

A human forebrain organoid model reveals the essential function of GTF2IRD1-TTR-ERK axis for the neurodevelopmental deficits of Williams Syndrome

Xingsen Zhao, Qihang Sun, Yikai Shou, Weijun Chen, Mengxuan Wang, Wenzheng Qu, Xiaoli Huang, Ying Li, Chao Wang, Yan Gu, Chai Ji ✉, Qiang Shu ✉, Xuekun Li ✉

The Children's Hospital, National Clinical Research Center for Child Health, School of Medicine, Zhejiang University, Hangzhou, China • The Institute of Translational Medicine, School of Medicine, Zhejiang University, Hangzhou, China • Binjiang Institute of Zhejiang University, Hangzhou, China • Center of Stem Cell and Regenerative Medicine, and Department of Neurology of the Second Affiliated Hospital, Zhejiang University School of Medicine, Hangzhou, China

 https://en.wikipedia.org/wiki/Open_access

 Copyright information

Abstract

Williams Syndrome (WS; OMIM#194050) is a rare disorder, which is caused by the microdeletion of one copy of 25-27 genes, and WS patients display diverse neuronal deficits. Although remarkable progresses have been achieved, the mechanisms for these distinct deficits are still largely unknown. Here, we have shown that neural progenitor cells (NPCs) in WS forebrain organoids display abnormal proliferation and differentiation capabilities, and synapse formation. Genes with altered expression are related to neuronal development and neurogenesis. Single cell RNA-seq (scRNA-seq) data analysis revealed 13 clusters in healthy control and WS organoids. WS organoids show an aberrant generation of excitatory neurons. Mechanistically, the expression of transthyretin (TTR) are remarkably decreased in WS forebrain organoids. We have found that GTF2IRD1 encoded by one WS associated gene *GTF2IRD1* binds to *TTR* promoter regions and regulates the expression of *TTR*. In addition, exogenous TTR can activate ERK signaling and rescue neurogenic deficits of WS forebrain organoids. *Gtf2ird1* deficient mice display similar neurodevelopmental deficits as observed in WS organoids. Collectively, our study reveals critical function of GTF2IRD1 in regulating neurodevelopment of WS forebrain organoids and mice through regulating TTR-ERK pathway.

eLife assessment

Zhao et al. report **valuable** adverse effects on cell proliferation, differentiation and gene expression, possibly linked to reduced binding activity of the transcription factor GTF2IRD1 to the transthyretin (TTR) promoter, in a human forebrain organoid model of Williams Syndrome (WS). The authors provide **incomplete** evidence of the effects of GTF2IRD1, a mutated gene in WS, on altering MAPK/ERK pathway activity, a well-recognized target in cell proliferation.

<https://doi.org/10.7554/eLife.98081.1.sa2>

Introduction

Williams Syndrome, also known as Williams-Beuren syndrome (WS; OMIM#194050) is a rare disorder with a prevalence of 1 in 7500 live births (Kozel et al., 2021 [DOI](#); Pober, 2010 [DOI](#)). WS is caused by the microdeletion of approximately 25-27 genes (termed as WS genes) on chromosome 7q11.23 and affects multiple systems including cardiovascular and neuronal systems (Collins, 2013 [DOI](#), 2018 [DOI](#); Lin et al., 2019 [DOI](#); Pober et al., 2008 [DOI](#)). Most individuals with WS displayed neuronal phenotypes including hypersociability, impaired myelination and intellectual disability (IQ<70) (Barak and Feng, 2016 [DOI](#); Barak et al., 2019 [DOI](#); Kozel et al., 2021 [DOI](#); Morris et al., 2020 [DOI](#)). fMRI neuroimaging and examination with post-mortem brain tissues of WS patients both showed abnormalities of brain structure of WS patients, including visuospatial construction deficit and increased length of dendrites, numbers of spine and branching points (Chailangkarn et al., 2016 [DOI](#); Kippenhan et al., 2005 [DOI](#); Meyer-Lindenberg et al., 2004 [DOI](#)).

Neural progenitor cells (NPCs) and neurons derived from the induced pluripotent stem cells (iPSC) of WS patient display deficits, such as increased apoptosis, aberrant morphological complexity and electrophysiological activity (Chailangkarn et al., 2016 [DOI](#); Khattak et al., 2015 [DOI](#)). The dysregulation of Wnt signaling pathway plays important roles in regulating the survival and development of neurons derived from induced pluripotent stem cells (iPSCs) of WS patient. Neuronal deletion of WS gene *Gtf2i* leads to severe behavioral deficits and reduced myelination in mice (Barak et al., 2019 [DOI](#)). Although single gene mutation of those 25-27 genes only causes partial phenotypes of WS, genotype-phenotype relationship analysis suggests that *GTF2IRD1*, *GTF2I*, *BAZ1B*, *VPS37D*, *STX1A*, *CLIP2*, *LIMK1*, *MLXIPL* and *ELN* could be the key genes for the phenotypes (Kozel et al., 2021 [DOI](#); Lek et al., 2016 [DOI](#)). *GTF2IRD1* encodes DNA binding protein of the Transcription factor (TF) II-I family and its deficiency contributes to neurological deficits of WS patients, such as the impaired motor coordination, visual-spatial constructive deficits and cognitive deficits (Carmona-Mora et al., 2015 [DOI](#); Corley et al., 2016 [DOI](#); Dai et al., 2009 [DOI](#); Enkhmandakh et al., 2009 [DOI](#); Kopp et al., 2020 [DOI](#); Makeyev and Bayarsaihan, 2009 [DOI](#); Morris et al., 2003 [DOI](#); O'Leary and Osborne, 2011 [DOI](#); Tassabehji et al., 2005 [DOI](#)). Despite this remarkable progress, the mechanisms for diverse neuronal defects of WS still remain largely unknown.

Due to the limited accessibility of human brain samples of WS patients, diverse cellular and animal models have been used to study the phenotypes and mechanisms of WS. Brain organoids are derived iPSCs under 3D cultural condition and exhibit unique advantages in modeling spatio-temporal features of brain development compared to 2D cultures including iPSCs. Therefore, brain organoids have been applied for studying human brain development and neurological disorders, including Fragile X syndrome and autism (Clevers, 2016 [DOI](#); Di Lullo and Kriegstein, 2017 [DOI](#); Kang et

al., 2021b [\[link\]](#); Kelava and Lancaster, 2016 [\[link\]](#); Li and Izpisua Belmonte, 2019 [\[link\]](#); Lyon, 2019 [\[link\]](#); Pasca, 2019 [\[link\]](#); Qian et al., 2019 [\[link\]](#)). To our knowledge, there have been no studies performed with brain organoids of WS.

In the present study, utilizing the iPSCs derived forebrain organoids, we found that NPCs in WS forebrain organoids displayed abnormal proliferation and differentiation, and synapse formation. Bulk RNA-seq revealed the altered gene expression in WS organoids, which is strongly related to neuronal development and neurogenesis. scRNA-seq data analysis revealed 13 clusters in healthy control and WS organoids, and WS NPCs showed an aberrant excitatory neurogenesis. Mechanistically, the expression of transthyretin (*TTR*) significantly decreased in WS forebrain organoids. *GTF2IRD1* encoded by one WS gene *GTF2IRD1* binds to *TTR* promoter regions and regulates the expression of *TTR*. Exogenous *TTR* can rescue neurogenic and neurodevelopmental deficits of WS forebrain organoids by activating ERK signaling. Taken together, our results reveal the critical neurogenesis features of WS forebrain organoids and provide novel insight into mechanisms underlying the abnormal neurodevelopment of WS.

Results

The forebrain organoids of William's syndrome patient display deficits of neurogenesis and neuronal differentiation

To study the neuronal deficits in William's syndrome (WS) patient, we isolated peripheral blood mononuclear cells (PBMCs) and urinary cells from healthy control (CTRL) and WS patients (Supplementary Figure 1A), respectively. After the clinical evaluations of phenotypes of three WS patients (Supplementary Figure 1B), target sequencing was performed and showed the hemizygous deletion of 23 genes in three WS patients (Supplementary Figures 1C, 1D). PBMCs and urinary cells of CTRL and WS patients were cultured *in vitro*, respectively (Supplementary Figure 2A). For reprogramming, cultured cells were infected with Sendai virus according to the manufacturer's protocol. Three induced pluripotent stem cells (iPSCs) lines were characterized for each CTRL and WS samples. iPSCs displayed typical morphology, normal karyotypes, were positive for alkaline phosphatase live staining and expressed canonical pluripotency markers SOX2, NANOG, SSEA4 and OCT4 (Supplementary Figures 2B-2F).

To assess the neurodevelopmental deficits of WS patients, we generated three dimensional (3D) forebrain organoids with CTRL and WS iPSCs using an established protocol, respectively, which could be well correlated with fetal human brain development (Figure 1A [\[link\]](#)) (Kang et al., 2021b [\[link\]](#); Qian et al., 2016 [\[link\]](#)). Considering day 56 (D56) forebrain organoids were well correlated with fetal brain development (Kang et al., 2021b [\[link\]](#); Kostovic et al., 2019 [\[link\]](#); Qian et al., 2016 [\[link\]](#)), our following experiments adopted D56 forebrain organoids if no specific annotation. We first performed immunofluorescence staining and observed that WS forebrain organoids showed a higher percentage of Ki67⁺/SOX2⁺ cells compared to that of CTRL organoids (Figures 1B, 1C [\[link\]](#)). Further, CTRL and WS organoids were pulsed with 5-ethynyl-2'-deoxyuridine (EdU) at a dosage of 10 μM for 2 hr, and we found that the percentage of EdU⁺/SOX2⁺ cells also significantly increased in WS organoids (Figures 1D, 1E [\[link\]](#)). Furthermore, CTRL and WS organoids were incorporated with EdU for 24 hours, and the cell cycle exiting of neural progenitor cells (NPCs) was assessed by analyzing the co-localization of EdU and Ki67. We observed that the percentage of Ki67⁺EdU⁺ cells was significantly decreased in WS organoids (Figures 1F, 1G [\[link\]](#)). Fewer TBR2⁺ intermediate neural progenitor cells (NPCs) localized at MAP2⁺ ventricular zone (VZ) like layer in WS forebrain organoids compared to the CTRL organoids (Figures 1H, 1I [\[link\]](#)). These results suggest an aberrant proliferating capability of NPCs in WS forebrain organoids.

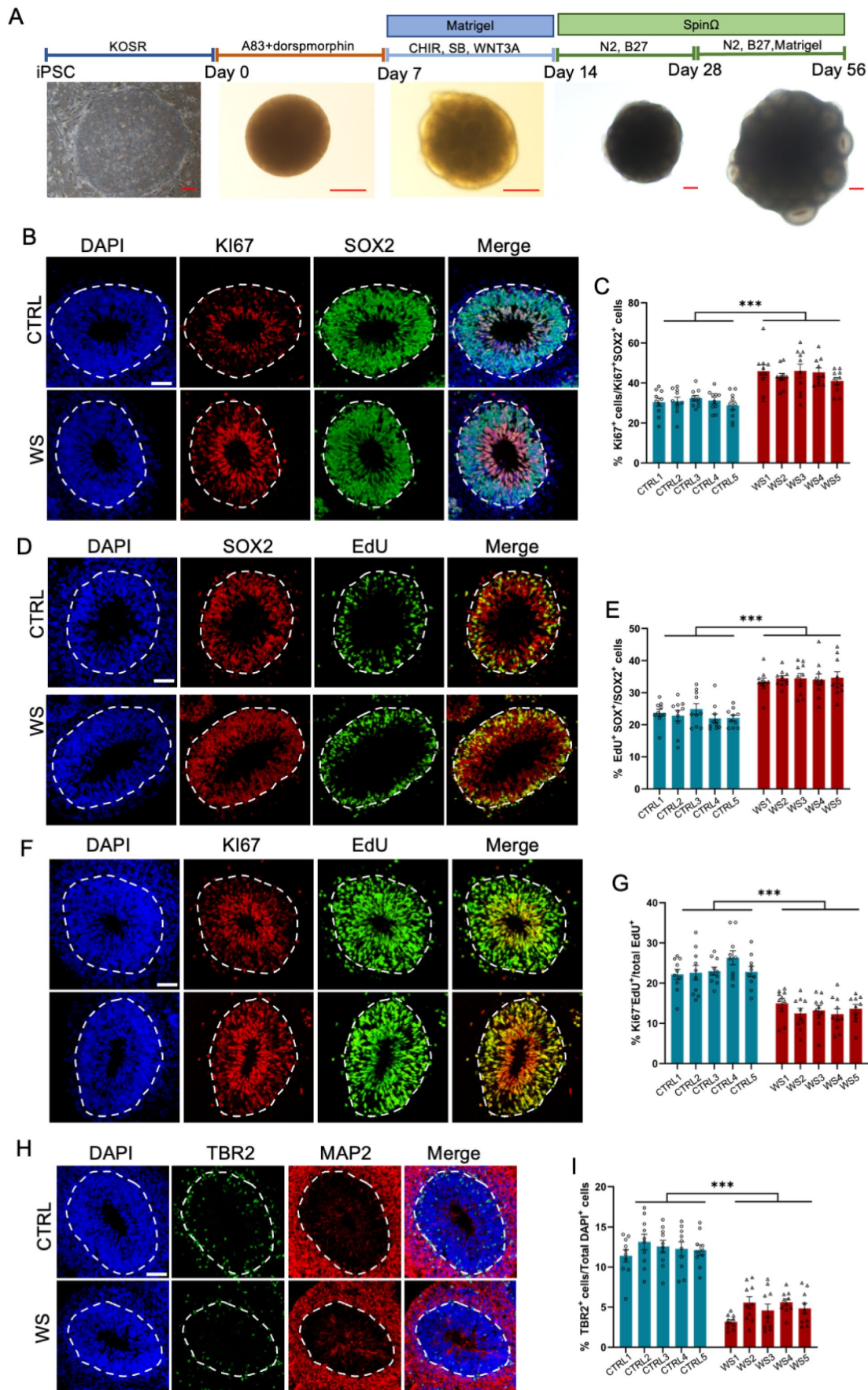


Figure 1.

WS neural progenitor cells display abnormal proliferating capability

(A) Schematic illustration of forebrain organoids culture process and representative brightfield images of organoids over development. Scale bar, 200 μ m.

(B) Representative images of KI67 and neural progenitor cells (NPCs) marker SOX2 immunofluorescence staining with healthy (CTRL) and WS patient (WS) forebrain organoids at day 56, respectively. The proliferating cells were positive for KI67 labeling, and NPCs were positive for SOX2 labeling. The white dotted line indicated the outer interface of the ventricular zone (VZ) region.

(C) Quantification results showed that the average proportion of KI67 positive (KI67⁺) cells among total NPCs (SOX2⁺) significantly increased in the VZ layer of WS forebrain organoids relative to CTRL.

(D) Representative images of EdU and SOX2 immunofluorescence staining with CTRL and WS forebrain organoids at day 56. EdU was applied for 2 hr.

(E) Quantification results showed that the average proportion of EdU⁺ cells among total SOX2⁺ NPCs significantly increased in the VZ layer of WS forebrain organoids compared to CTRL.

(F) Representative images of EdU and KI67 immunofluorescence staining with CTRL and WS forebrain organoids at day 56, respectively. EdU was applied for 24 hr.

(G) Quantification results show that the average proportion of KI67⁺EdU⁺ cells among total EdU⁺ cells significantly decreased in the VZ layer of WS forebrain organoids compared to CTRL. EdU was applied for 24 hr. KI67⁺EdU⁺ cells were counted, that is, the proportion of cells exiting the cell cycle.

(H) Representative images of TBR2, a marker for intermediate precursor cells (IPCs), and neuronal marker MAP2 immunofluorescence staining of CTRL and WS forebrain organoids, respectively.

(I) Quantification results showed that the average proportion of TBR2⁺ IPCs remarkably decreased in WS forebrain organoids compared to CTRL.

For (B)–(I), five IPS lines of three healthy control (Ctrl) and three WS patients (WS) were adopted for the study, respectively. 3 to 5 organoids were analyzed from each IPS line and 10 representative rosettes of organoids were analyzed in each line. values represent mean $\pm$ SEM. * p <0.05, ** p <0.01, *** p , 0.001, one-way ANOVA followed by Dunnett's multiple-comparison test. Scale bar, 50 μ m.

Next, we examined the differentiation capability of NPCs. We found that upon the differentiation, the expression of neuronal precursor and immature neuronal cell marker doublecortin positive (DCX⁺) cells at SOX2⁺ ventricle (VZ)-like layer significantly decreased in WS forebrain organoids (Figures 2A, 2B). The relative thickness of the VZ layer indicated by SOX2⁺ aberrantly increased, but the thickness of cortical plate (CP) layer indicated by MAP2⁺ abnormally decreased (Figures 2C–2E). The total number (Figures 2F, 2G) and the proportion of neuronal marker CTIP2⁺ cells in CP like layer (Figures 2F, 2H) were significantly decreased in WS forebrain organoids. The total number (Figures 2I, 2J) and the proportion of neuronal marker TBR1⁺ cells in CP like layer (Figures 2I, 2K) were also significantly decreased in WS forebrain organoids. These results suggest a dysregulated neuronal differentiation of NPCs in WS forebrain organoids.

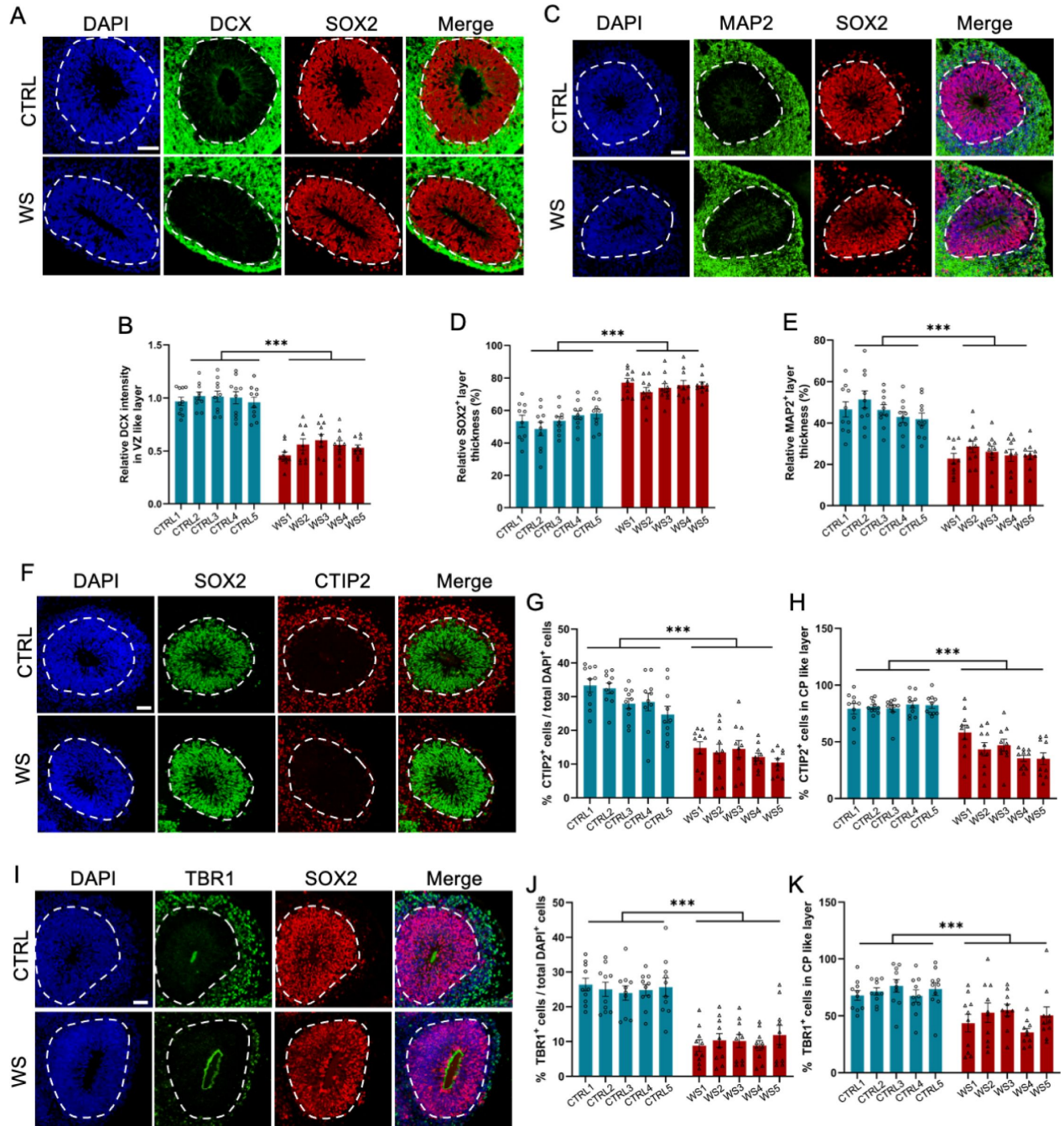


Figure 2

WS forebrain organoids exhibit the reduced neuronal differentiation

- (A) Representative images of DCX and SOX2 immunofluorescence staining of CTRL and WS forebrain organoids, respectively.
- (B) Quantification results showed that the intensity of DCX signal significantly decreased in SOX2⁺ VZ like layers of WS forebrain organoids compared to CTRL.
- (C) Representative images of MAP2 and SOX2 immunofluorescence staining of CTRL and WS forebrain organoids, respectively.
- (D, E) Quantification results showed that the thickness of VZ like layer (SOX2⁺MAP2⁻) significantly increased (D), but the thickness of cortical plate (CP) like layer (MAP2⁺) CP like layer significantly decreased (E) in WS forebrain organoids compared to CTRL.
- (F) Representative images of SOX2 and neuronal marker CTIP2 immunofluorescence staining of CTRL and WS forebrain organoids, respectively.
- (G) Quantification results showed that the proportion of CTIP2⁺ neurons significantly decreased in WS forebrain organoids compared to CTRL.
- (H) Quantification results showed that the relative thickness of SOX2⁺ VZ like layer significantly increased, while the the relative thickness of CTIP2⁺ CP like layer significantly decreased in WS forebrain organoids compared to CTRL.
- (I) Representative images of SOX2 and neuronal marker TBR1 immunofluorescence staining of CTRL and WS forebrain organoids, respectively.
- (J) Quantification results showed that the proportion of total TBR1⁺ neurons significantly decreased in WS forebrain organoids compared to CTRL.
- (K) Quantification results showed that the proportion of TBR1⁺ neurons significantly decreased in TBR1⁺ CP like layer of WS forebrain organoids compared to CTRL.

For (A-K), five IPS lines of Ctrl and WS were adopted for the study, respectively. 3 to 5 organoids were adopted from each IPS line and 10 representative rosettes of organoids were analyzed in each line. values represent mean $\pm$ SEM. *p<0.05, **p<0.01, ***p, 0.001, one-way ANOVA followed by Dunnett's multiple-comparison test. Scale bar, 50 μ m.

WS forebrain organoids display the aberrant expression of neurodevelopmental genes

Next, we performed bulk RNA sequencing (RNA-seq) with both CTRL and WS forebrain organoids. In CTRL forebrain organoids. **Figure 3A** [↗](#) showed the FPKM values of 23 WS-associated genes, and other three genes *LAT2*, *TREM270* and *FKBP6* showed very low expression (FPKM less than 1). The expression of these 23 WS genes significantly decreased in WS forebrain organoids (**Figure 3B** [↗](#)). In addition, RNA-seq data analysis revealed 1761 differentially expressed genes (DEGs): 1003 down-regulated and 758 up-regulated (**Figure 3C** [↗](#), supplemental Table 1). Gene Ontology (GO) analysis with all DEG showed enrichment for neuronal development and neurogenesis (**Figure 3D** [↗](#)), and interaction network of top 30 genes related to neurogenesis was shown (**Figure 3E** [↗](#)). GO analysis also revealed the enrichment of up-regulated genes for cell proliferation and of

down-regulated genes for neuronal differentiation, synaptic signaling and neurogenesis (**Figures 3F, 3G** [↗](#)), respectively. In addition, qRT-PCR results the decreased levels of NPC markers *SOX2*, *NESTIN*, *PAX6* and *HES5* (**Figures 3H** [↗](#)) and the increased levels of neuronal differentiation markers, such as *TBR1*, *TBR2*, *CTIP2* and *SATB2* (**Figures 3I** [↗](#)) in WS organoids. WB assay and quantification results showed that the levels of *SOX2*, *NESTIN*, *PAX6* and *HES5* were significantly increased (**Figures 3J-3N** [↗](#)) and the levels of *TBR1*, *SATB2* and *CTIP2* (**Figures 3O** [↗](#)-**3R** [↗](#)) were significantly decreased in WS organoids compared to CTRL. Collectively, these data suggested the aberrant expression of neurogenic and neurodevelopmental genes in WS forebrain organoids.

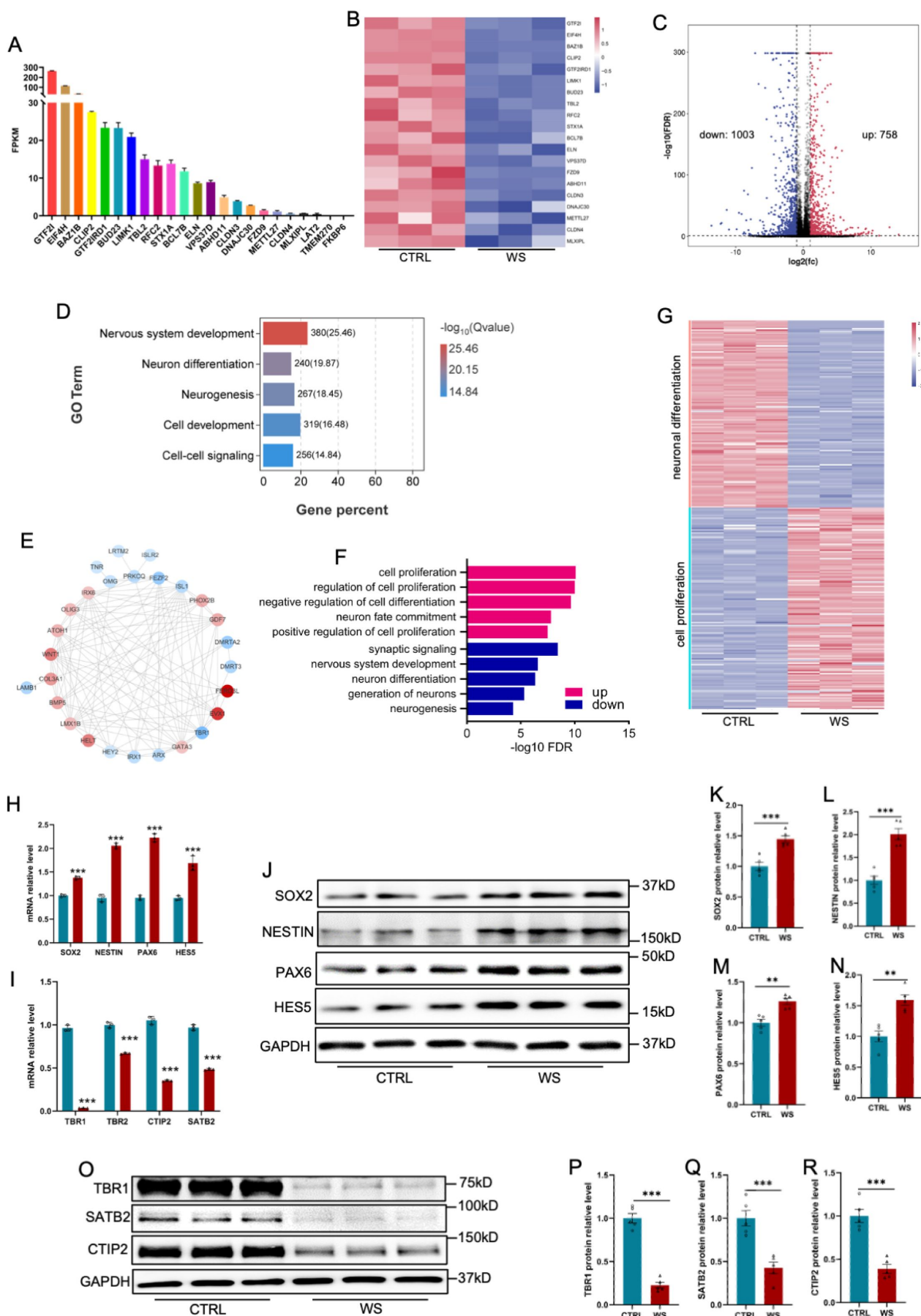


Figure 3.

WS forebrain organoids display the altered expression of neurogenic genes

(A) Fragments per kilobase of transcript per million mapped reads (FPKM) values of 23 WS genes in Day 56 CTRL organoids.

(B) Heatmap analysis of 23 WS genes in both CTRL and WS brain organoids.

(C) Volcano plot of the differentially expressed genes (DEGs). Bulk RNA sequencing (bulk RNA-seq) revealed 758 up-regulated and 1003 down-regulated genes in WS brain organoids compared with CTRL organoids, respectively. Fold change > 2 and p value < 0.05 were considered as significance.

(D) Gene Ontology (GO) analysis of DEGs showed the enrichment in terms related to neuronal differentiation and neurogenesis.

(E) Interactome plot of top 27 genes of neurogenesis GO term was presented. Red dots representing up regulated genes and blue dots representing down regulated genes.

(F) GO analysis of DEGs in WS organoids. Red bar represents terms enriched by up regulated genes, and blue bar represents terms enriched by down regulated genes.

(G) Heatmap of cell proliferation and neuronal differentiation related genes in CTRL and WS organoids

(H, I) qRT-PCR results showed the increased expression of NPCs markers including SOX2, NESTIN, PAX6 and HES5 (H) and the decreased expression of neuronal markers including TBR1, TBR1, CTIP2 and SATB2 (I) in WS organoids compared to Ctrl group. Values represent mean $\pm$ SEM; *p < 0.05, **p < 0.01, ***p < 0.001; unpaired Student's t test.

(J-N) WB assay (J) and quantification results showed the increased expression of SOX2 (K), NESTIN (L), PAX6 (M), HES5 (N) in WS organoids compared to CTRL group. Values represent mean $\pm$ SEM; *p < 0.05, **p < 0.01, ***p < 0.001; unpaired Student's t test.

(O-R) WB assay (O) and quantification results of TBR1 (P), CTIP2 (Q), SATB2 (R) in WS organoids compared to CTRL group. Values represent mean $\pm$ SEM; n=5 independent experiments; *p < 0.05, **p < 0.01, ***p < 0.001; unpaired Student's t test.

scRNA-seq reveals the abnormal generation of excitatory neurons in WS forebrain organoids

To gain further insight into the specific transcriptome alterations in different cell types of WS, we performed 10X genomics chromium single-cell RNA-Seq (scRNA-Seq) with WS and CTRL forebrain organoids. After quality control, total 96969 cells were obtained from CTRL and WS samples, and were analyzed together using unsupervised clustering (Figure 4A, supplementary Figure 4A). Uniform manifold approximation and projection (UMAP) identified 13 cell clusters in CTRL and WS forebrain organoids, which were then annotated using the expression of cell-type-specific markers (Figure 4B, supplementary Figures 4B, 4C). We observed that the proportions of deeper layer projection neuron (DL_PN) and upper layer projection neuron (UL_PN) clusters were significantly decreased in WS forebrain organoids compared to CTRL, while the proportions of other clusters did not show significant difference between CTRL and WS organoids (Figures 4C, 4E, supplementary Figure 4D). According to the markers for the cluster characterization

including *BCI11B* and *FEZF2*, DL_PN and UL_PN were excitatory neurons (Paulsen et al., 2022 [↗](#)). scRNA-seq data also revealed the differentially expressed genes (DEGs) in 6 distinct clusters including cycling progenitor, oRG, IPC, newborn DL_PN, DL_PN and UL_PN, which were related to the development of excitatory neurons (**Figure 4F** [↗](#)). GO analysis with DEGs in DL_PN and UL_PN clusters showed the enrichment for neuronal development and neuronal differentiation (**Figures 4G, 4H** [↗](#)). Further, GO analysis with DEGs in cycling progenitor, oRG, IPC and new born_DL_PN clusters showed the enrichment for neurogenesis, cell division and cell migration terms, etc (Supplementary Figures 4E-4I). In particular, UMAP distribution maps and heatmaps of WS-related genes showed a distinct expression pattern of WS-deleted genes in cell clusters (**Figure 4I** [↗](#)). Collectively, these results suggested the deficits of excitatory neurogenesis in WS forebrain organoids.

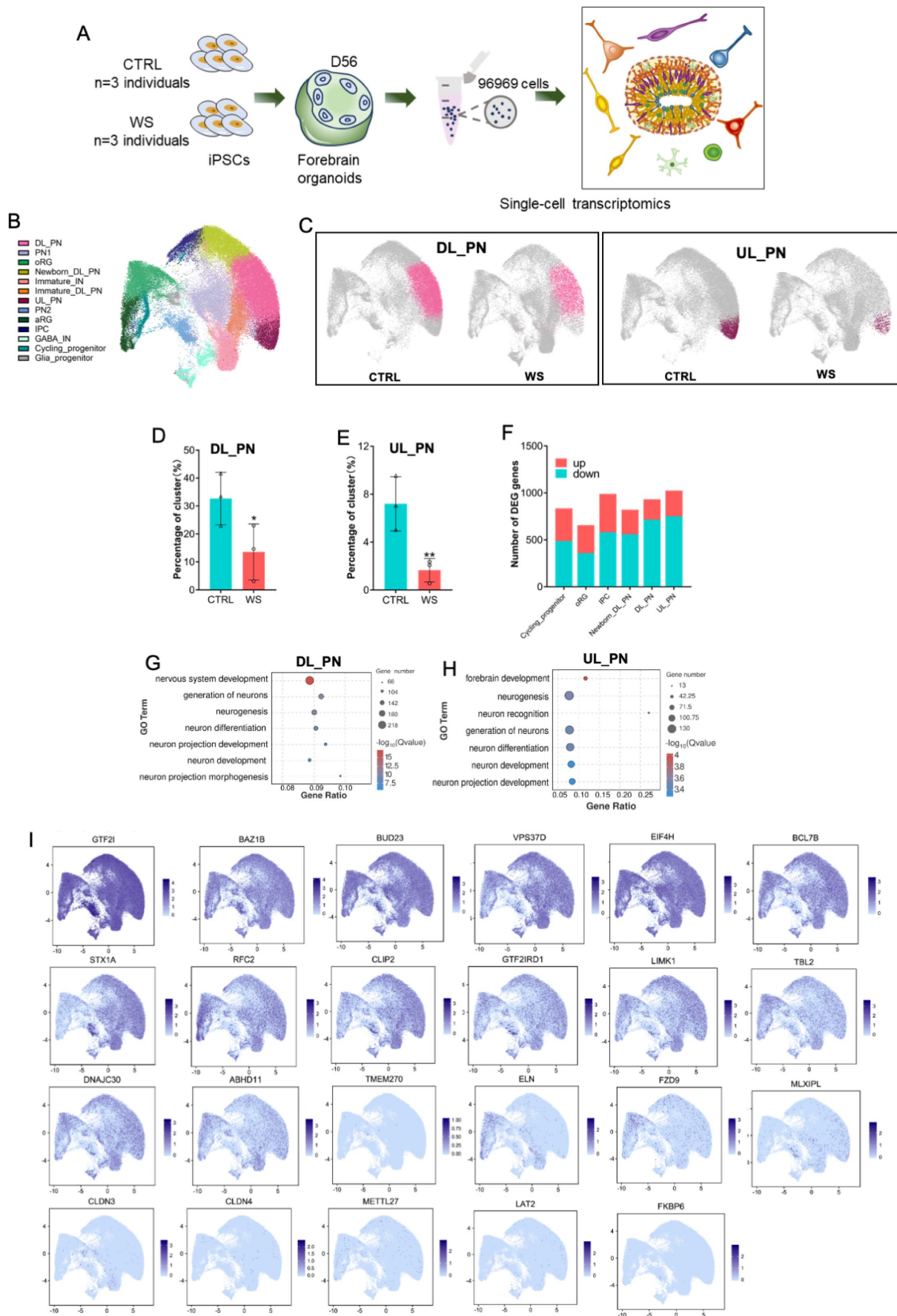


Figure 4.

scRNA-seq reveals the abnormal neuronal differentiation in WS forebrain organoids

- (A) Schematic illustration of the scRNA-seq and histological analysis of both CTRL and WS forebrain organoids.
- (B) UMAP plot of cell types detected in both CTRL and WS forebrain organoids. DL_PN, deep layer projection neurons; UL_PN, upper layer projection neurons; IN, interneurons; aRG, apical radial glia; oRG, outer radial glia; IPC, intermediate progenitor cells; GABA IN, GABAergic interneurons.
- (C) UMAP plot of DL_PN (left) and UL_PN (right) in both CTRL and WS forebrain organoids
- (D, E) Quantification of the percentages of DL_PN and UL_PN in both CTRL and WS forebrain organoids. Values represent mean $\pm$ SEM; * $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$; unpaired Student's t test.
- (F) Number of DEGs in the indicated cell types in WS organoids. Fold change > 1.28 and p value < 0.05 were considered as significance
- (G, H) GO analysis of DEGs in the DL_PN (I) and UL_PN (J) cell types in WS organoids.
- (I) UMAP visualization of the expression of WS genes in CTRL forebrain organoids.

Given the aberrant production of the excitatory neurons in WS forebrain organoids, we next analyzed the developmental pseudotime trajectories for cycling progenitor, oRG, IPC, newborn DL_PN, DL_PN, and UL_PN clusters of CTRL and WS organoids (Figure 5A, 5B). The pseudotime of excitatory neuronal trajectories in WS organoids analysis showed a remarkable delayed development compared to CTRL organoids (Figure 5C). Pseudotime uniform manifold approximation analysis revealed that the excitatory neuronal lineage displayed an increased distribution towards the start point trajectory and a decreased distribution towards the end point trajectory in WS organoids (Figure 5D). The varieties of gene expression along pseudotime trajectories was shown by heatmap (Figure 5E). Furthermore, the expression of NPC marker genes *SOX2* and *TBR2* exhibited increasing trend at early stage of trajectories, but the expression of neuronal marker genes *NHLH1*, *TBR1*, *CTIP2*, and *SATB2* showed decreasing trend at middle and later stages of trajectories in WS organoids (Figure 5F). Collectively, these data suggested an aberrant neurodevelopmental timeline in WS organoids.

The deficiency of *GTF2IRD1* reduces *Transthyretin* in WS brain organoids

Next, we aim to reveal the mechanism underlying the neuronal deficits of WS organoids. Integrated analysis with down-regulated genes identified both by bulk RNA-seq data and scRNA-seq, respectively, revealed one overlapped gene *transthyretin* (*TTR*) (Figure 6A), whereas there is no overlapped genes were identified in up-regulated genes by bulk RNA-seq data and scRNA-seq, respectively (Supplementary Figure 5A). *TTR* is required for the transportation of thyroxine and retinol and plays an essential function in the development of the central nervous system (Fleming et al., 2009; Gomes et al., 2016; Magalhaes et al., 2021; Santos et al., 2010). Interestingly, a previous study has showed a decreased expression of *TTR* in the brain of WS patients (Supplementary Figure 5B) (Barak et al., 2019). scRNA-seq data analysis showed that *TTR* was widely expressed in all cell clusters of CTRL organoids, but significantly decreased in WS organoids (Figures 6B, 6C). Immunofluorescence staining, qRT-PCR and WB assay results confirmed the remarkable decrease of *TTR* in WS brain organoids relative to Ctrl (Figures

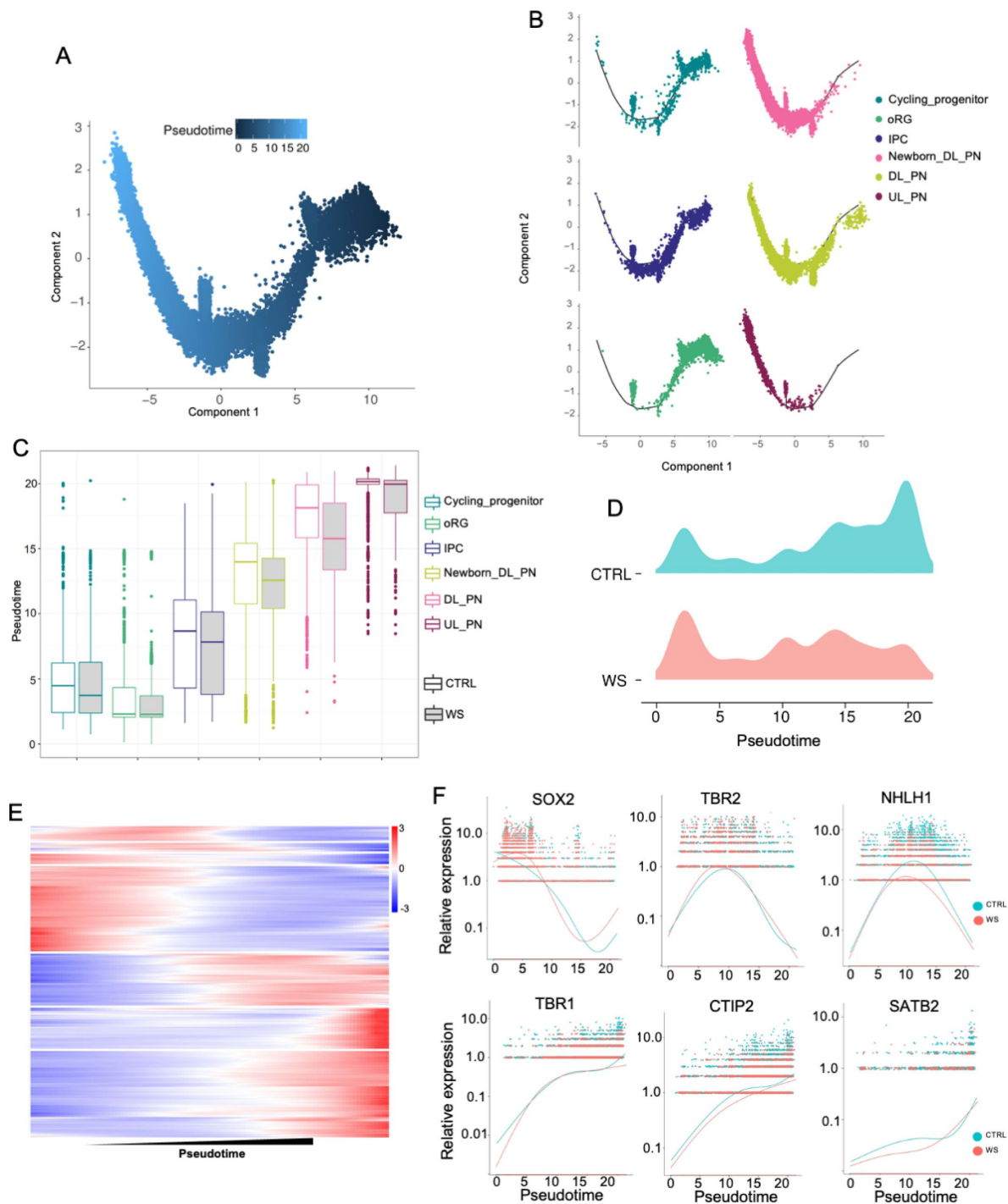


Figure 5.

WS forebrain organoids exhibit aberrant developmental trajectory of excitatory neurons

- (A) Pseudo-time distribution of cell trajectories of excitatory neurons related cell clusters.
 (B) Distribution of cell cluster in Pseudotime trajectories.
 (C) Boxplot showing the distribution of pseudotime within each cell type in both CTRL and WS-derived forebrain organoids.
 (D) Mountain maps of pseudotime uniform manifold approximation in both CTRL and WS forebrain organoids.
 (E) Heatmap of pseudotime line-dependent gene expression.
 (F) Expression of *SOX2*, *TBR2*, *NHLH1*, *TBR1*, *CTIP2* and *SATB2* ordered by Monocle analysis in pseudotime.

6D [↗](#)–**6G** [↗](#), supplementary Figure 5C). Of note, RNA-seq data analysis showed that down-regulated genes enriched for MAPK signaling pathway (**Figure 6H** [↗](#)). WB assay results showed the significant reduction of GTF2IRD1 and p-ERK/ERK in WS brain organoids (**Figures 6I** [↗](#)–**6L** [↗](#)). These results indicate a reduced expression of TTR and defects of MAPK/ERK signaling in WS brain organoids.

Next, we aim to uncover the mechanism of how TTR was regulated. Two genes among the deleted 23 genes in WS patients, *GTF2I* and *GTF2IRD1*, are two transcription factors and were widely expressed in distinct cell clusters in CTRL organoids revealed by scRNA-seq (as shown in **Figure 4I** [↗](#)). We speculated whether *GTF2I* and *GTF2IRD1* regulated the expression of TTR, and then performed knockdown (KD) of *GTF2I* and *GTF2IRD1* with shRNAs in HEK293T cells, respectively. We observed that the depletion of *GTF2IRD1* but not *GTF2I* led to a significant decrease of *TTR* (Supplementary Figures 5D, 5E). These results collectively suggest that *GTF2IRD1* deficiency indeed reduces the expression TTR.

Exogenous TTR rescues the neuronal deficits in WS brain organoids

Next, we aim to examine whether TTR application could rescue the neuronal deficits in WS brain organoids. WS brain organoids at day 28 were exposed to TTR recombinant protein (55 µg/ml) and collected for assays at day 56 (Supplementary Figure 6A). WB assay and quantification results showed TTR exposure significantly increased the level p-ERK (**Figures 7A** [↗](#)–**7C** [↗](#)), which could be significantly inhibited by the supplement of TTR inhibitor receptor-associated protein (RAP), a ligand inhibiting the internalization of TTR (**Figures 7D** [↗](#)–**7F** [↗](#)). Both TTR only and TTR plus RAP did not affect the level of total ERK (**Figures 7C**, **7F** [↗](#)). RAP Immunofluorescence staining and quantification results showed that both TTR exposure remarkably enhanced the percentages of CTIP2⁺ cells compared to untreated WS organoids (**Figures 7G** [↗](#), **7H**). TTR exposure also significantly increased the percentages of TBR1⁺ cells (**Figures 7I**, **7J** [↗](#)). These results suggest that TTR can rescue the neuronal deficits of WS forebrain organoids.

Gtf2ird1 deficiency induces neurodevelopmental deficits in mice

Finally, we aim to examine whether *Gtf2ird1* deficiency also induces neurodevelopmental deficits in mice. *Gtf2ird1*^{+/+} (Wild-type, WT), *Gtf2ird1*^{+/-} (heterozygous, Het) and *Gtf2ird1*^{-/-} (knockout, KO) mice were adopted for the study. Immunofluorescence staining with brain sections and quantification results showed that *Gtf2ird1*^{+/-} and *Gtf2ird1*^{-/-} mice had the increased BrdU⁺ (**Figures 8A**, **8B** [↗](#)) and Ki67⁺Sox2⁺ (**Figures 8C**, **8D** [↗](#)) neuronal progenitor cells in VZ/SVZ regions, respectively, but had the decreased CTIP2⁺ (**Figures 8E**, **8F** [↗](#)), STAB⁺ cells (**Figures 8E**, **8G** [↗](#)) and TBR1⁺ (**Figures 8H**, **8I** [↗](#)) in IZ/CP regions relative to WT mice, respectively. In addition, WB assay and quantification results showed that the protein levels of *Gtf2ird1*, p-ERK and TTR (**Figures 8J** [↗](#)–**8N** [↗](#)) were significantly decreased in the cortex of *Gtf2ird1*^{+/-} and *Gtf2ird1*^{-/-} mice relative to WT mice, respectively.

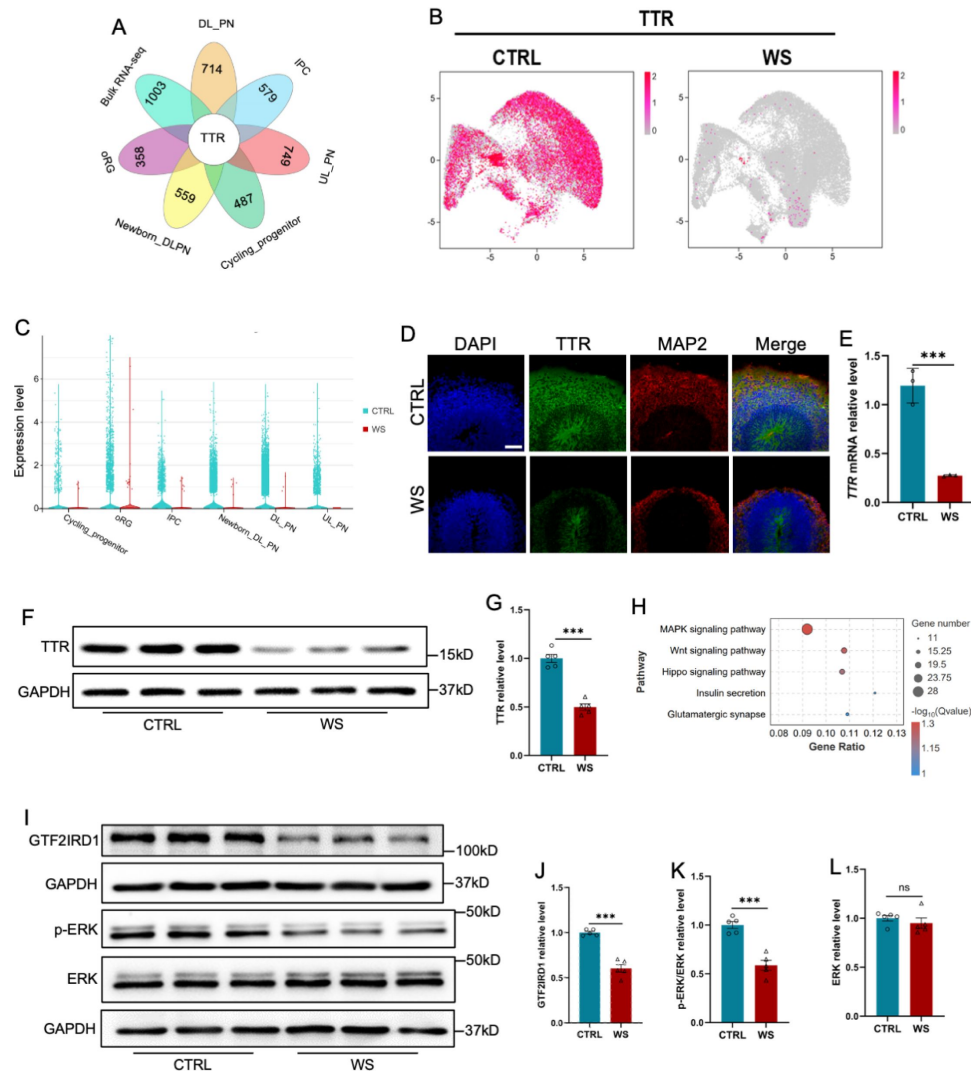


Figure 6.

The deficiency of *GTF2IRD1* leads to the down-regulation of *Transthyretin* and inhibition of ERK signaling

(A) Integrated analysis of down-regulated genes in 6 clusters relating to the generation of excitatory neurons and down-regulated genes revealed by bulk RNA-seq.

(B) UMAP visualization of the expression of *TTR* in CTRL and WS forebrain organoids, respectively.

(C) *Transthyretin* (*TTR*) displayed the decreased expression in 6 clusters relating to the generation of excitatory neurons.

(D) Representative images of *TTR* immunofluorescence staining with in CTRL and WS forebrain organoids, respectively. Scale bar, 100 μ m.

(E) qRT-PCR results showed that *TTR* level significantly decreased in WS forebrain organoids compared to Ctrl group. Values represent mean $\pm$ SEM; n=3 independent experiments; *p < 0.05, **p < 0.01, ***p < 0.001; unpaired Student's t test.

(F, G) WB assay (F) and quantification results (G) showed that the level of *TTR* was significantly decreased in WS brain organoids compared to Ctrl. Values represent mean $\pm$ SEM; n=5 independent experiments; *p < 0.05, **p < 0.01, ***p < 0.001; unpaired Student's t test.

(H) KEGG analysis showed that down-regulated genes enriched for MAPK pathway, etc.

(I-K) WB assay (I) and quantification results showed that the level of phosphorylated ERK (p-ERK) was significantly decreased (J), but the level of total ERK (K) was not changed in WS brain organoids compared to Ctrl. Values represent mean $\pm$ SEM; n=5 independent experiments; *p < 0.05, **p < 0.01, ***p < 0.001; unpaired Student's t test.

(L, M) WB assay (L) and quantification results (M) showed that the level of *GTF2IRD1* was significantly decreased in WS brain organoids compared to Ctrl. Values represent mean $\pm$ SEM; n=5 independent experiments; *p < 0.05, **p < 0.01, ***p < 0.001; unpaired Student's t test.

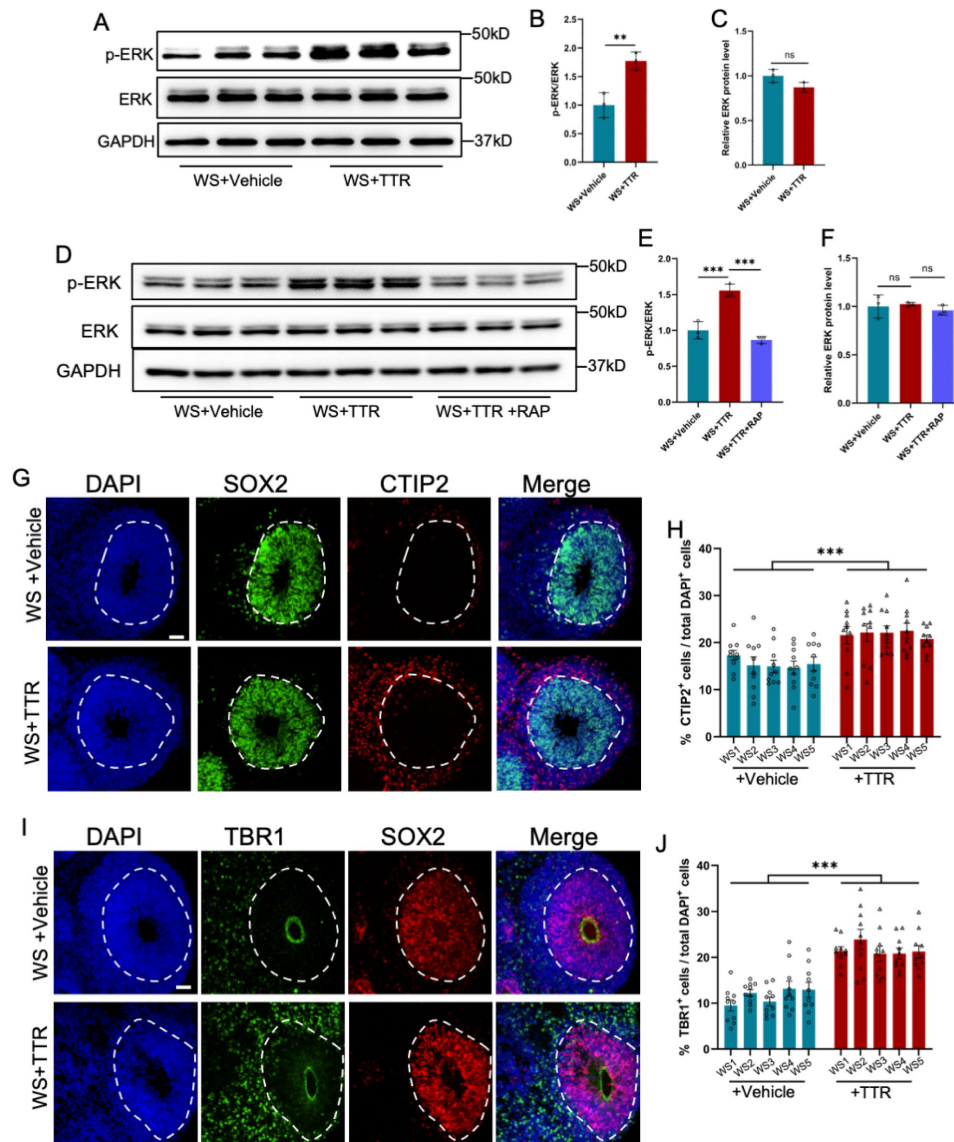


Figure 7.

Recombinant TTR rescues neurodevelopmental defects of WS organoids

(A-C) WB assay (A) and quantification results showed that TTR exposure enhanced the level of p-ERK in WS brain organoids (B), while the level of total ERK was not affected (C). Values represent mean $\pm$ SEM; n=3; *p < 0.05, **p < 0.01, ***p < 0.001; unpaired Student's t test.

(D-F) Western blot (D) and quantification results showed that TTR exposure enhanced the level of p-ERK (E), which could be inhibited by receptor-associated protein (RAP) in WS brain organoids (F). Values represent mean $\pm$ SEM; n=3 independent experiments; *p < 0.05, **p < 0.01, ***p < 0.001; unpaired Student's t test.

(G) Representative images of SOX2 and CTIP2 immunofluorescence staining with WS-derived forebrain organoids under different culture conditions. Scale bar, 50 μ m.

(H) Quantification results showed that TTR exposure significantly increased the proportion of CTIP2⁺ neurons in WS-derived forebrain organoids compared to CTRL. Values represent mean $\pm$ SEM; n=30-35 VZ-like regions of 3-5 organoids from three WS patients in each group. *p < 0.05, **p < 0.01, ***p < 0.001; unpaired Student's t test.

(I) Representative images of SOX2 and TBR1 immunofluorescence staining with WS-derived forebrain organoids under different culture conditions. Scale bar, 50 μ m.

(J) Quantification results showed that TTR exposure significantly increased the proportion of TBR1⁺ neurons in WS-derived forebrain organoids. Values represent mean $\pm$ SEM; n = 30-35 VZ-like regions of 3-5 organoids from three WS patients in each group. *p < 0.05, **p < 0.01, ***p < 0.001; unpaired Student's t test.

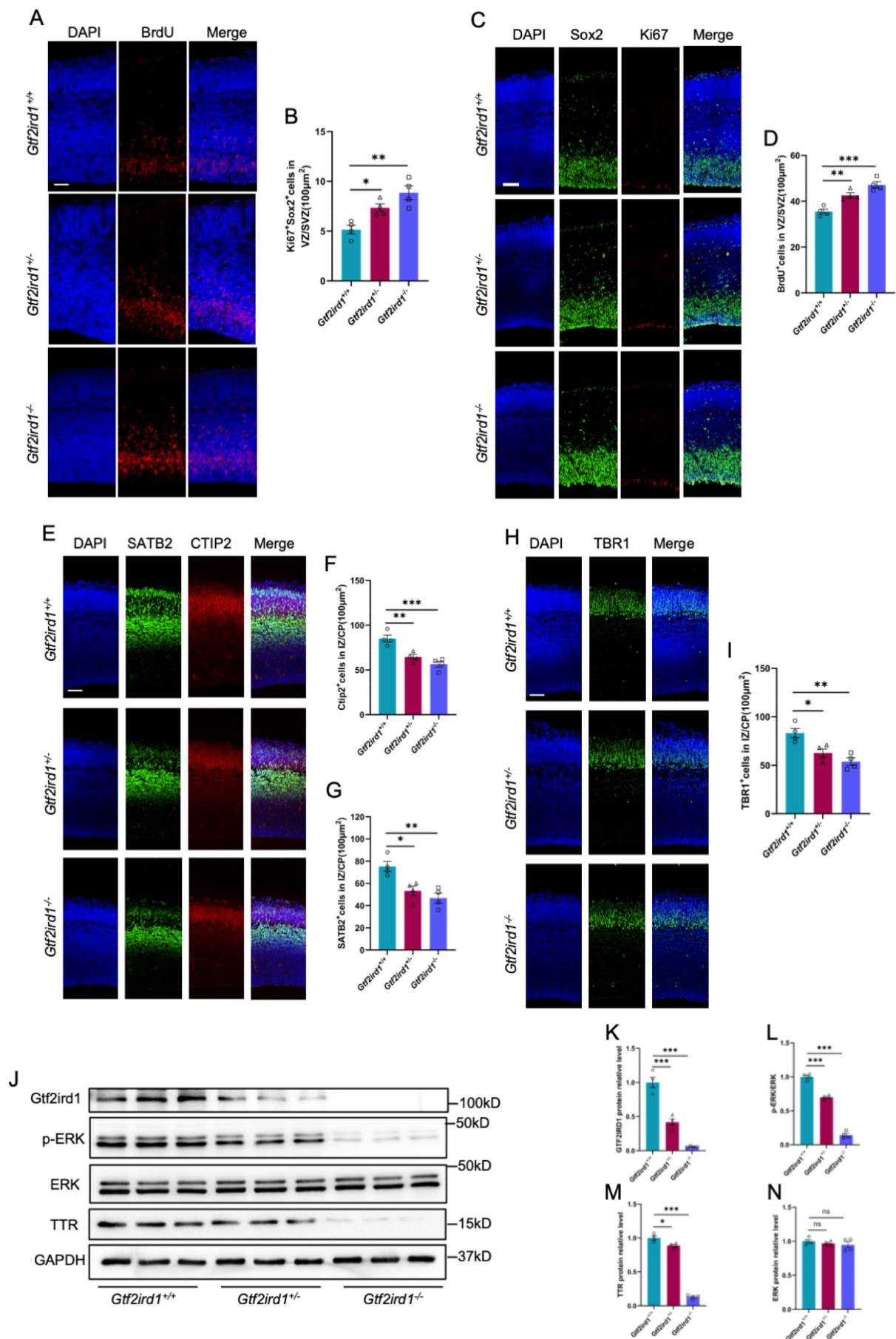


Figure 8.

***Gtf2ird1* deficient mice display neurodevelopmental deficits and the reduced TTR**

(A, B) Representative images of BrdU immunofluorescence staining (A) and quantification results (B) showed the increased BrdU⁺ eNPCs in VZ/SVZ of *Gtf2ird1*^{+/-} (heterozygous, Het) and *Gtf2ird1*^{-/-} (knockout, KO) mice compared to *Gtf2ird1*^{+/+} (Wild-type, WT) mice, respectively. Pups were injected with BrdU at E15.5 and sacrificed 1.5 hr later. Values represent mean ± SEM; n=4 animals for each group; *p < 0.05, **p < 0.01, ***p < 0.001; unpaired Student's t test. Scale bar, 50 μm.

(C, D) Representative images of Sox2 and Ki67 immunofluorescence staining (C) and quantification results (D) showed the increased Ki67⁺Sox2⁺ eNPCs in VZ/SVZ of Het and KO mice compared to WT mice, respectively. Values represent mean ± SEM; n=4 animals for each group; *p < 0.05, **p < 0.01, ***p < 0.001; unpaired Student's t test. Scale bar, 50 μm.

(E-G) Representative images of neuronal marker SATB2 and CTIP2 immunofluorescence staining (E) and quantification results showed the decreased Ctip2⁺ (F) and SATB2⁺ (G) neurons of Het and KO mice compared to WT mice, respectively. Values represent mean ± SEM; n=4 animals for each group; *p < 0.05, **p < 0.01, ***p < 0.001; unpaired Student's t test. Scale bar, 50 μm.

(H, I) Representative images of neuronal marker Tbr1 immunofluorescence staining (H) and quantification results (I) showed the decreased Tbr1⁺ neurons in IZ/CP of Het and KO mice compared to WT mice, respectively. Values represent mean ± SEM; n=4 animals for each group; *p < 0.05, **p < 0.01, ***p < 0.001; unpaired Student's t test. Scale bar, 50 μm.

(J-N) WB assay (J) and quantification results showed that the levels of *Gtf2ird1* (K), p-ERK (L) and TTR (M) were significantly decreased, while the level of total ERK (N) was not altered in the cortical tissues of Het and KO mice compared to WT mice, respectively. Values represent mean ± SEM; n=4 animals for each group; *p < 0.05, **p < 0.01, ***p < 0.001; unpaired Student's t test.

Next, we examined the effects of *Gtf2ird1* on the proliferation and differentiation of embryonic neural progenitor cells of mouse (eNPCs) *in vitro*. eNPCs were isolated from the forebrains of WT, Het and KO mice, respectively. BrdU incorporation assay and quantification results showed that the percentage of BrdU⁺Nestin⁺/Nestin⁺ (Figures 9A, 9B) and the percentage of Ki67⁺Sox2⁺/Sox2⁺ (Figures 9C, 9D) were significantly increased in Het and KO eNPCs, respectively. Upon the differentiation, Het and KO eNPCs produced fewer Tuj1⁺ neurons and more GFAP⁺ astrocytes relative to WT eNPCs (Figures 9E-9G), respectively. qRT-PCR results consistently showed the altered expression of *Gtf2ird1*, *TTR*, *Ki67*, *Nestin* and *Sox2* in proliferating eNPCs (Supplemental Figure 7A) and *Gtf2ird1*, *TTR*, *Tuj1* and *Gfap* (Supplemental Figure 7B) in differentiated eNPCs, respectively. We then further performed acute knockdown of *GTF2IRD1* (KD) in eNPCs and found that *GTF2IRD1* KD significantly reduced the mRNA level of *TTR* (Figures 9H, 9I). WB assay and quantification results showed that *GTF2IRD1* KD also significantly reduced the protein level of TTR (Figures 9J-9L). In addition, *GTF2IRD1* KD significantly inhibited neuronal differentiation of mouse eNPCs (Supplementary Figures 7C, 7D).

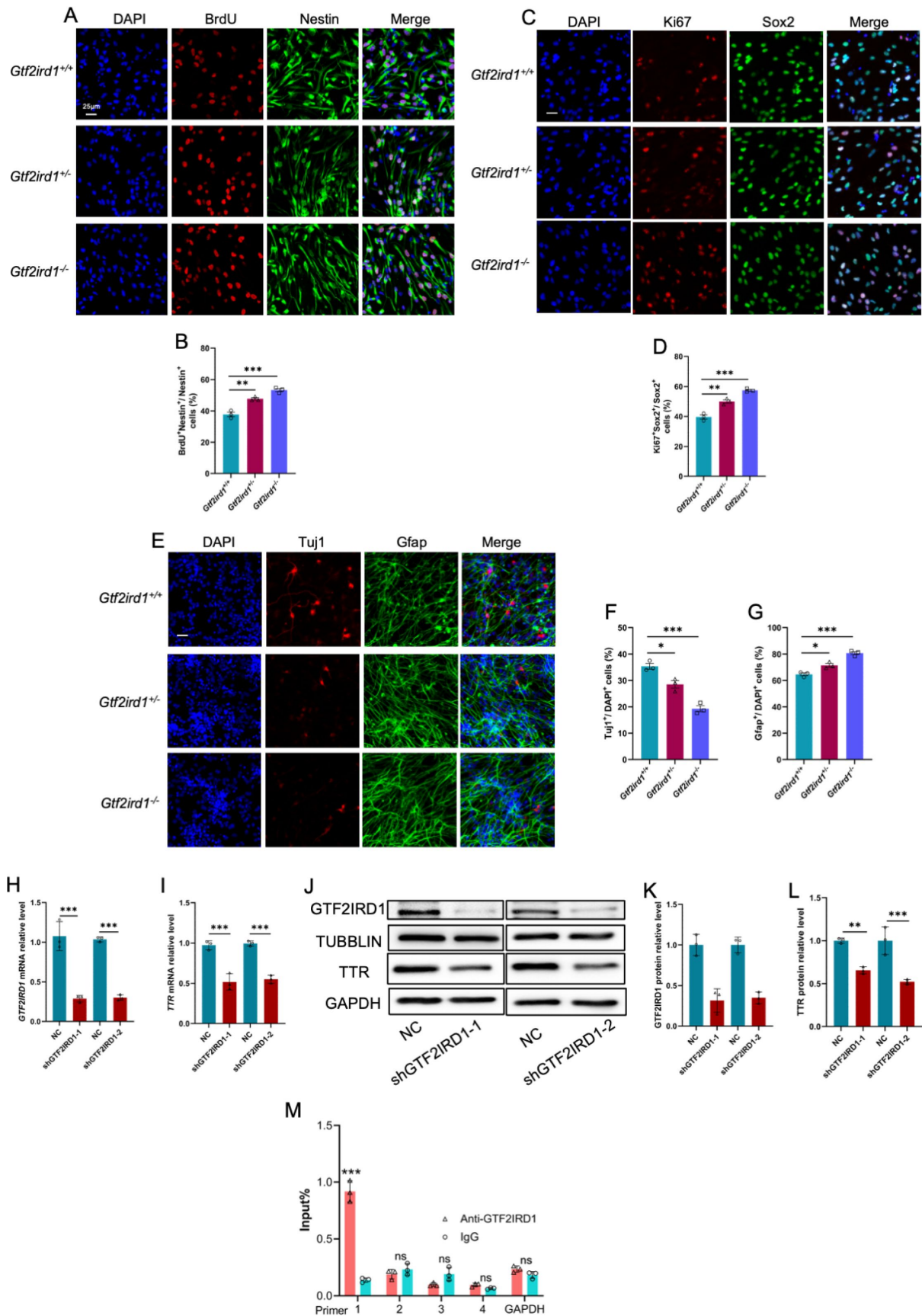


Figure 9.

Gtf2ird1 regulates the proliferation and differentiation of eNPCs *in vitro*

(A) Representative images of BrdU and Nestin immunofluorescence staining with cultured eNPCs from WT, Het and KO mice, respectively. Scale bar, 25 μ m.

(B) Quantification results showed that the percentage of BrdU⁺/BrdU⁺Nestin⁺ eNPCs was significantly increased in Het and KO mice relative to WT cells, respectively. Values represent mean $\pm$ SEM; n=3 independent experiments; *p < 0.05, **p < 0.01, ***p < 0.001; unpaired Student's t test.

(C) Representative images of Ki67 and Sox2 immunofluorescence staining with cultured eNPCs from WT, Het and KO mice, respectively. Scale bar, 25 μ m.

(D) Quantification results showed that the percentage of Ki67⁺/Ki67⁺Sox2⁺ eNPCs was significantly increased in Het and KO mice relative to WT cells, respectively. Values represent mean $\pm$ SEM; n=3 independent experiments; *p < 0.05, **p < 0.01, ***p < 0.001; unpaired Student's t test.

(E) Representative images of Tuj1 and Gfap immunofluorescence staining with cultured eNPCs from WT, Het and KO mice, respectively. Scale bar, 25 μ m.

(F, G) Quantification results showed that the number of Tuj1⁺ neurons was significantly decreased (**F**), but the number of Gfap⁺ astrocytes was significantly increased (**G**) upon the differentiation of eNPCs in Het and KO mice relative to WT cells, respectively. Values represent mean $\pm$ SEM; n=3 independent experiments; *p < 0.05, **p < 0.01, ***p < 0.001; unpaired Student's t test.

(H, I) qRT-PCR results showed that acute knockdown of *Gtf2ird1* significantly reduced the level of *Gtf2ird1* (**H**) and *TTR* (**I**) in WT eNPCs compared to scramble group (NC), respectively. Values represent mean $\pm$ SEM; n=3 independent experiments; *p < 0.05, **p < 0.01, ***p < 0.001; unpaired Student's t test.

(J-L) WB assay (**J**) and quantification results showed that acute knockdown of *Gtf2ird1* significantly reduced the level of Gtf2ird1 (**K**) and TTR (**L**) in WT eNPCs, respectively. Values represent mean $\pm$ SEM; n=3 independent experiments; *p < 0.05, **p < 0.01, ***p < 0.001; unpaired Student's t test.

(M) Chromatin immunoprecipitation followed qPCR (ChIP-qPCR) showed that Gtf2ird1 binds to the promoter regions of TTR. Values represent mean $\pm$ SEM; n=3 independent experiments; *p < 0.05, **p < 0.01, ***p < 0.001; unpaired Student's t test.

Finally, we examined whether GTF2IRD1 could bind to the promoter region of TTR to regulate the expression of TTR. Chromatin immunoprecipitation followed by qPCR results with eNPCs showed that GTF2IRD1 indeed binds to the promoter region of TTR (**Figure 9M** [↗](#)). Collectively, these results suggested that *Gtf2ird1* deficiency leads to abnormal neurogenesis through regulating TTR in mice.

Discussion

In the present study we established a forebrain organoid model and identified aberrant neurogenesis deficits in WS organoids (Supplemental Figure 8). Bulk RNA-seq revealed the altered gene expression related to neuronal development. scRNA-seq revealed an altered developmental trajectory and aberrant excitatory neurogenesis in WS organoids. Mechanistically, we showed that

transthyretin (TTR) had a significantly decreased expression in WS organoids. GTF2IRD1 encoded by one WS gene *GTF2IRD1* regulated the expression of TTR. Furthermore, exogenous TTR could rescue the neurogenesis deficits of WS organoids. Collectively, our data highlight the essential function of GTF2IRD1-TTR axis for mammalian neuronal development and provide novel insights into mechanisms underlying the abnormal neurogenesis in WS brain.

Individuals with WS clinically exhibited diverse neuronal deficits including hypersociability, autistic deficits, impaired myelination, intellectual disability and abnormal brain structure due to the altered neuronal development (Kozel et al., 2021 [↗](#); Osborne, 2010 [↗](#); Poher, 2010 [↗](#)). Distinct models have been applied to investigate the mechanisms underlying these defects (Alesi et al., 2021 [↗](#); Barak and Feng, 2016 [↗](#); Barak et al., 2019 [↗](#); Chailangkarn et al., 2016 [↗](#); Corley et al., 2016 [↗](#); Dai et al., 2009 [↗](#); Enkhmandakh et al., 2009 [↗](#); Kopp et al., 2019 [↗](#); Kopp et al., 2020 [↗](#); Kozel et al., 2021 [↗](#); Mervis et al., 2012 [↗](#); Morris et al., 2020 [↗](#); Osborne, 2010 [↗](#); Tassabehji et al., 2005 [↗](#)). Brain organoids derived iPSCs under 3D culturing condition have been providing a unique model to investigate human brain development and neurological diseases (Amin and Pasca, 2018 [↗](#); Benito-Kwiecinski and Lancaster, 2019 [↗](#); Clevers, 2016 [↗](#); Di Lullo and Kriegstein, 2017 [↗](#); Kelava and Lancaster, 2016 [↗](#); Lancaster et al., 2013 [↗](#); Li and Izpisua Belmonte, 2019 [↗](#); Pasca, 2019 [↗](#); Trujillo and Muotri, 2018 [↗](#)). Utilizing the forebrain organoids together with scRNA-seq, our study has revealed the aberrant capabilities of proliferation and differentiation of WS NPCs. Of note, our study revealed for the first time an altered developmental trajectory and a remarkably reduced excitatory neurogenesis in WS organoids. Therefore, our findings provide a new insight regarding the deficits of neurogenesis and neuronal development in WS brain.

Genotype-phenotype relationship analysis suggests that among 25-27 WS genes, each gene could be responsible for distinct phenotypes and could contribute to WS phenotype at varying degrees (Kozel et al., 2021 [↗](#); Lek et al., 2016 [↗](#); Tassabehji, 2003 [↗](#)). For example, LIMK1, CYLN2, DSCAM, PAK1, GTF2I and BAZ1B contributed for neurological phenotypes (Barak et al., 2019 [↗](#); Lalli et al., 2016 [↗](#); Pinelli et al., 2020 [↗](#); Todorovski et al., 2015 [↗](#); van Hagen et al., 2007 [↗](#)), ELN for blood vessel development (Li et al., 1998 [↗](#)), and DNAJC30 for mitochondrial function (Tebbenkamp et al., 2018 [↗](#)). As a member of the transcription factor II (TF-II), GTF2IRD1 regulates gene expression and involves multiple processes, such as embryonic development, cell cycle and neuronal development (Enkhmandakh et al., 2009 [↗](#); Kopp et al., 2020 [↗](#); O'Leary and Osborne, 2011 [↗](#); Palmer et al., 2007 [↗](#); Tassabehji et al., 2005 [↗](#); Thompson et al., 2007 [↗](#); van Hagen et al., 2007 [↗](#)). Our findings reveal a wide expression of *GTF2IRD1* in all identified cell clusters. Intriguingly, the deletion of *GTF2IRD1*, but not *GTF2I*, caused a remarkable decrease of *transthyretin* (TTR). TTR is widely expressed in the neuronal system and promotes neuronal development (Buxbaum et al., 2008 [↗](#); Gomes et al., 2016 [↗](#); Li et al., 2011 [↗](#); Magalhaes et al., 2021 [↗](#)). TTR deficient mice displayed a reduced neurogenesis and increased oligodendrogenesis (Alshehri et al., 2020 [↗](#); Vancamp et al., 2019 [↗](#)). Our results showed that *Gtf2ird1* deficiency also induces abnormal neurogenesis and neurodevelopmental deficits and reduces TTR in mice. Consistently, TTR had decreased expression in the postmortem frontal cortex of WS patients (Barak et al., 2019 [↗](#)). Our results also show that TTR can rescue neuronal deficits of NPCs in WS organoids through activating ERK signaling. Therefore, our findings reveal a novel mechanism underlying the aberrant neurogenesis and neuronal development in WS brain.

In summary, our study has showed the abnormal neurogenesis and neuronal differentiation, especially the aberrant excitatory neurogenesis in WS organoids. Our study has revealed that the dysfunction of GTF2IRD1-TTR axis plays essential roles for the neurodevelopmental deficits of WS brain. Our findings also suggest that TTR could be a potential target for the treatment of WS at clinic.

Methods

Animals

Gtf2ird1^{+/-} mice were purchased from Model Animal Research Center (Nanjing, China). *Gtf2ird1*^{+/-} male mice were crossed with *Gtf2ird1*^{+/-} female mice to generate *Gtf2ird1*^{+/+} (wild-type), *Gtf2ird1*^{+/-} (heterozygous, Het) and *Gtf2ird1*^{-/-} (homozygous, KO) mice. Animals were in C57BL/6 genetic background and housed in the animal center of Zhejiang University under 12-hour light/12-hour dark conditions with free access to food and water. The genotypes of animals were determined with PCR. The used primers included: 5'TGGAAGCCCAGTGACTACTTG and 5'ACATAGGCATAAGGGCCCAG (for wild-type band), and 5'CAATGAGAGCGTCTTCGTGAT and 5'ACCATTGAAAAGTAGAGTAGAATGC (for knockout band). All animal experiments were performed following with the protocols approved by the Zhejiang University Animal Care and Ethics Committee.

The establishment and culture of iPSCs

Peripheral blood samples of three male patients with WS and three healthy males were collected at the Children's Hospital, School of Medicine, Zhejiang University following the protocol approved by the ethics committee of Zhejiang university Medical University Children's hospital ([2016]NO.326). After the isolation of peripheral blood mononuclear cells (PBMCs) and urinary cells, respectively, the reprogramming was carried out following the manufacturer's protocol (ThermoFisher, Cat# A16517). Briefly, around 1X10⁵ PBMCs were transduced using the Sendai reprogramming vectors at appropriate MOI in 24-well plates. For feeder-free culture, iPSCs were cultured with mTeSR 1 medium (STEMCELL) on Matrigel (Corning). For feeder-dependent culture, iPSCs were cultured with DMEM/F12 medium (Corning) consisting of 20% KnockOut Serum Replacement (Gibco), 1X GlutaMAX (Gibco), 1X MEM Non-Essential Amino Acids (Gibco), 100 μM 2-Mercaptoethanol (Sigma), 10 ng/mL human FGF-basic (PeproTech) on the inactivated mouse embryonic fibroblasts (MEF). The information of used reagents could be found in Table S2.

The culture of forebrain organoids

The generation and maintenance of forebrain organoids were carried out as described previously with moderation (Kang et al., 2021b; Qian et al., 2016). iPSCs colonies on MEF were digested with 1 mg/mL Collagenase Type IV (Gibco) at 37°C for 1 hour. After the digestion, the colonies were resuspended with the forebrain first medium (DMEM/F12, 20% KnockOut Serum Replacement, 1x GlutaMAX, 1x MEM Non-Essential Amino Acids, 100μM 2-Mercaptoethanol, 2μM A83-01, 2μM Dorsomorphin) and cultured in low-attachment 6-well plate (Corning) for 4 days. On day 5 and day 6, the medium was halfly replaced with forebrain second medium containing DMEM/F12 (Corning), 1X GlutaMAX (Gibco), 1X MEM Non-Essential Amino Acids (Gibco), 1X N2 (Gibco), 1μM SB-431542 (STEMCELL), 1μM CHIR99021 (STEMCELL), 4ng/mL WNT-3A (R&D Systems), 10μg/mL Heparin (Sigma). On day 7, EBs were embedded with Matrigel using forebrain second medium in order to induce the neuroepithelium. On day 14, organoids were mechanically separated and transferred to 12-well spin Ω bioreactor for culture, and the medium was changed to forebrain third medium, consisting of DMEM/F-12 (Corning), 1X GlutaMAX (Gibco), 1X MEM Non-Essential Amino Acids (Gibco), 1X N2 (Gibco), 1XB27 (Gibco), 2.5μg/mL insulin (Sigma), 1X P/S (Gibco), 100μM 2-Mercaptoethanol (Sigma). From day 35 to day 56, 1% Matrigel was added to the forebrain third medium to remodel the extracellular matrix (ECM) in 12-well spinning bioreactors.

Targeted panel sequencing

DNA was extracted from the peripheral blood of healthy control and WS patients, and purified with GeneJET Whole Blood Genomic DNA Purification Mini Kit (Fisher Scientific). DNA purity was identified by NanoDrop® spectrophotometers (Thermo Fisher, MA, USA). DNA concentration was quantified by Qubit® DNA Assay Kit in Qubit® 3.0 Fluorometer (Life Technologies, CA). For each sample, a total of 1 µg of genomic DNA was used for library preparation with the NimbleGen SeqCap EZ Human Exome V3 following manufacturer's instructions (Basel, Swiss). Library concentration was measured by Qubit® DNA Assay Kit in Qubit® 3.0, and insert size was detected by the Agilent Bioanalyzer 2100 system (Agilent Technologies, CA, USA). The clustering of the index coded samples was performed on a cBot Cluster Generation System according to the manufacturer's recommendations (Illumina, USA). After cluster generation, the library preparations were sequenced on an Illumina HiSeq Ten platform with 150 bp paired end module. The panel kit was customized from Agilent Technologies, Design ID: 3249861, Cat#5190-4816, Species: H.sapiens (UCSC hg19, GRCh37).

Total RNA isolation and quantitative real-time PCR

Total RNA was extracted with TRIzol reagent following the manufacturer's protocol (Thermo Fisher Scientific). The concentration of RNA was quantified using a NanoDrop spectrophotometer 2000 (Thermo Fisher Scientific). 0.5 mg of total RNA was used for reverse transcription, and standard real-time qPCR assays were performed using SYBR Green (Vazyme) in triplicates. The results were analyzed using the $\Delta\Delta C_t$ method. The information of used primers could be found in Table S3.

Bulk RNA-seq and data analysis

RNA quality was determined using Agilent 2100 Bioanalyzer. After enriched by Oligo(dT) beads, mRNA was fragmented into short fragments using fragmentation buffer and reverse transcribed into cDNA using NEBNext Ultra RNA Library Prep Kit for Illumina (NEB, USA). Purified double-stranded cDNA fragments were end-repaired, base added and ligated to Illumina sequencing adapters. The ligation reaction was purified with the AMPure XP Beads(1.0X). Ligated fragments were subjected to size selection by agarose gel electrophoresis followed by PCR amplification. Library quality was assessed on the Agilent Bioanalyzer 2100 system (Agilent Technologies Inc.) and sequenced on an Illumina HiSeq platform (Illumina NovaSeq6000).

To obtain high quality clean reads, raw data of fastq format were processed by removing reads containing adapter, reads containing ploy-N and low-quality reads from raw data. FPKM (Fragments Per Kilobase of transcript sequence per Millions base pairs sequenced) of each transcript was calculated based on the length of the gene and reads count mapped to this gene. Transcripts with the parameter of false discovery rate (FDR) below 0.05 and absolute fold change ≥ 2 were considered differentially expressed transcripts.

Single cell RNA-sequencing (scRNA-seq)

Three pairs of Day 56 forebrain organoids derived from iPSCs of three CTRL and WS patients were dissociated with 3 mL Tryp-LE (Sigma-Aldrich) for 15 minutes at 37°C, respectively. A single-cell suspension was subsequently collected at a cell density of 1000 cells/µL, and were added to a 10X Genomics GemCode Single-cell instrument to generates a single-cell Gel Bead-In-Emulsion (GEMs). The leftover biochemical reagents and primers were removed with silane magnetic beads. Full-length, barcoded cDNAs were then amplified by PCR to generate sufficient amount of DNA for library construction. Libraries were sequenced by Chromium Next GEM Single Cell 3' Reagent Kits v3.1. Single Cell 5' 16 bp 10x Barcode and 10 bp UMI were encoded in Read 1, while Read 2 was

used to sequence cDNA fragment. Sample index sequences were incorporated as the i7 index read. Read 1 and Read 2 were standard Illumina® sequencing primer sites used in paired-end sequencing.

scRNA-seq data analysis

10X Genomics Cell Ranger software (version 3.1.0) was used for the conversion of raw BCL files to FASTQ files, alignment and counts quantification. Reads with low-quality barcodes and UMIs were filtered out and then mapped to the reference genome (human GRCh38). Reads were uniquely mapped to the transcriptome and intersecting an exon at least 50% were considered for UMI counting. Before quantification, the UMI sequences would be corrected for sequencing errors, and valid barcodes were identified based on the Empty Drops method (Lun et al., 2019 [\[1\]](#)). The cell by gene matrices were produced via UMI counting and cell barcodes calling. The cell by gene matrices for each sample were individually imported to Seurat version 3.1.1 for downstream analysis (Butler et al., 2018 [\[2\]](#)). Cells with unusually high number of UMIs 8000) or mitochondrial gene percent 10%) were filtered out. We also excluded cells with less than 500 or more than 4000 genes detected. Expression value of each gene in given cluster were compared against the rest of cells using Wilcoxon rank sum test. Cell clusters were annotated to known biological cell types using canonical cell marker genes (Paulsen et al., 2022 [\[3\]](#)). Significant upregulated genes were determined based on 1) at least 1.28 fold increased in the target cluster; 2) expressed in more than 25% of the cells in the target cluster; 3) p value is less than 0.05.

Gene ontology (GO) analysis was performed using the DAVID database as described previously (Chen et al., 2021 [\[4\]](#)). Each enriched GO function term is represented by a node and the node size is proportional to the number of genes in its corresponding function term in the enrichment maps. Similar GO functions are categorized as one cluster. The function term and the number of genes in each cluster are labelled.

Isolation and culture of embryonic mouse NPCs

Embryonic mouse neural progenitor cells (eNPCs) were isolated and cultured as described previously (Li et al., 2017, Li et al., 2021). Briefly, the cerebral cortex of mice (Embryonic day 14, Day 14) was dissected and treated with 0.1% Trypsin for 5 min in 37°C. Then cells were cultured with DMEM/F-12 medium with 2% B27 (minus vitamin A), 1% antibiotic-antimycotic, 20 ng/mL FGF-2, 20 ng/mL EGF, and in a 5% CO₂ incubator at 37°C. eNPCs were maintained in proliferating conditions for 2 weeks. For the differentiation assay, eNPCs were cultured with DMEM/F-12 medium containing 2% B27 (minus vitamin A), 2 mM L-glutamine, 1% antibiotic-antimycotic, 1 μM retinoic acid and 5 μM forskolin (differentiation medium) for 48 hrs. For BrdU incorporation assay, BrdU was supplemented at the final concentration of 5 μM for 8 hrs. At the scheduled time point, eNPCs were fixed with 4% paraformaldehyde (PFA) followed by immunostaining with proper antibodies.

Culture of HEK293T cells and plasmid transfection

HEK293T cells were cultured with DMEM medium containing 10% FBS, 2 mM L-glutamine, and 1% antibiotic-antimycotic in a 5% CO₂ incubator at 37°C, and medium was replaced every day. After the cell density reaches about 70%, half volume of medium was changed with fresh medium 6 h before the transfection. 4 μg of plasmids and 6 μl of Lipo2000 were mixed with 100 μl of Opti-MEM at room temperature for 10 minutes, respectively. Reagents were mixed together and further reacted at room temperature for 10 minutes. The mixture was added to one well of a 6-well plate. The medium was replaced with fresh medium 8-10 hrs after transfection. Cells were harvested for assays 48 hrs post transfection.

Immunofluorescence staining

Organoids were collected and fixed with 4% paraformaldehyde for 4 hours. After washed with 1X PBS and dehydrated with 30% sucrose solution at 4°C, brain organoids were embedded with O.C.T (SAKURA) and sections of 10 µm thickness were prepared with a cryostat (Leica).

Embryonic day 15.5 (E15.5) pups were hypothermic anesthetized with ice, and transcardially perfused with cold phosphate buffer saline (PBS) followed by cold 4% paraformaldehyde (PFA). Brain samples were removed, and post-fixed with 4% PFA overnight and completely dehydrated with 30% sucrose at 4°C. Brain samples were embedded with OCT (4583, SAKURA) and 30-µm thickness sections were prepared with a cryostat (CM1950, Leica). For BrdU assay, E15.5 pups were injected with BrdU (0.1 mg/10 g body weight) and sacrificed 1.5 hr later.

To perform immunofluorescence staining, section samples were incubated with blocking buffer for 1 hr and applied with proper primary antibodies at 4°C overnight. The next day, samples were washed with 1x PBS followed by the application of proper secondary antibodies. Images were taken with a confocal microscope (Olympus, FV3000). The quantification of the thickness and cell number of were performed with imageJ software as described previously (Kang et al., 2021a [\[link\]](#); Qian et al., 2016 [\[link\]](#)). For stratification and thickness analysis of brain organoid, ventricular zone (VZ) layers were defined as SOX2⁺ and CTIP2⁻ or TBR1⁻ (MAP2⁻) regions. The cortical plate (CP) layer is defined as CTIP2⁺ or TBR1⁺ or MAP2⁺ regions.

Click-iT™ EdU labeling

Organoids were applied with 10 µM EdU for 2 h or 24 h, respectively. At the scheduled timepoint, cells were washed with 1X PBS, and subsequently fixed with 4% paraformaldehyde and dehydrated with 30% sucrose. Brain organoid sections were prepared with a cryostat (Leica). EdU staining was performed following the manufacturer's manual (Invitrogen). Primary and secondary antibodies were applied. Images were taken with a confocal microscope (Olympus, FV3000).

Plasmid transfection and lentivirus packaging

Scramble shRNA (5'-TTCTCCGAACGTGTCACGT-3'), shRNAs targeting human *GTF2I* (5'-CGGATGAGTGATAGTCTGTA-3'), human *GTF2IRD1* (5'-GCGCCATAGTATCCACTTCAT-3') and mouse *Gtf2ird1* (#1, 5'-GTGCCCTACAAGAGAATCAAA-3'; #2, 5'-GTGAAGCTCTGGGCATCAAAAT-3') were cloned into lentivirus vector, respectively. Lentiviruses were packaged, and used to infect HEK293T cells and eNPCs. 48 hrs later, the cells were collected or fixed with 4% paraformaldehyde for the following experiments.

Western blot

The collected organoids and cell samples were treated with RIPA buffer (Abcam) containing 1X protease inhibitor cocktail (MCE). Protein concentration was measured with a Biophotometer (Eppendorf), and after denaturation, 20-40 µg proteins were subjected to SDS-polyacrylamide gel electrophoresis. Signals were detected with Tanon Detection system (Tanon) and the intensities of the immunoblot bands were normalized to Gapdh or Tubulin. The information of primary and secondary antibodies used can be found in Table S2. Western blot images were analyzed using ImageJ software.

TTR exposure

WS brain organoids at day 28 were exposed to TTR recombinant protein (55 µg/ml) and collected for assays at day 56 followed by immunostaining. Some organoids were also treated with TTR only, TTR plus RAP (one TTR inhibitor) (55 µg/ml) and collected for assays at day 56 followed by western

blot assays.

Chromatin immunoprecipitation-qPCR assay

Chromatin immunoprecipitation (ChIP) experiment was performed following the manufacturer's protocol (Beyotime Biotechnology, China). Briefly, 250 μ L of 37% formaldehyde was added to cultured cells for cross-link for 10 minutes at 37°C. The reaction was then immediately terminated with 125 mM glycine solution for 5 min. Then cells were washed with PBS containing 1 mM and resuspended in 200 μ L SDS lysis buffer containing 1 mM PMSF in an ice bath for 10 min. Samples were sonicated into DNA fragments of 200-600 bp size using a sonicator. 20 μ L sample was kept as input control, and the left was incubated with anti-IgG (Proteintech) and anti-GTF2IRD1 antibody (RD system) overnight at 4°C, respectively. The next day, after centrifugation of sample, pellets were washed with Low Salt Immune Complex Wash Buffer, High Salt Immune Complex Wash Buffer, LiCl Immune Complex Wash Buffer and TE Buffer. Samples were de-crosslinked and DNA was purified by a PCR purification kit according to the manual (Beyotime biotechnology, China). qPCR reaction was performed to measure enrichment with 4 pairs of primers designed within the promoter region of *TTR*, and *GAPDH* was used as a internal control. Fold enrichment was calculated by the comparative threshold cycling method using Equation $2^{-\Delta\Delta C_t}$. Primer sequences were provided in Table S2.

Statistical Analysis

Data were presented as mean $\pm$ SEM. Statistical analysis between group differences was performed with unpaired Student's t test or one way ANOVA using Graph Prism software (version 9.0, GraphPad). For multiple group comparisons, a one-way ANOVA followed by Dunnett's multiple-comparison test. *P* value less than 0.05 was considered as statistical significance. Replicate information is indicated in the figure legends.

Data Availability

Bulk RNAs-seq and scRNAs-seq data have been deposited in the National Genomics Data Center and the accession number HRA002966 (<https://ngdc.cncb.ac.cn/gsa-human> .

Study approval

This study was approved by the ethics committee of Zhejiang university Medical University Children's hospital ([2016]NO.326).

Acknowledgements

This work was supported by the National Natural Science Foundation of China (grants 82371182 to X.L.) and the National Key Research and Development Program of China (2017YFE0196600 to X.L.).

Author Contributions

X.L. and Q.S. conceptualized the project. X.Z., Q.S. and Y.S. performed iPSCs and organoids culture, biochemical assay and analyzed organoids immunostaining data with the help of M.W., X.H., Y.L., C.W. and Y.G. W.C. and C.J. performed clinical evaluation, and collected urine and blood samples of

Ctrl and WS patients. W.Q. analyzed the target sequencing data. X.Z. and W.Q. analyzed bulk RNA-seq data, scRNA-seq data, and did qRT-PCR, western blot and *in utero* electroporation with the help of X.H. X.L. wrote the manuscript. All authors reviewed and approved the final manuscript.

Declaration of Interests

The authors declare no competing interests.

References

1. Alesi V. *et al.* (2021) **Atypical 7q11.23 deletions excluding ELN gene result in Williams-Beuren syndrome craniofacial features and neurocognitive profile** *Am J Med Genet A* **185**:242–249
2. Alshehri B., Pagnin M., Lee J.Y., Petratos S., Richardson S.J (2020) **The Role of Transthyretin in Oligodendrocyte Development** *Scientific reports* **10**
3. Amin N.D., Pasca S.P (2018) **Building Models of Brain Disorders with Three-Dimensional Organoids** *Neuron* **100**:389–405
4. Barak B., Feng G (2016) **Neurobiology of social behavior abnormalities in autism and Williams syndrome** *Nat Neurosci* **19**:647–655
5. Barak B. *et al.* (2019) **Neuronal deletion of Gtf2i, associated with Williams syndrome, causes behavioral and myelin alterations rescuable by a remyelinating drug** *Nat Neurosci* **22**:700–708
6. Benito-Kwiecinski S., Lancaster M.A (2019) **Brain Organoids: Human Neurodevelopment in a Dish** *Cold Spring Harbor perspectives in biology*
7. Butler A., Hoffman P., Smibert P., Papalexi E., Satija R (2018) **Integrating single-cell transcriptomic data across different conditions, technologies, and species** *Nat Biotechnol* **36**:411–420
8. Buxbaum J.N., Ye Z., Reixach N., Friske L., Levy C., Das P., Golde T., Masliah E., Roberts A.R., Bartfai T (2008) **Transthyretin protects Alzheimer's mice from the behavioral and biochemical effects of Abeta toxicity** *Proc Natl Acad Sci U S A* **105**:2681–2686
9. Carmona-Mora P. *et al.* (2015) **The nuclear localization pattern and interaction partners of GTF2IRD1 demonstrate a role in chromatin regulation** *Hum Genet* **134**:1099–1115
10. Chailangkarn T. *et al.* (2016) **A human neurodevelopmental model for Williams syndrome** *Nature* **536**:338–343
11. Chen J. *et al.* (2021) **Ogt controls neural stem/progenitor cell pool and adult neurogenesis through modulating Notch signaling** *Cell reports* **34**
12. Clevers H (2016) **Modeling Development and Disease with Organoids** *Cell* **165**:1586–1597
13. Collins R.T. (2013) **Cardiovascular disease in Williams syndrome** *Circulation* **127**:2125–2134
14. Collins R.T (2018) **Cardiovascular disease in Williams syndrome** *Curr Opin Pediatr* **30**:609–615
15. Corley S.M., Canales C.P., Carmona-Mora P., Mendoza-Reinosa V., Beverdam A., Hardeman E.C., Wilkins M.R., Palmer S.J (2016) **RNA-Seq analysis of Gtf2ird1 knockout epidermal tissue provides potential insights into molecular mechanisms underpinning Williams-Beuren syndrome** *BMC Genomics* **17**

16. Dai L. *et al.* (2009) **Is it Williams syndrome? GTF2IRD1 implicated in visual-spatial construction and GTF2I in sociability revealed by high resolution arrays** *Am J Med Genet A* **149**:302–314
17. Di Lullo E., Kriegstein A.R. (2017) **The use of brain organoids to investigate neural development and disease** *Nat Rev Neurosci* **18**:573–584
18. Enkhmandakh B., Makeyev A.V., Erdenechimeg L., Ruddle F.H., Chimge N.O., Tussie-Luna M.I., Roy A.L., Bayarsaihan D (2009) **Essential functions of the Williams-Beuren syndrome-associated TFII-I genes in embryonic development** *Proc Natl Acad Sci U S A* **106**:181–186
19. Fleming C.E., Mar F.M., Franquinho F., Saraiva M.J., Sousa M.M (2009) **Transthyretin internalization by sensory neurons is megalin mediated and necessary for its neuritogenic activity** *J Neurosci* **29**:3220–3232
20. Gomes J.R., Nogueira R.S., Vieira M., Santos S.D., Ferraz-Nogueira J.P., Relvas J.B., Saraiva M.J (2016) **Transthyretin provides trophic support via megalin by promoting neurite outgrowth and neuroprotection in cerebral ischemia** *Cell Death Differ* **23**:1749–1764
21. Kang Y. *et al.* (2021) **A human forebrain organoid model of fragile X syndrome exhibits altered neurogenesis and highlights new treatment strategies** *Nat Neurosci* **24**:1377–1391
22. Kang Y. *et al.* (2021) **A human forebrain organoid model of fragile X syndrome exhibits altered neurogenesis and highlights new treatment strategies** *Nat Neurosci*
23. Kelava I., Lancaster M.A (2016) **Stem Cell Models of Human Brain Development** *Cell Stem Cell* **18**:736–748
24. Khattak S. *et al.* (2015) **Human induced pluripotent stem cell derived neurons as a model for Williams-Beuren syndrome** *Molecular brain* **8**
25. Kippenhan J.S., Olsen R.K., Mervis C.B., Morris C.A., Kohn P., Meyer-Lindenberg A., Berman K.F (2005) **Genetic contributions to human gyrification: sulcal morphometry in Williams syndrome** *J Neurosci* **25**:7840–7846
26. Kopp N., McCullough K., Maloney S.E., Dougherty J.D (2019) **Gtf2i and Gtf2ird1 mutation do not account for the full phenotypic effect of the Williams syndrome critical region in mouse models** *Hum Mol Genet* **28**:3443–3465
27. Kopp N.D., Nygaard K.R., Liu Y., McCullough K.B., Maloney S.E., Gabel H.W., Dougherty J.D (2020) **Functions of Gtf2i and Gtf2ird1 in the developing brain: transcription, DNA binding and long-term behavioral consequences** *Hum Mol Genet* **29**:1498–1519
28. Kostovic I., Sedmak G., Judas M (2019) **Neural histology and neurogenesis of the human fetal and infant brain** *Neuroimage* **188**:743–773
29. Kozel B.A., Barak B., Kim C.A., Mervis C.B., Osborne L.R., Porter M., Pober B.R (2021) **Williams syndrome** *Nat Rev Dis Primers* **7**
30. Lalli M.A. *et al.* (2016) **Haploinsufficiency of BAZ1B contributes to Williams syndrome through transcriptional dysregulation of neurodevelopmental pathways** *Hum Mol Genet* **25**:1294–1306

31. Lancaster M.A., Renner M., Martin C.A., Wenzel D., Bicknell L.S., Hurles M.E., Homfray T., Penninger J.M., Jackson A.P., Knoblich J.A (2013) **Cerebral organoids model human brain development and microcephaly** *Nature* **501**:373–379
32. Lek M. *et al.* (2016) **Analysis of protein-coding genetic variation in 60,706 humans** *Nature* **536**:285–291
33. Li D.Y., Faury G., Taylor D.G., Davis E.C., Boyle W.A., Mecham R.P., Stenzel P., Boak B., Keating M.T (1998) **Novel arterial pathology in mice and humans hemizygous for elastin** *J Clin Invest* **102**:1783–1787
34. Li M., Izpisua Belmonte J.C (2019) **Organoids - Preclinical Models of Human Disease** *N Engl J Med* **380**:569–579
35. Li X., Masliah E., Reixach N., Buxbaum J.N (2011) **Neuronal production of transthyretin in human and murine Alzheimer's disease: is it protective?** *J Neurosci* **31**:12483–12490
36. Lin C.J., Staiculescu M.C., Hawes J.Z., Cocciolone A.J., Hunkins B.M., Roth R.A., Lin C.Y., Mecham R.P., Wagenseil J.E (2019) **Heterogeneous Cellular Contributions to Elastic Laminae Formation in Arterial Wall Development** *Circ Res* **125**:1006–1018
37. Lun A.T.L., Riesenfeld S., Andrews T., Dao T.P., Gomes T., participants in the 1st Human Cell, Atlas J., Marioni J.C. (2019) **EmptyDrops: distinguishing cells from empty droplets in droplet-based single-cell RNA sequencing data** *Genome Biol* **20**
38. Lyon L (2019) **Building brains: using brain organoids to study neural development and disease** *Brain* **142**
39. Magalhaes J., Eira J., Liz M.A (2021) **The role of transthyretin in cell biology: impact on human pathophysiology** *Cell Mol Life Sci* **78**:6105–6117
40. Makeyev A.V., Bayarsaihan D (2009) **New TFII-I family target genes involved in embryonic development** *Biochem Biophys Res Commun* **386**:554–558
41. Mervis C.B. *et al.* (2012) **Duplication of GTF2I results in separation anxiety in mice and humans** *Am J Hum Genet* **90**:1064–1070
42. Meyer-Lindenberg A., Kohn P., Mervis C.B., Kippenhan J.S., Olsen R.K., Morris C.A., Berman K.F (2004) **Neural basis of genetically determined visuospatial construction deficit in Williams syndrome** *Neuron* **43**:623–631
43. Morris C.A., Braddock S.R., Council On G (2020) **Health Care Supervision for Children With Williams Syndrome** *Pediatrics* **145**
44. Morris C.A. *et al.* (2003) **GTF2I hemizyosity implicated in mental retardation in Williams syndrome: genotype-phenotype analysis of five families with deletions in the Williams syndrome region** *Am J Med Genet A* **123**:45–59
45. O'Leary J., Osborne L.R (2011) **Global analysis of gene expression in the developing brain of Gtf2ird1 knockout mice** *PLoS One* **6**

46. Osborne L.R (2010) **Animal models of Williams syndrome** *Am J Med Genet C Semin Med Genet* **154**:209–219
47. Palmer S.J., Tay E.S., Santucci N., Cuc Bach T.T., Hook J., Lemckert F.A., Jamieson R.V., Gunnning P.W., Hardeman E.C (2007) **Expression of Gtf2ird1, the Williams syndrome-associated gene, during mouse development** *Gene Expr Patterns* **7**:396–404
48. Pasca S.P. (2019) **Assembling human brain organoids** *Science* **363**:126–127
49. Paulsen B. *et al.* (2022) **Autism genes converge on asynchronous development of shared neuron classes** *Nature* **602**:268–273
50. Pinelli M. *et al.* (2020) **A small 7q11.23 microduplication involving GTF2I in a family with intellectual disability** *Clin Genet* **97**:940–942
51. Pober B.R (2010) **Williams-Beuren syndrome** *N Engl J Med* **362**:239–252
52. Pober B.R., Johnson M., Urban Z (2008) **Mechanisms and treatment of cardiovascular disease in Williams-Beuren syndrome** *J Clin Invest* **118**:1606–1615
53. Qian X. *et al.* (2016) **Brain-Region-Specific Organoids Using Mini-bioreactors for Modeling ZIKV Exposure** *Cell* **165**:1238–1254
54. Qian X., Song H., Ming G.L (2019) **Brain organoids: advances, applications and challenges** *Development* **146**
55. Santos S.D., Lambertsen K.L., Clausen B.H., Akinc A., Alvarez R., Finsen B., Saraiva M.J (2010) **CSF transthyretin neuroprotection in a mouse model of brain ischemia** *J Neurochem* **115**:1434–1444
56. Tassabehji M (2003) **Williams-Beuren syndrome: a challenge for genotype-phenotype correlations** *Hum Mol Genet* **12**:R229–237
57. Tassabehji M. *et al.* (2005) **GTF2IRD1 in craniofacial development of humans and mice** *Science* **310**:1184–1187
58. Tebbenkamp A.T.N. *et al.* (2018) **The 7q11.23 Protein DNAJC30 Interacts with ATP Synthase and Links Mitochondria to Brain Development** *Cell* **175**:1088–1104
59. Thompson P.D., Webb M., Beckett W., Hinsley T., Jowitt T., Sharrocks A.D., Tassabehji M (2007) **GTF2IRD1 regulates transcription by binding an evolutionarily conserved DNA motif 'GUCE'** *FEBS Lett* **581**:1233–1242
60. Todorovski Z., Asrar S., Liu J., Saw N.M., Joshi K., Cortez M.A., Snead O.C., Xie W., Jia Z. (2015) **LIMK1 regulates long-term memory and synaptic plasticity via the transcriptional factor CREB** *Mol Cell Biol* **35**:1316–1328
61. Trujillo C.A., Muotri A.R (2018) **Brain Organoids and the Study of Neurodevelopment** *Trends Mol Med* **24**:982–990
62. van Hagen J.M. *et al.* (2007) **Contribution of CYLN2 and GTF2IRD1 to neurological and cognitive symptoms in Williams Syndrome** *Neurobiol Dis* **26**:112–124

63. Vancamp P., Gothie J.D., Luongo C., Sebillot A., Le Blay K., Butruille L., Pagnin M., Richardson S.J., Demeneix B.A., Remaud S. (2019) **Gender-specific effects of transthyretin on neural stem cell fate in the subventricular zone of the adult mouse** *Scientific reports* 9

Editors

Reviewing Editor

Michelle Antoine

National Institute on Alcohol Abuse and Alcoholism, Bethesda, United States of America

Senior Editor

Sacha Nelson

Brandeis University, Waltham, United States of America

Reviewer #1 (Public Review):

Summary:

Zhao et al. used the human forebrain organoid model, transgenic mice model, and embryonic neural progenitor cells to investigate the mutation previously identified in Williams Syndrome. They found abnormal proliferation and differentiation induced by this mutation, as well as altered expression profiles corresponding with aberrant cell clusters. This is regulated through the binding of GTF2IRD1 to transthyretin (TTR) promoter regions and tested on three models mentioned above on neurodevelopmental deficits.

Strengths:

Authors have applied both cell culture, organoid culture and in vivo model to test the previously reported mutation found in Williams Syndrome. They investigated cell behavior including proliferation and differentiation, while using the NGS technique to identify potential signaling pathways that are highly involved and can serve as a candidate to save the phenotype.

<https://doi.org/10.7554/eLife.98081.1.sa1>

Reviewer #2 (Public Review):

Summary:

The study by Xingsen Zhao et al on "A human forebrain organoid model reveals the essential function of GTF2IRD1-TTR-ERK axis for the neurodevelopmental deficits of Williams Syndrome" presents a forebrain organoid model for WS and has identified defects in neurogenesis. The authors have performed scRNAseq from these patients' derived forebrain organoids showing upregulation expression in genes related to cell proliferation while genes involved in neuronal differentiation were downregulated. The major findings presented in this study are an increase in the size of SOX2+ ventricular zone in WS forebrain organoids with an altered developmental trajectory and aberrant excitatory neurogenesis. The study also presents evidence that transthyretin (TTR) has a reduced expression in WS organoids, and its expression is regulated by the transcription factor -GTF2IRD1. The authors then go on to identify mechanistic details of TTR function on MAPK/ERK pathway which has been known to be involved in brain development. Overall, this is a well-constructed study revealing the function of one of the key genes that is deleted in WS and provides novel insights into mechanisms underlying the abnormal neurogenesis in WS brain.

Strengths:

WS patients have neurocognitive disorders which most likely stem from defects in early neurodevelopment. This study has investigated a WS forebrain organoid model with scRNAseq and identified differences in cell proliferation and differentiation. This study has presented some new evidence regarding the function and regulation of TTR and its regulator GTF2IRD1 during brain development.

Weaknesses:

Though the evidence presented for the mechanism of action of TTR on the MAPK pathway is unclear and lacks depth. It would require identifying downstream targets of TTR and how it interacts with the MAPK pathway.

<https://doi.org/10.7554/eLife.98081.1.sa0>