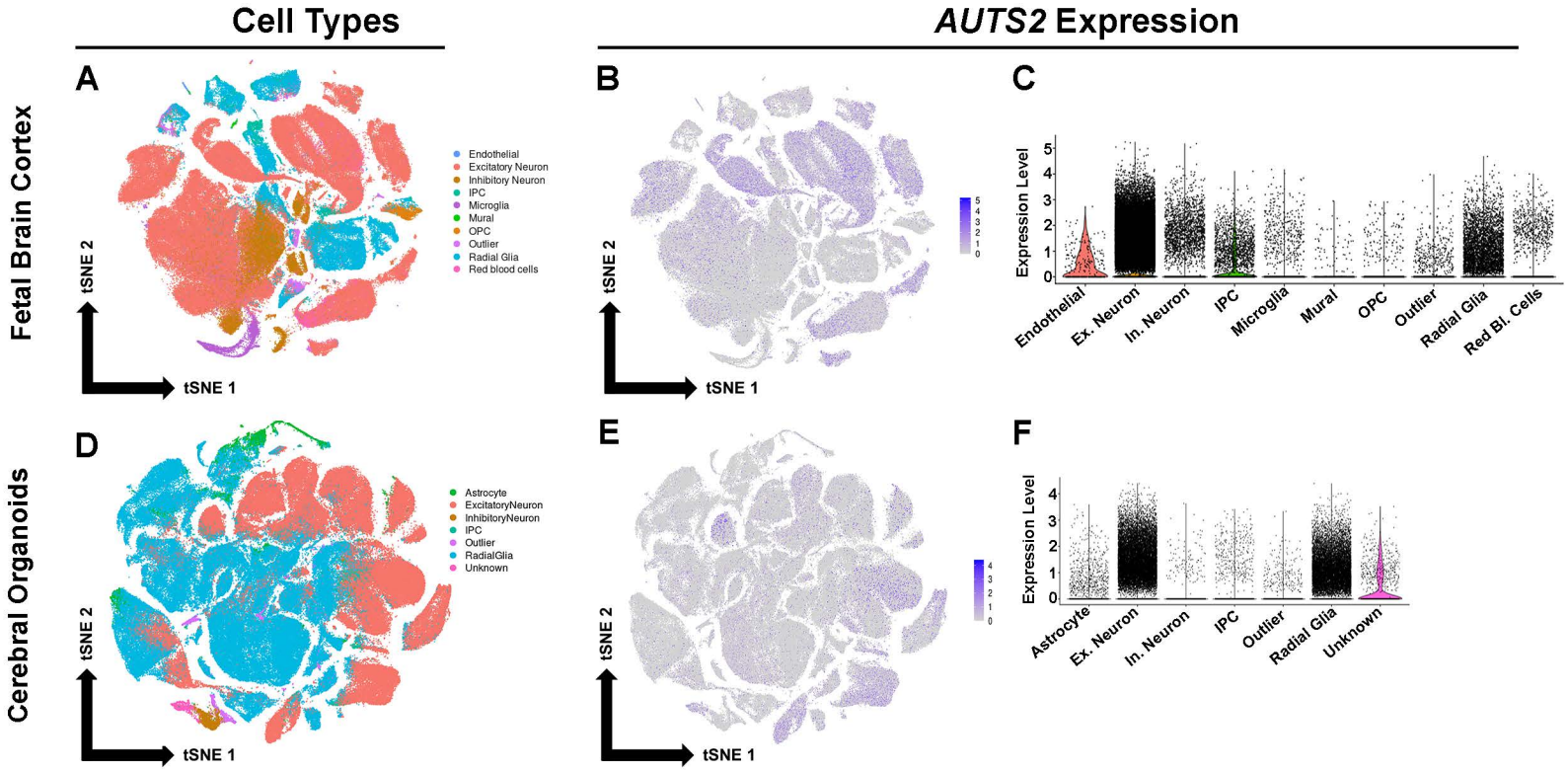
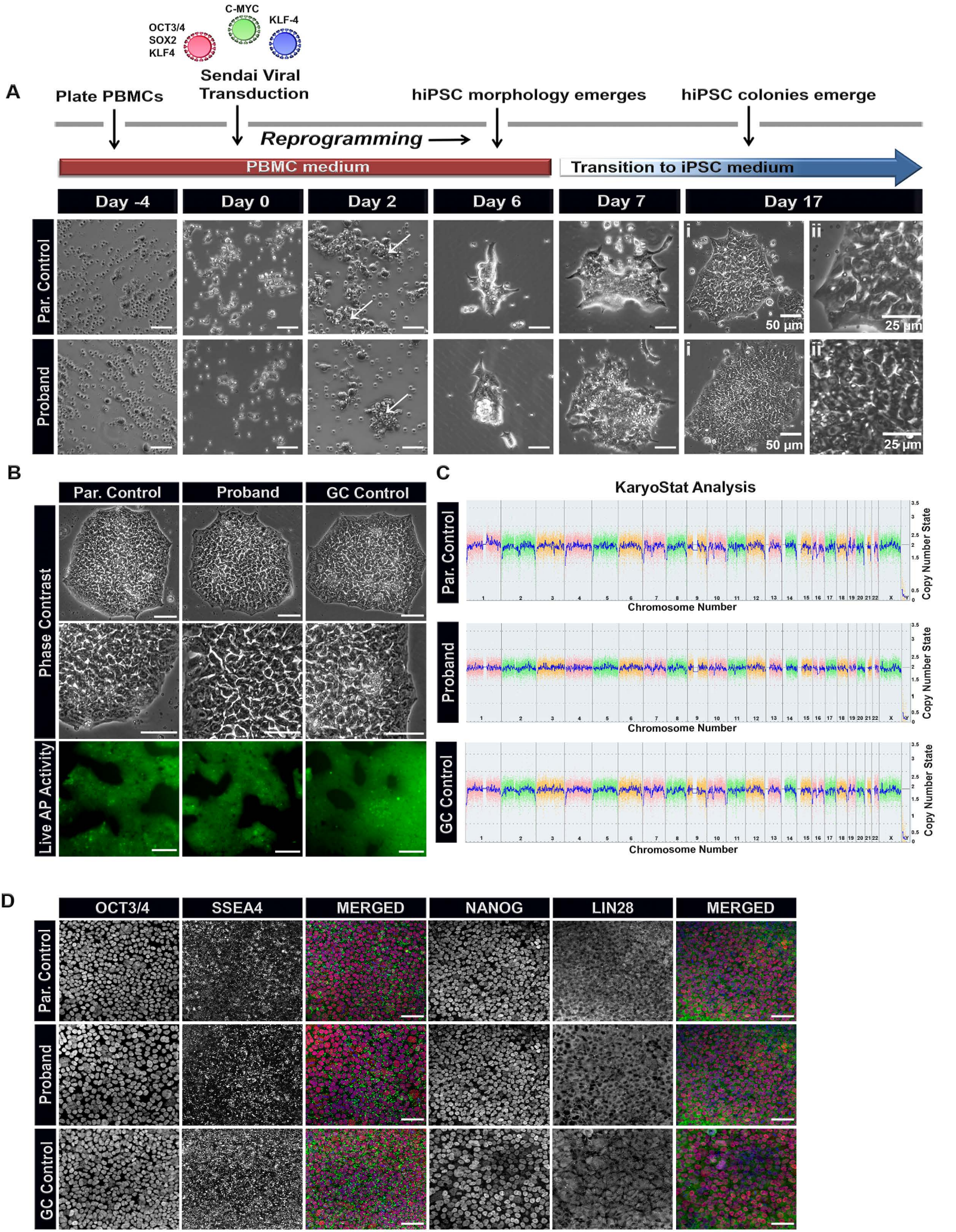
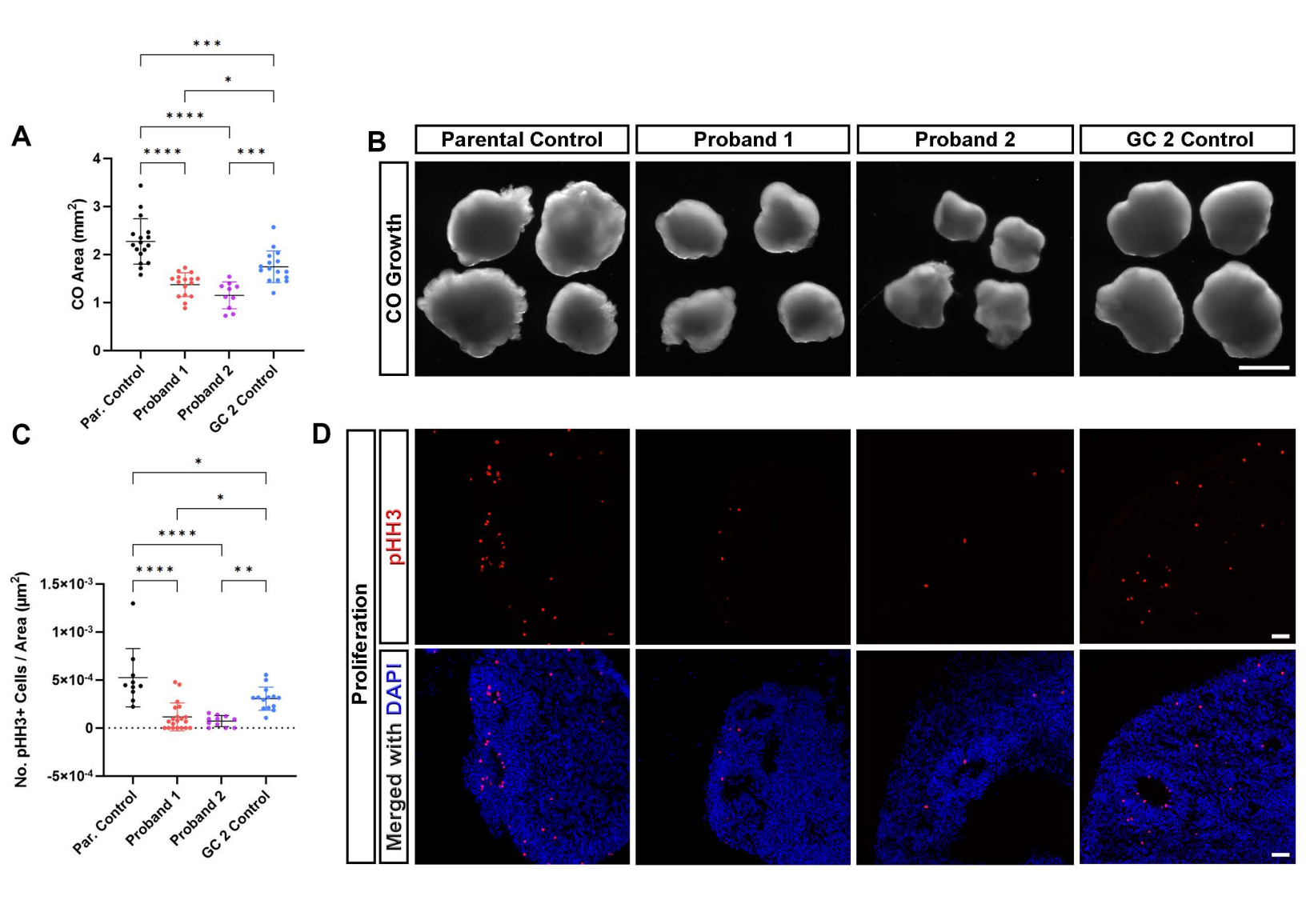
Supplementary Figure 1. Characterization of *AUTS2* expression at single cell resolution in the human fetal brain and COs. (**A**) tSNE plot of single cell fetal brain cortex dataset showing nine cell types: endothelial cells, excitatory neurons, inhibitory neurons, intermediate progenitor cells (IPCs), microglia, oligodendrocyte progenitor cells (OPC), radial glia and red blood cells. Additional cluster of 'Outlier' cells demarcated by original authors. 189,409 cells shown. (**B,C**) Feature and violin plots show *AUTS2* expression in several neuronal and progenitor cell types within fetal cortex. (**D**) tSNE plot of CO dataset with five cell types: astrocytes, excitatory neurons, inhibitory neurons, IPCs, and radial glia. Additional clusters of 'Unknown' and 'Outlier' cells demarcated by original authors. 235,121 cells shown. (**E, F**) Feature and violin plots also show *AUTS2* expression in neuronal and progenitor cell types within COs.


Cell Types
AUTS2 Expression
Fetal Brain Cortex
Cerebral Organoids
A
B
C
D
E
F
tSNE 1
tSNE 2
Endothelial
Excitatory Neuron
Inhibitory Neuron
IPC
Microglia
Mural
OPC
Outlier
Radial Glia
Red blood cells
Astrocyte
ExcitatoryNeuron
InhibitoryNeuron
IPC
Outlier
RadialGlia
Unknown
Expression Level
Endothelial
Ex. Neuron
In. Neuron
IPC
Microglia
Mural
OPC
Outlier
Radial Glia
Red Bl. Cells
Astrocyte
Ex. Neuron
In. Neuron
IPC
Outlier
Radial Glia
Unknown

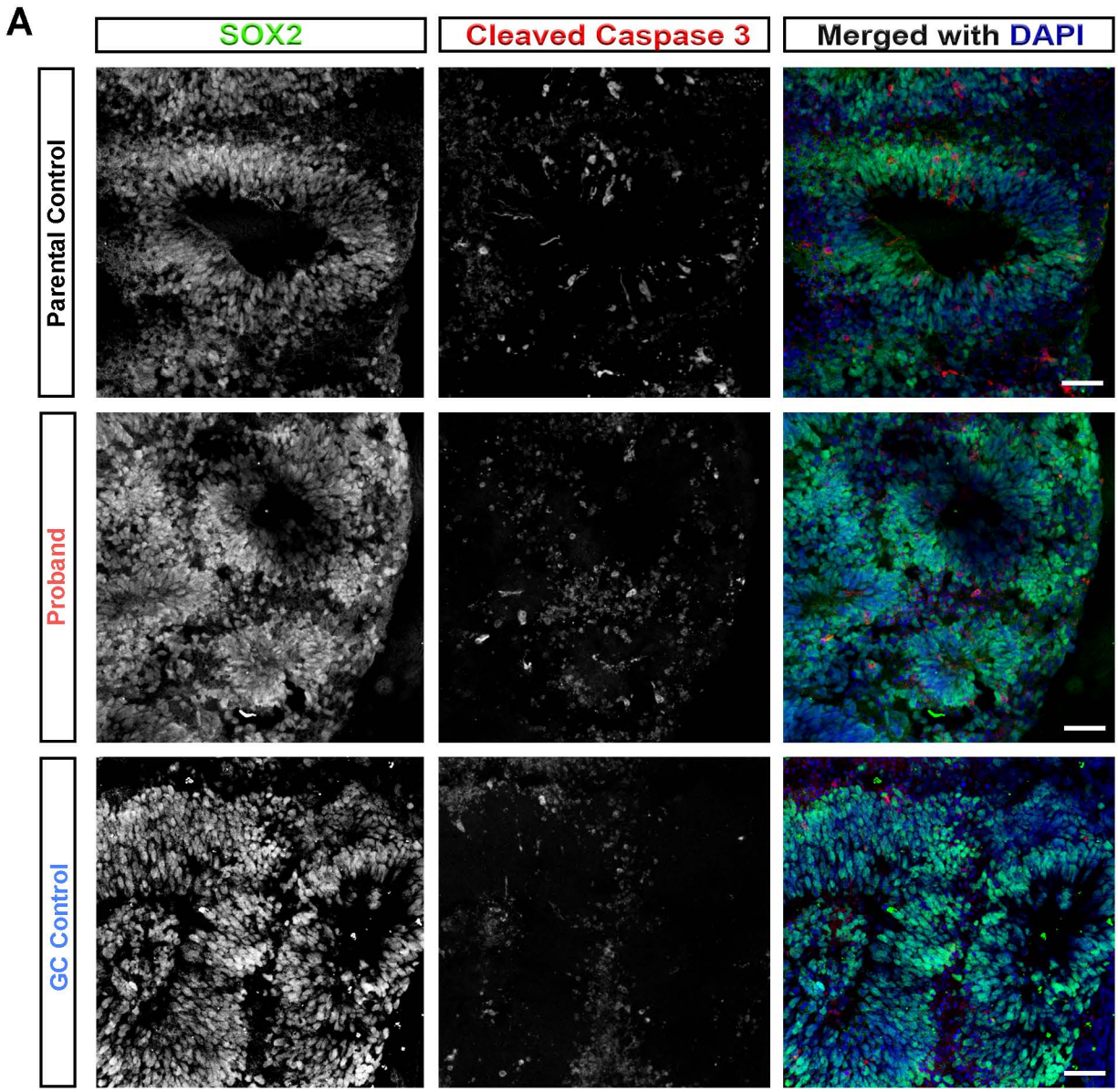
Supplementary Figure 2. Reprogramming of *AUTS2*^{T534P} proband and parental control PBMCs into hiPSCs and molecular characterization. (**A**) Schematic showing a timeline of major steps during the reprogramming process of PBMCs into hiPSCs. At day -4, PBMCs are seeded and then infected with Yamanaka transcription factors via Sendai virus. hiPSC morphology emerges around day 6 post-infection and hiPSC colonies emerge days later. Scale bar = 50 µm and 25 µm for d-4 to d17i and d17ii, respectively. (**B**) Phase contrast images and live alkaline phosphatase (AP) activity of hiPSC colonies from parental control, proband, and GC control lines showing morphological and functional activity characteristics of human pluripotent stem cells. Scale bar = 100 µm and 50 µm for upper and lower phase contrast images, respectively. Scale bar = 100 µm for alkaline phosphatase images. (**C**) Karyotyping (KaryoStat, Thermofisher) analysis showing chromosomal stability within all hiPSC lines. (**D**) Immunofluorescence analysis of key pluripotency markers (OCT3/4, SSEA4, NANOG, and LIN28) in all hiPSC lines. Co-staining of OCT3/4 with SSEA4 or NANOG with LIN28 shown in grayscale and merged colored images shown to the right of individual channels. DAPI is shown in blue. Scale bar = 50 µm.

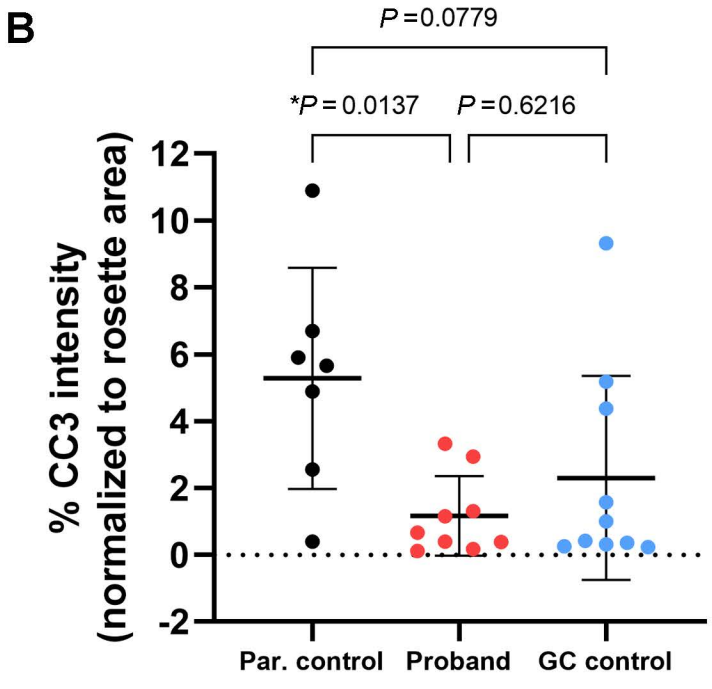

A
OCT3/4
SOX2
KLF4
C-MYC
KLF-4
Sendai Viral
Transduction
Plate PBMCs
Reprogramming
hiPSC morphology emerges
hiPSC colonies emerge
PBMC medium
Transition to iPSC medium
Day -4
Day 0
Day 2
Day 6
Day 7
Day 17
Par. Control
Proband
i
ii
50 µm
25 µm
B
Par. Control
Proband
GC Control
Phase Contrast
Live AP Activity
C
KaryoStat Analysis
Par. Control
Proband
GC Control
Copy Number State
Chromosome Number
D
OCT3/4
SSEA4
MERGED
NANOG
LIN28
MERGED
Par. Control
Proband
GC Control

Supplementary Figure 3. *AUTS2*^{T534P} COs derived from multiple iPSC clones show reduced growth and proliferative deficits. (**A**) Quantification of day 30 CO area, showing a statistically significant growth reduction in both proband 1 (original proband clone) and proband 2 COs, which is rescued with an additonal gene corrected iPSC clone (GC 2 control, see Supplementary Table 4). (**B**) Representative images of parental control, proband 1, proband 2, and GC 2 COs at Day 30. Scale bar = 1 mm. (**C-D**) Parental and gene corrected COs show proliferating neural progenitors identified as phospho-Histone H3+ (pHH3; mitosis marker) compared to proband 1 and proband 2 COs, which show a reduction (quantified in **C**, representative images in **D**). Scale bar = 50 μm. DAPI (blue) stains nuclei. All data are shown as the mean ± standard deviation (SD). Statistical analyses in panels a and d were performed using one-way ANOVA with Tukey's multiple comparisons test.

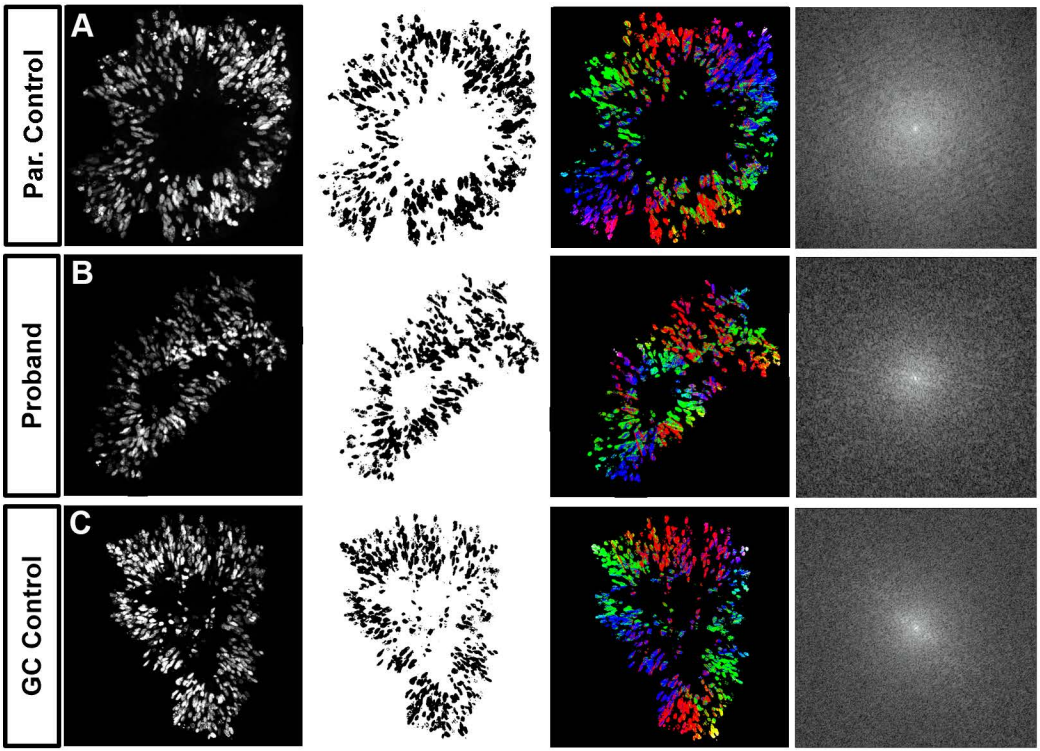

A
***
*
****
****
***
CO Area (mm2)
4
3
2
1
0
Par. Control
Proband 1
Proband 2
GC 2 Control
B
Parental Control
Proband 1
Proband 2
GC 2 Control
CO Growth
C
*
*
****
****
**
No. pHH3+ Cells / Area (µm2)
1.5×10-3
1×10-3
5×10-4
0
-5×10-4
Par. Control
Proband 1
Proband 2
GC 2 Control
D
Proliferation
pHH3
Merged with DAPI

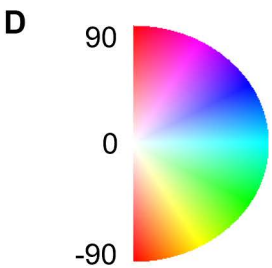
Supplementary Figure 4. Apoptotic cell death analysis in *AUTS2* proband and control COs. (**A**) CC3 expression shown in grayscale, which identifies apoptotic cells, is observed within a small subset of neural progenitor cells that are SOX2 positive (middle and left panels, respectively). Merged images are shown in the right panels, scale bar = 50 μm. (**B**) Quantification of cells that are CC3+, normalized to rosette area. All data are shown as the mean ± SD. Statistical analyses were performed using one-way ANOVA with Tukey's multiple comparisons test.


A
SOX2
Cleaved Caspase 3
Merged with DAPI
Parental Control
Proband
GC Control


B
P = 0.0779
*P = 0.0137
P = 0.6216
% CC3 intensity
(normalized to rosette area)
12
10
8
6
4
2
0
-2
Par. control
Proband
GC control

Supplementary Figure 5. Proband and control iPSCs show similar proliferation rates. Cell viability was measured using the MTT assay. iPSC cell absorbance values were subtracted from the average value from the blanks and plotted as absorbance against cell density. All data are shown as the mean ± SD. Statistical analyses were performed using a simple linear regression to determine goodness of fit (R^2) and significance of the slope (P = 0.23, indicating that the differences between the slopes are not statistically significant between groups).

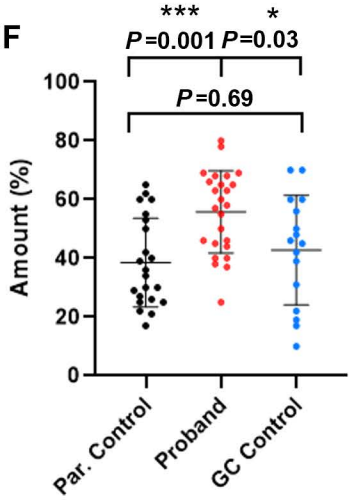


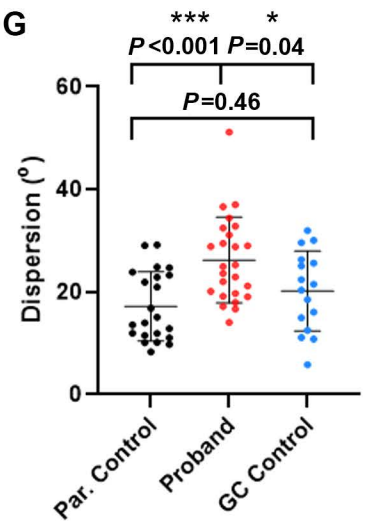


D

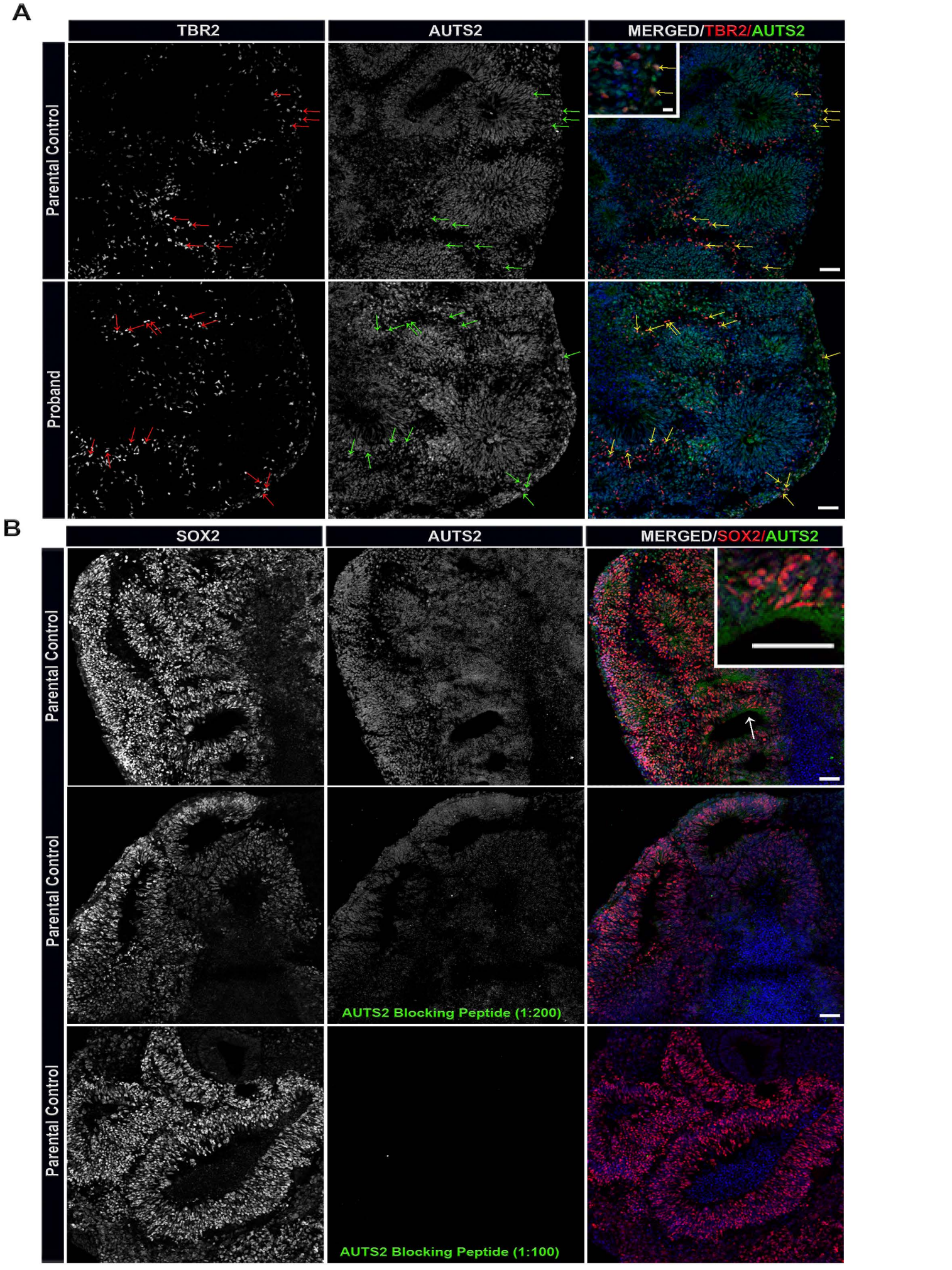
E

Genotype	Direction (°)	Dispersion (°)	Amount
Par. Control (a)	58.36	9.79	0.21
Proband (b)	-73.29	33.58	0.51
GC control (c)	55.15	22.44	0.45

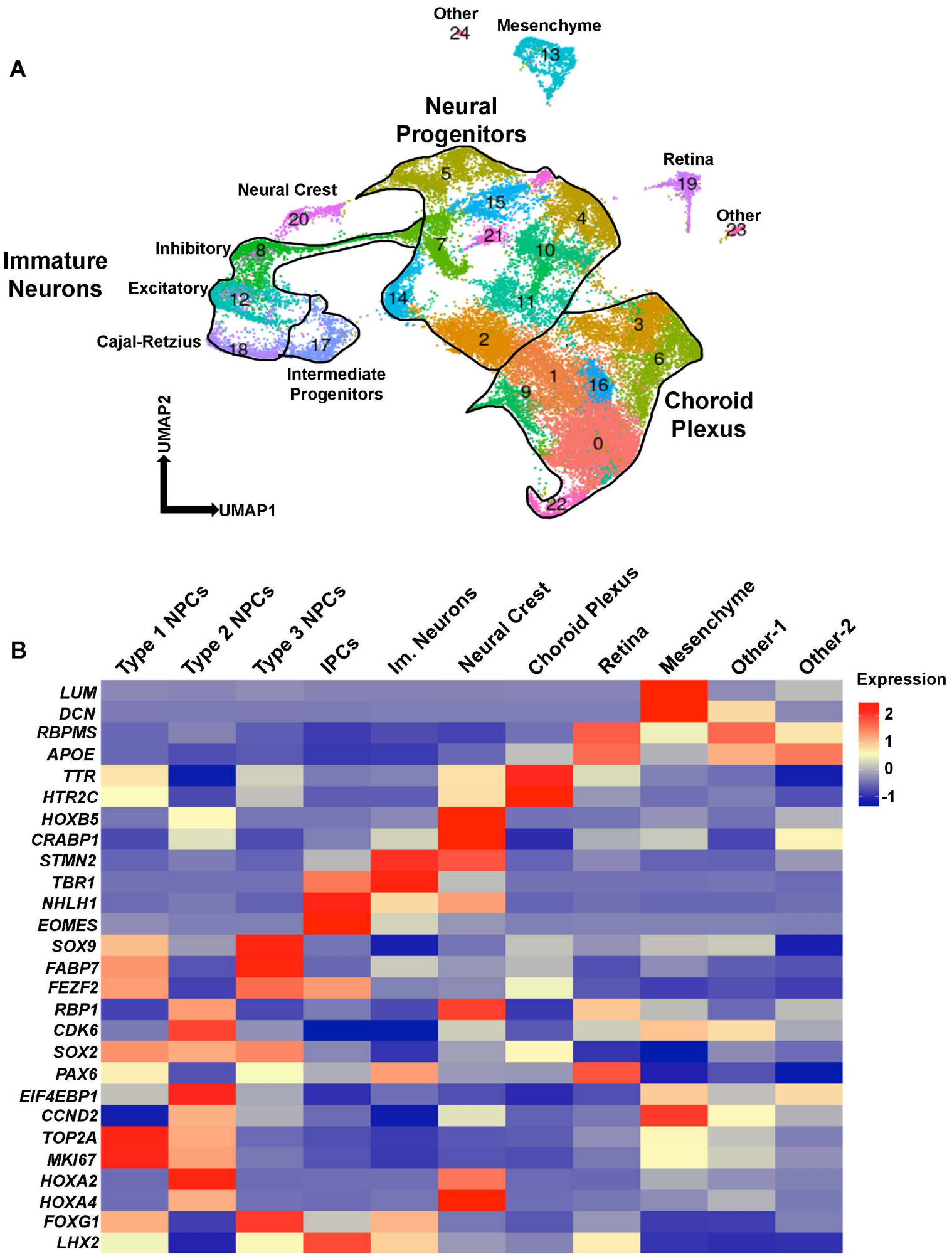




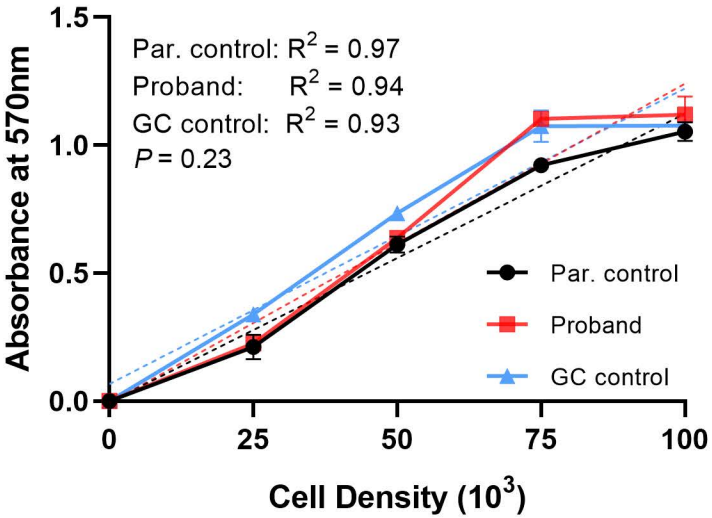
Supplementary Figure 6. *AUTS2* proband COs show disrupted rosette structures. (**A-C**) Representative images showing how cell polarity was investigated using the ImageJ plug-in 'directionality' (detailed in Materials & Methods): (**A**) parental control; (**B**) proband; (**C**) gene corrected (GC) control. From left to right, first panels show a single rosette identified with EdU+ to mark proliferating neural progenitors. Second panels show the same rosette from (**A**) after applying a threshold. Third panel shows an 'orientation map' utilizing the threshold image. The orientation map assigns each cell a color based upon its angle relative to the horizontal (as per the color wheel shown in panel (**D**)). Fourth panels show the Fourier power spectra of each input image and analyzed in polar coordinates using spatial filters, quantifying preferred orientation across the 2D image. (**E**) Table shows data output from the 'directionality' plug-in: direction, dispersion, and amount. Data generated form the representative images shown in (**A-C**), as indicated. (**F**) Compiled data of the amount (%), representing the proportion of cells in a specified orientation. (**G**) Compiled data of the dispersion (°), reporting the standard deviation of that Gaussian function fitted to the peak. All data are shown as the mean ± SD. Statistical analyses were performed using one-way ANOVA with Tukey's multiple comparisons test (n ≥ 16 rosettes quantified across a minimum of 4 independent organoids per group and 1 independent experiment performed). *$P \leq 0.05$; ** $P \leq 0.01$ *** $P \leq 0.001$; **** $P \leq 0.0001$; ns=not significant.


A
TBR2
AUTS2
MERGED/TBR2/AUTS2
Parental Control
Proband
B
SOX2
AUTS2
MERGED/SOX2/AUTS2
Parental Control
Parental Control
AUTS2 Blocking Peptide (1:200)
Parental Control
AUTS2 Blocking Peptide (1:100)

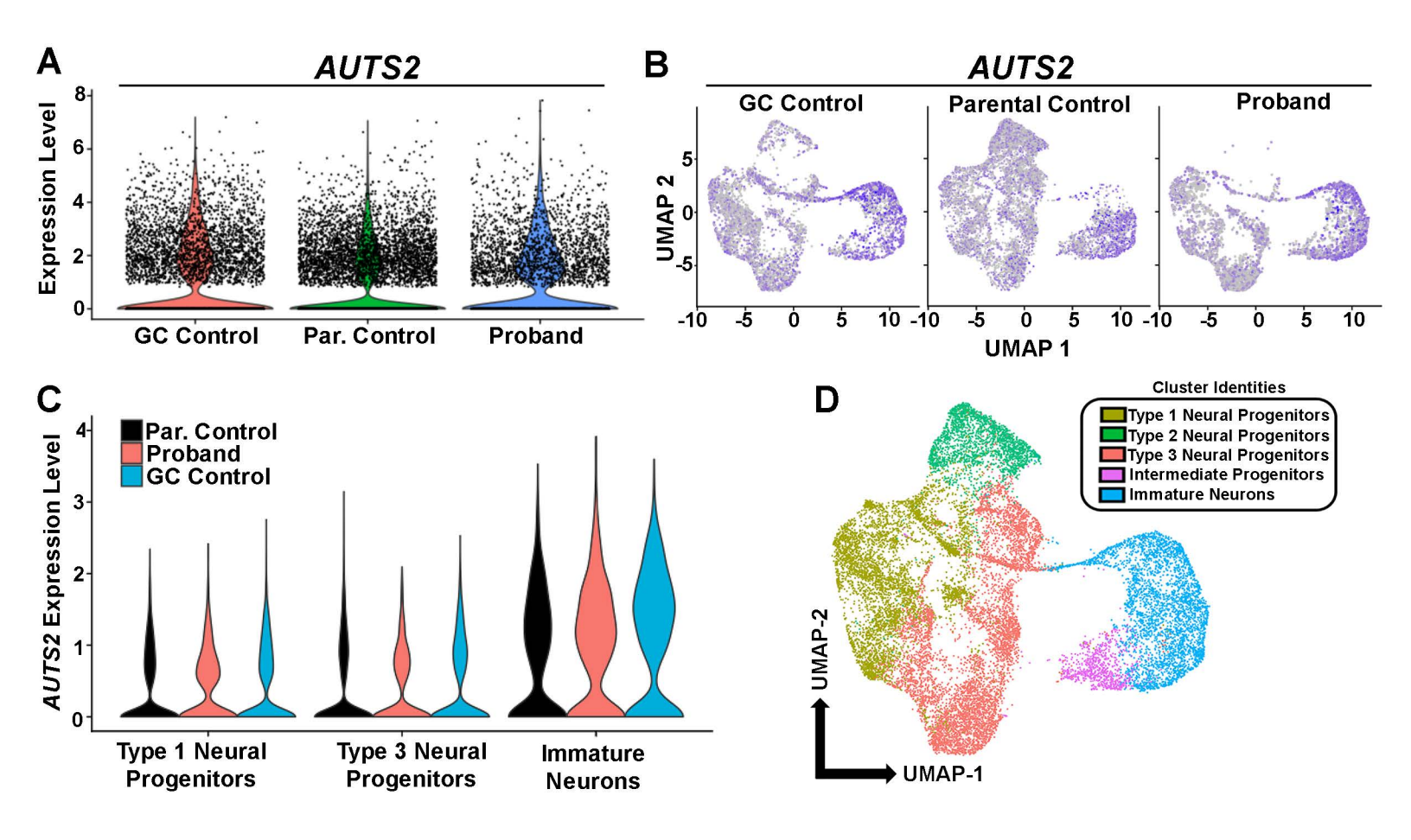
Supplementary Figure 7. Co-expression of intermediate and neural progenitor cell markers with AUTS2 in day 30 COs. (**A**) TBR2 expression shown in grayscale (as depicted by red arrows), which identifies intermediate progenitor cells that are located outside of neural rosette structures. AUTS2 expression is observed within neural progenitor cells and within a subset of intermediate progenitors, which are TBR2+ and AUTS2+ as denoted by green arrows showing AUTS2 expression in grayscale and yellow arrows showing co-localization of TBR2 and AUTS2 in merged images shown in right panels (inset shows high power magnification image of AUTS2 and TBR2 nuclear colocalization, scale bar = 25 μm). All other scale bars = 50 μm. (**B**) SOX2 expression (shown in grayscale) co-localizing with AUTS expression (shown in grayscale) within neural progenitor cells. Merged images are shown in the right panels. Inset in the merged panel show both cytoplasmic AUTS2 expression (as depicted by a white arrow) within ventricular zone-like structures and nuclear expression within neural progenitor cells in COs, scale bar = 50 μm. Specificity of AUTS2 staining in COs was demonstrated through use of a blocking peptide directed against the AUTS2 antibody. Scale bar for all other panels = 50 μm.


A
Other
24
Mesenchyme
13
Neural
Progenitors
Retina
19
Other
23
Neural Crest
20
5
15
4
21
7
10
Inhibitory
8
Immature
Neurons
Excitatory
12
14
11
3
2
Cajal-Retzius
18
17
6
1
16
9
Intermediate
Progenitors
Choroid
Plexus
0
22
UMAP2
UMAP1
B
Type 1 NPCs
Type 2 NPCs
Type 3 NPCs
IPCs
Im. Neurons
Neural Crest
Choroid Plexus
Retina
Mesenchyme
Other-1
Other-2
Expression
2
1
0
-1
LUM
DCN
RBPMS
APOE
TTR
HTR2C
HOXB5
CRABP1
STMN2
TBR1
NHLH1
EOMES
SOX9
FABP7
FEZF2
RBP1
CDK6
SOX2
PAX6
EIF4EBP1
CCND2
TOP2A
MKI67
HOXA2
HOXA4
FOXG1
LHX2

Supplementary Figure 8. Single cell RNA sequencing analysis of day 30 COs. (**A**) Unbiased clustering of single cell data reveals 24 unique cell clusters. (35,633 cells shown). These clusters were categorized into the major cell types: immature neurons (inhibitory, excitatory, and Cajal-Retzius/RELN+), progenitors (intermediate and neural progenitors), choroid plexus, retina, and mesenchyme. Additionally, the dataset contained two uncategorized 'Other' clusters comprised of low-quality cells. Only key neuronal and progenitor cell types were used in downstream analyses, as described in Figure 6 and 7. (**B**) Average expression of canonical markers for major cell types. Choroid plexus: *TTR*, *HTR2C*, pan-neural progenitors: *SOX2*, Type 1 neural progenitors: *MKI67*, *TOP2A*, Type 2 neural progenitors: *HOXA2,CDK6*, *OLIG3*, Type 3 neural progenitors: *SOX9* and *FABP7*, immature neurons: *STMN2*, *TBR1*, mesenchyme: *LUM*, *DCN*, intermediate progenitors: *EOMES*, *NHLH1*, retina: *RBPMS*, *APOE*, neural crest: *CRABP1*, *HOXB5*.

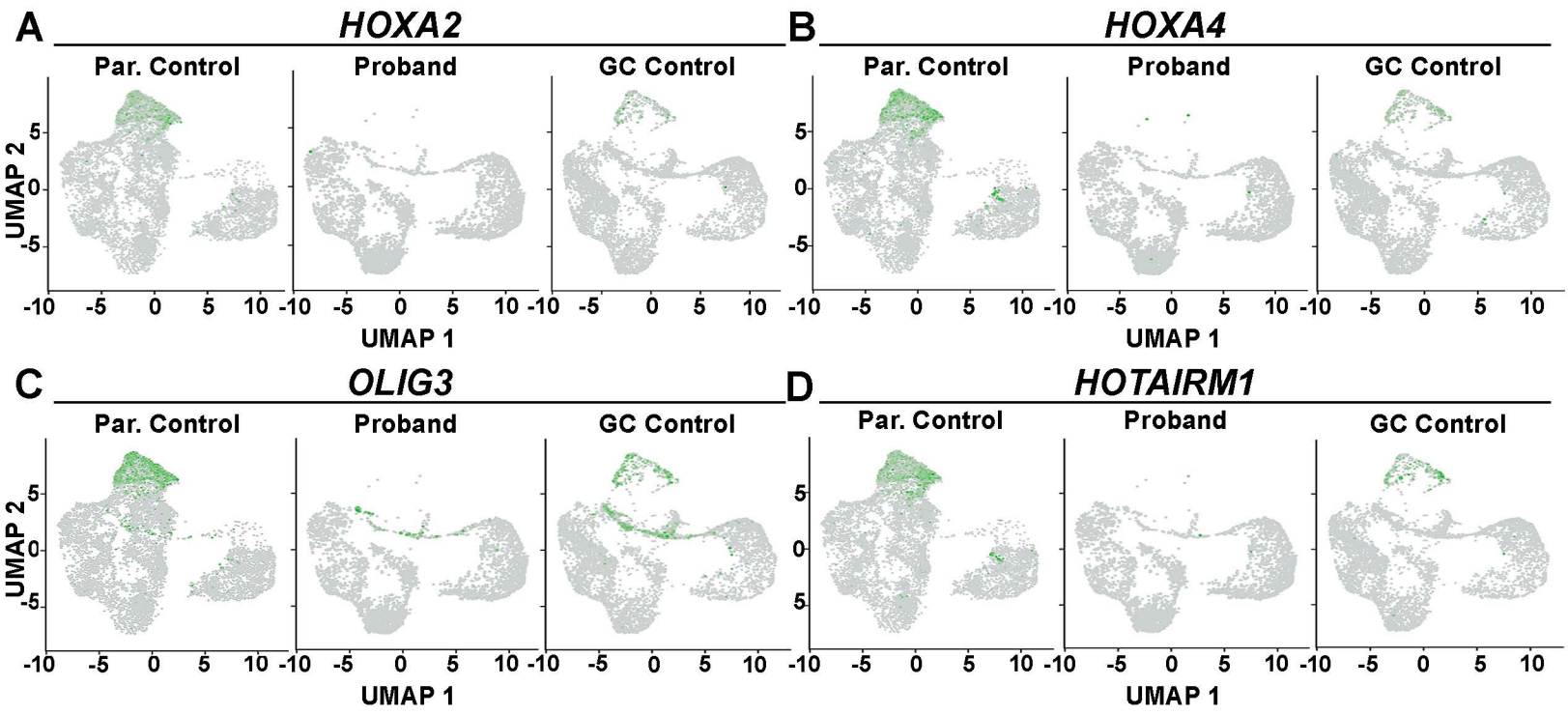

Par. control: R2 = 0.97
Proband: R2 = 0.94
GC control: R2 = 0.93
P = 0.23
Par. control
Proband
GC control
Absorbance at 570nm
Cell Density (10^3)
1.5
1.0
0.5
0.0
0
25
50
75
100

Supplementary Figure 9. Characterization of *AUTS2* expression in proband and control COs at day 30. (**A**) Violin plots of total *AUTS2* expression across all cell types in GC control, parental control, and proband COs. (**B**) Feature plots showing expression of *AUTS2* in GC control, parental control, and proband COs. (**C**) Violin plots of *AUTS2* expression in Type 1-3 neural progenitors, immature neurons, and intermediate progenitors in GC control, parental control, and proband COs. (**D**) UMAP plot showing Type 1-3 neural progenitors, immature neurons, and intermediate progenitors for reference.

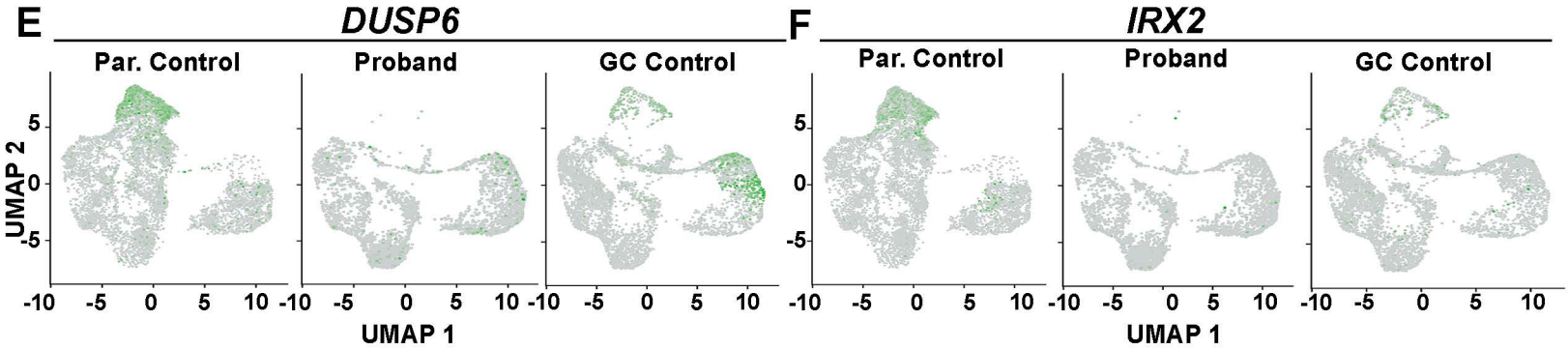

A
AUTS2
Expression Level
GC Control
Par. Control
Proband
B
AUTS2
GC Control
Parental Control
Proband
UMAP 2
UMAP 1
C
Par. Control
Proband
GC Control
AUTS2 Expression Level
Type 1 Neural Progenitors
Type 3 Neural Progenitors
Immature Neurons
D
Cluster Identities
Type 1 Neural Progenitors
Type 2 Neural Progenitors
Type 3 Neural Progenitors
Intermediate Progenitors
Immature Neurons
UMAP-2
UMAP-1

Supplementary Figure 10. Type 2 NPC marker gene expression patterns in proband and control COs at day 30. (**A-D**) Violin plots of hindbrain-specifying marker genes in GC control, parental control, and proband COs. (**E-F**) Violin plots of midbrain/hindbrain boundary-specifying marker genes in GC control, parental control, and proband COs. (**G-H**) Violin plots of midbrain-specifying marker genes in GC control, parental control, and proband COs.

Hindbrain-Specifying Marker Genes



Midbrain/Hindbrain Boundary-Specifying Marker Genes



Midbrain-Specifying Marker Genes