DSCAM/PAK1 pathway suppression reverses neurogenesis deficits in iPSC-derived cerebral organoids from patients with Down syndrome

Xiao-Yan Tang,1 Lei Xu,1 Jingshen Wang,2 Yuan Hong,1 Yuanyuan Wang,2 Qian Zhu,1 Da Wang,1 Xin-Yue Zhang,1 Chun-Yue Liu,2 Kai-Heng Fang,1 Xiao Han,1 Shihua Wang,3 Xin Wang,3 Min Xu,1 Anita Bhattacharyya,4 5 Xing Guo,2 6 Mingyan Lin,2 and Yan Liu1

1Department of Stem Cell and Neural Regeneration, State Key Laboratory of Reproductive Medicine, School of Pharmacy, and 2Department of Neurobiology, School of Basic Medical Sciences, Nanjing Medical University, Nanjing, Jiangsu, China. 3State Key Laboratory of Cellular Stress Biology, Fujian Provincial Key Laboratory of Neurodegenerative Disease and Aging Research, Institute of Neuroscience, School of Medicine, Xiamen University, Xiamen, China. 4Waisman Center and 5Department of Cell and Regenerative Biology, School of Medicine and Public Health, University of Wisconsin, Madison, Wisconsin, USA. 6Department of Endocrinology, Sir Run Run Hospital, Nanjing Medical University, Nanjing, Jiangsu, China.

Down syndrome (DS), caused by trisomy of chromosome 21, occurs in 1 of every 800 live births. Early defects in cortical development likely account for the cognitive impairments in DS, although the underlying molecular mechanism remains elusive. Here, we performed histological assays and unbiased single-cell RNA-Seq (scRNA-Seq) analysis on cerebral organoids derived from 4 euploid cell lines and from induced pluripotent stem cells (iPSCs) from 3 individuals with trisomy 21 to explore cell-type–specific abnormalities associated with DS during early brain development. We found that neurogenesis was significantly affected, given the diminished proliferation and decreased expression of layer II and IV markers in cortical neurons in the subcortical regions; this may have been responsible for the reduced size of the organoids. Furthermore, suppression of the DSCAM/PAK1 pathway, which showed enhanced activity in DS, using CRISPR/Cas9, CRISPR interference (CRISPRi), or small-molecule inhibitor treatment reversed abnormal neurogenesis, thereby increasing the size of organoids derived from DS iPSCs. Our study demonstrates that 3D cortical organoids developed in vitro are a valuable model of DS and provide a direct link between dysregulation of the DSCAM/PAK1 pathway and developmental brain defects in DS.

Introduction

Down syndrome (DS), caused by trisomy of chromosome 21, is the most frequent genetic cause of birth defects and cognitive abnormalities, with an occurrence of 1 of 800 live births (1). Decades of research using transgenic mouse models and postmortem human tissues have revealed that the characteristics of DS include reduced brain weight, cerebral atrophy, a thinner cortex, impaired neurogenesis, and altered cortical lamination (2–6). Moreover, in vitro culturing of neural progenitor cells (NPCs) isolated from DS fetuses revealed impaired proliferation (7) and reduced neurogenesis (8–10), which was informative in dissecting the mechanisms underlying early brain defects in DS. Nevertheless, the above results were based on inaccessible pathological specimens, so there is a need to better clarify the pathogenesis of DS by using a readily available model of human origin. The advent of human induced pluripotent stem cells (iPSCs) presents unprecedented opportunities to establish human cellular models for investigating neurodevelopmental diseases. Using human PSC technology, our previous reports demonstrated that DS iPSC–derived neurons had significant synaptic deficits (11) as well as impaired migration and neurite projections (12). Despite the accumulated knowledge in the past 2 decades regarding neural development in DS, the mechanisms underlying the impaired cortical development in DS are still unclear.

Recent advances in cerebral organoid culturing systems have opened new avenues to study human developmental disorders (13–24). Cerebral organoids are 3D structures reminiscent of human brain regions, including the cerebral cortex and subventricular zone. Recently, several independent studies established cerebral organoids to clarify the pathogenesis of microcephaly, autism, Miller-Dieker syndrome, and other neurodevelopmental disorders (14, 15, 20, 21, 25–29), but there have been no reports on the pathological mechanism underlying delayed cortical development in DS.

Among the protein-coding genes in HSA21 (human chromosome 21), DS cell adhesion molecule (DSCAM) encodes a cell adhesion molecule involved in neuronal generation, maturation, dendrite morphology, and neuronal wiring (30, 31), which are important for brain development. Triplication of the DSCAM gene deregulated the activity of p21-activated kinase 1 (PAK1) and phosphorylated PAK1 (p-PAK1), resulting in neuronal connectivity dysfunction in immortalized cells from trisomy 16 (Ts16) fetal mice (32). PAK1, a gene downstream of DSCAM, plays a role in cortical development by regulating the proliferation of neural progenitors (33). Nevertheless, it is unclear whether the DSCAM/PAK1 pathway regulates the development of the cerebral cortex in DS.

Here, we established 3D cerebral organoid culture to investigate the mechanisms associated with abnormal cerebral development in DS. To resolve confounding factors owing to the asynchronous and heterogeneous appearance of disease phenotypes in vitro culture,
we performed high-resolution single-cell transcriptomic analyses to uncover the cell-type–specific molecular pathology of DS. Our data showed that DS iPSC-derived cerebral organoids partially recapitulated the abnormalities observed in DS mouse models and postmortem DS brain samples, including a reduced proliferation rate and abnormal neurogenesis. Furthermore, we showed that a thinner cortex and proliferation deficits in DS organoids could be reversed by knocking out 1 locus of the 3 DSCAM alleles. Likewise, the small-molecule inhibitor FRAX486 could rescue the neuropathological phenotypes by regulating the expression of PAK1. Our findings may provide a potential target for prenatal intervention for DS.

Results

Single-cell RNA-Seq reveals altered neural development in trisomy 21 organoids. To generate organoids from human PSCs, we modified the protocol of cerebral organoid generation described in previous reports (refs. 34–37, Figure 1A, and Supplemental Figure 1; supplemental material available online with this article; https://doi.org/10.1172/JCI135763DS1). After continuous culturing for 30–60 days, the cerebral organoids showed stratified neuroepithelium-like architecture expressing neural progenitor markers on day 30 and cortical layer markers from days 50 to 70 (Supplemental Figure 1). Moreover, the cortical upper-layer markers could be examined after 100–115 days of differentiation (Supplemental Figure 1).

Next, we generated organoids from 5 iPSC lines from 3 patients with DS (DS1, 2DS, and DSP), 3 euploid iPSC lines (IMR90-4, hESC-03, and DS2U), and 1 human embryonic stem cell (hESC) line (H9) (Figure 1A). Similar to euploid organoids, trisomy 21 organoids displayed regionalization and cortical layers (Figure 1, B–D). To gain insight into the cell-type–specific transcriptomic changes in trisomy and euploid organoids, we profiled cerebral organoids (n = 65,342 cells from 7 human PSC [hPSC] lines) on day 30 and day 70 using 10× genomics chromosome single-cell RNA-Seq (scRNA-Seq) (Figure 1A and Supplemental Table 3). Unbiased clustering identified 8 major cell types (Figure 1E), which were then annotated according to the expression of known cell-type markers (Supplemental Figure 2A). To verify the regional identity of cells in cerebral organoids, we mapped the scRNA-Seq data onto 3D ISH data from the Allen Brain Atlas by using the VoxHunt algorithm. We found that clusters in cerebral organoids highly mapped onto the dorsal forebrain of the E13.5 mouse brain (Figure 1F). In addition, our samples showed a maximum correlation with transcriptomes from post-conception week (pcw) 8 to pcw 16 samples of the developing human brain (Supplemental Figure 2B). We then compared our scRNA-Seq transcriptomic data with the BrainSpan database. Remarkably, organoids showed a significant positive correlation with postmortem fetal tissues at pcw 8 and 9 (Figure 1G). By comparing the proportion of cells in each cluster, we found that cell diversity was similar across lines and groups (Supplemental Figure 2C). The box plot between pseudotime and subclusters supported our speculation that developmental stage classification was positively correlated with pseudotime (Supplemental Figure 2D), while a delayed developmental state from the progenitor-to-neuron trajectory was found in trisomy 21 organoids in comparison with the developmental state of euploid organoids on day 30 (Figure 1, H and I). Gene ontology (GO) analysis showed that the significantly differentially expressed genes (DEGs) among all the clusters were enriched in biological processes such as neurogenesis, forebrain development, and neural precursor cell proliferation (Figure 1J). Consistent with this, pathway enrichment analyses indicated that neurogenesis-related pathways were markedly altered (Supplemental Figure 2E).

Taken together, these data show that neural development in DS cerebral organoids was significantly altered compared with that in euploid organoids.

Disrupted chromatin accessibility underlies impaired transcription in cerebral organoids derived from trisomy iPSCs. Large genomic changes can result in the disruption of several aspects of chromatin conformation (38), such as the distribution of chromatin accessibility. To test whether global chromatin accessibility was affected in DS, we profiled the genome-wide chromatin accessibility of day-30 cerebral organoids using the assay for transposase-accessible chromatin by sequencing (ATAC-Seq). In general, the distribution of chromatin accessibility remained unchanged in DS cerebral organoids (Supplemental Figure 3A). However, we observed dramatic and widespread increases in chromatin accessibility across the promoter regions of chromosome 21 in DS, which conformed with the characteristics of trisomy 21 (Figure 2, A and B). Many regions on other chromosomes also showed significant changes in accessibility, with 1785 increased and 1695 decreased differentially open chromatin regions (dOCRs) identified in organoids derived from trisomy iPSCs compared with those derived from euploid iPSCs (Figure 2C). Among them, OCRs associated with the markers PAX6, GLPER, PTCCH1, LM01, VCA1, and WNT7A showed a decrease in chromatin openness at their promoters in the trisomy samples (Supplemental Figure 3B). By identifying the transcription factor motifs enriched in the trisomy- and euploid-specific accessible regions, we found that many genes regulated by SOX2, Is1, and Rfx5 had decreased accessibility in the trisomy samples (Supplemental Figure 3C). This result was consistent with our finding that genes near dOCRs were enriched in neurogenesis and nervous system development (Supplemental Figure 3D).

To examine whether disruption of chromatin accessibility would be responsible for transcriptomic changes, we performed bulk RNA-Seq analyses of day-30 cerebral organoids in the 2 groups. Differential expression profiling of 104 upregulated genes and 91 downregulated genes is displayed as a heatmap in Figure 2D (see also Supplemental Table 5). Consistent with the findings of the scRNA-Seq data, the DEGs between the trisomy- and euploid-derived organoids showed significant enrichment of genes related to nervous system development, cell proliferation, and neurogenesis (Supplemental Figure 3, E and F, and Supplemental Tables 6 and 7). Notably, the expression of Ki67 and PAX6, which are involved in regulating proliferation, was decreased in the trisomy organoids (Supplemental Figure 3, G and H). We then mapped the 20 differentially expressed proteins related to neurogenesis and 29 proteins related to cell proliferation using the STRING database (Supplemental Figure 3I). Not surprisingly, we observed a positive correlation between the change in a differentially accessible peak and the fold change in expression of its nearest gene (Spearman’s coefficient r = 0.165; Figure 2E). GO analyses of the coordinately upregulated and downregulated genes were enriched among biological processes such as central nervous system neuron differentiation, neuron migration, and glutamate receptor signaling (Figure 2F).

Taken together, these results suggested that at least part of the transcriptomic changes in DS are linked to the altered accessibility of their adjacent chromatin.
Reduced proliferation of NPCs results in smaller DS-derived cortical organoids. To verify the neuropathological phenotypes observed in the RNA-Seq data, we compared the proliferation of DS and control organoids. Strikingly, in contrast to euploid embryonic bodies (EBs) on day 7, trisomy EBs showed a reduced perimeter length (Figure 3, A and B). Moreover, the size of the trisomy 21 organoids was obviously smaller than that of the euploid organoids, and the former had significantly decreased expansion rates (Figures 3, C and D, and Supplemental Figure 4C). To further examine the abnormal structures in day-30 trisomy 21 organoids, we assessed the architecture of neuroepithelial reduced proliferation of NPCs results in smaller DS-derived cortical organoids.
could influence the size of the forebrain (39, 40). To test whether
This phenotype is consistent with previous observations of a reduc-
trisomy 21 organoids had reductions in all parameters (Figure 3, F–K).
(Figure 3E). We found that, compared with the euploid organoids, the
loop area, and loop tissue area in the trisomy and euploid organoids
the diameter of the loops, and the size of the ventricle-like area, total
loops with respect to the length of the apical and basal membranes,
the diameter of the loops, and the size of the ventricle-like area, total
loop area, and loop tissue area in the trisomy and euploid organoids
(Figure 3E). We found that, compared with the euploid organoids, the
trisomy 21 organoids had reductions in all parameters (Figure 3, F–K).
This phenotype is consistent with previous observations of a reduc-
ion in brain size in patients with DS (2, 4–6).
Defects in NPC proliferation during early neural development
could influence the size of the forebrain (39, 40). To test whether
altered proliferation of ventricular zone (VZ) NPCs may delay the
organoid expansion of trisomy, we quantified the percentages of
Kif6°, Edu°, and PAX6° cells in the VZ-like region 30 days after the
initiation of differentiation (Figure 4, A–C and E–G). In accordance
with the reduction in organoid size, we found not only markedly
decreased proliferation in the trisomy cortical VZ-like regions but
also fewer SOX2° progenitor cells located in the region (day 30: DS1,
83.76% ± 1%; 2DS3, 82.31% ± 1.02%; DSP, 81.96% ± 0.88%; DS2U,
90.01% ± 0.77%; ihtc-03, 88.53% ± 0.77%; IMR90-4, 88.16% ±
0.77%; and H9, 88.79% ± 0.90%; Figure 4, D and H), whereas the
expression of apoptosis markers was not significantly altered at the
same time point (Supplemental Figure 4, D and E). These results
suggested that decreased proliferation of trisomy NPCs was responsi-
ble for the smaller size of the trisomy 21 organoids.
We next assessed the generation of different subtypes of cortical
neurons using the validated markers 50 days after differentiation
initiation. We found that the percentage of CTIP2° cells was decreased
in the DS group compared with the euploid control group (DS1,
17.01% ± 1.87%; IMR90-4, 31.1% ± 2.17%), whereas the percentage of
TBR1° cells was unchanged (Supplemental Figure 4, F–H). To further
study the later developmental stages in DS, we performed scRNA-
Seq and histological analysis of day-70 cerebral organoids (Figure
5A). Uniform manifold approximation and projection (UMAP) visu-
alization of subclusters of glutamatergic neurons showed that the
composition of cortical mature deep-layer and mature upper-layer
neurons was dramatically decreased in the trisomy 21 organoids
(Figure 5, B and C). Moreover, the proportion of cells expressing
cortical upper-layer markers, such as BRN2 and SATB2, was decreased
in trisomy glutamatergic neurons (Figure 5D). Indeed, our histology
results showed that the percentages of CTIP2° and SATB2° cells
were significantly decreased by day 70 in the DS-derived organoids,
Figure 3. Reduced size and expansion rates of organoids from patients with DS. (A) Bright-field microscopic images of trisomy 21 and euploid EBs on day 7. Scale bar: 250 μm. (B) Quantification of EB perimeters on day 7. At least 25 EBs were analyzed for each cell line; n ≥ 3 independent experiments. Data represent the mean ± SEM. ***P < 0.001, by 1-way ANOVA followed by Dunnett’s multiple-comparison test. (C) Bright-field microscopic images of trisomy 21 and euploid organoids at different developmental time points. Scale bar: 250 μm. (D) Quantification of the organoid area of trisomy 21 and euploid organoids on days 9, 12, 15, and 17 after differentiation reflected a reduction in the expansion rate of trisomy 21 organoids compared with euploid organoids. Organoids (n ≥ 16) from 3 independent biological replicate experiments were analyzed for each cell line. Data represent the mean ± SEM. ****P < 0.0001, by 2-way ANOVA followed by Sidak’s multiple-comparison test. (E) Schematic overview of the different parameters of neuroepithelial loops in organoids 30 days after the induction of differentiation. Shown are loop tissue area (top middle), total loop area (top right), ventricle area (bottom left), basal membrane length (bottom middle), and apical membrane length (bottom right). (F–K) Quantification of a series of parameters in neuroepithelial loops of trisomy 21 and euploid organoids on day 30. Organoids (n ≥ 16) from 3 independent biological replicate experiments were analyzed for each cell line. Data represent the mean ± SEM. ***P < 0.001, by 1-way ANOVA followed by Dunnett’s multiple-comparison test.
in dorsal neural progenitors. Remarkably, we found that there was increased expression of Ki67, PAK6, and SOX2 in the VZ-like region in the DSCAM-KD groups (Figure 7, D and E), suggesting enhanced proliferation upon KD of DSCAM. Importantly, the reduced DSCAM gene dosage in the trisomy 21 organoids restored the numbers of CTIP2+ neurons (DS1: 17.11% ± 1.20%; DSCAM-KD 2-1-12: 26.86% ± 1.62%; DSCAM-KD 2-1-6: 26.88% ± 1.62%) and SATB2+ neurons (DS1: 10.14% ± 0.76%; DSCAM-KD 2-1-12: 16.54% ± 1.44%) to numbers similar to those in the euploid organoids (Figure 7, F–I).

To confirm the effects of DSCAM in DS, we further downregulated DSCAM in DS1 by performing a CRISPR interference (CRISPRi) experiment (Supplemental Figure 6, A–D). The expression levels of DSCAM, PAK1, and p-PAK1 were robustly increased in DSCAM-KD cerebral organoids (Supplemental Figure 6, E–G). Furthermore, the perimeter of day-7 EBs, the size of the VZ in the organoids, cell proliferation, and cortical neurogenesis were effectively elevated in the CRISPRi-based DSCAM-KD groups (Supplemental Figure 7, A–H).

Taken together, these results demonstrated that the proliferation and neurogenesis deficits in trisomy 21 organoids can be rescued by regulating the DSCAM gene.

FRAX486 rescued the abnormal proliferation and neurogenesis of DS organoids. Considering that the DSCAM/PAK1 pathway is involved in neurodevelopment and is altered in DS (32, 33), we investigated whether the defects could be rescued by applying small
molecules targeting this pathway (Figure 8A). Indeed, pretreatment of DS1 organoids with FRAX486, an inhibitor that regulates the phosphorylation of PAK1, reduced the protein levels of p-PAK1 (Figures 8, B and D), without changing the expression levels of total PAK1 (Figure 8, C and E). Of note, we did not find significant changes in the protein levels of PAK1 or p-PAK1 in euploid organoids that were pretreated with FRAX486 (Supplemental Figure 8, A–H). Furthermore, we tested whether inhibition of PAK1 with FRAX486 could ameliorate the defective neurogenesis of DS organoids. Indeed, we observed a partial rescue of the abnormal architecture of neuroepithelial loops (Figure 8, F and G, and Supplemental Figure 8, I–K). In addition, FRAX486 with effectively increased the proliferation of NPCs after 30 days of differentiation (Ki67: DS1, 18.32% ± 2.19%; DS1 plus FRAX486, 24.85% ± 0.95%; PAX6: DS1, 70.05% ± 3.84%; DS1 plus FRAX486, 81.50% ± 1.13%; and SOX2: DS1, 81.14% ± 0.97%; DS1 plus FRAX486, 90.01% ± 0.77%; Figure 8, H and I). Furthermore, PAK1 inhibition via FRAX486 treatment subsequently increased the expression of CTIP2 (50 days after the initiation of differentiation, DS1: 17.37% ± 1.11%; DS1 plus FRAX486: 27.92% ± 1.67%) and SATB2 (70 days after the initiation of differentiation, DS1, 11.17% ± 0.6%; DS1 plus FRAX486, 14.57% ± 0.75%), suggesting the rescue of neurogenesis (Figure 8, J–M).

Discussion
In this study, we evaluated cortical developmental defects in DS using a patient iPS-derived cerebral organoid model. The DS organoids showed reduced proliferation in the VZ, decreased neuron distribution in the cortical plate, and smaller cerebral organoids. Transcriptomic and Western blot results revealed that DSCAM and PAK1 may contribute to these defects. Moreover, genetic

Figure 5. Neurogenesis studies of cerebral organoids. (A) Schematic illustrating the single-cell transcriptomic and histological analysis of trisomy 21 and euploid organoids. (B) UMAP visualization of scRNA expression in glutamatergic neuron subclusters of trisomy 21 and euploid organoids after 70 days of in vitro differentiation. Chart on the right shows the comparisons of cell composition between the trisomy 21 and euploid organoids after 70 days of differentiation. DL, deep layer; UL, upper layer. (C) Average expression (avg.exp.) levels of representative markers in each glutamatergic neuron subcluster are shown according to scaled expression scores. (D) Histogram of the log, fold change of average expression levels and percentage (pct.) of expression for significantly different genes in the glutamatergic neuron subcluster. (E) Images show decreased maturation of CTIP2+ neurons on day 70 in trisomy 21 organoids compared with euploid organoids. Scale bar: 50 μm. (F) Quantification of the proportion of CTIP2+ cells in both trisomy and euploid organoids on day 70. n = 13–20 neural tube–like regions in at least 5 organoids per cell line. Data represent the mean ± SEM. ***P < 0.001, by Student’s t test. (G) Images show decreased maturation of SATB2+ neurons on day 70 in trisomy 21 organoids compared with euploid organoids. Scale bar: 25 μm. (H) Quantification of the proportion of SATB2+ cells in trisomy and euploid organoids on day 70. n = 9–11 neural tube–like regions in at least 5 organoids per cell line. Data represent the mean ± SEM. **P < 0.01, by Student’s t test.
GABAergic interneurons (primarily CR+ and SST+ neurons), which might be associated with the abnormal development of DS neurons. However, the role of DSCAM in cortical neurogenesis in primates has not been studied. Its downstream gene PAK1 has been reported to play an essential role in regulating actin cytoskeleton dynamics, dendritic spine morphogenesis, and cortical neurogenesis in a mouse model (33, 53). Here, we used both genetic correction and small-molecule inhibitors to rescue NPC proliferation and neurogenesis defects in DS organoids. Our work indicated that the DSCAM/PAK1 pathway might be associated with the abnormal development of DS neurons.

Our cerebral organoid models provide a new avenue to study the early developmental deficits in DS. By using transcriptomic analysis, CRISPR-based gene correction, and small-molecule intervention, we demonstrated that the malformation of cortical development in DS was attributed to reduced neuronal proliferation of progenitors and was accompanied by neurogenesis deficits. Among them, dysfunction of the DSCAM/PAK1 pathway has been shown to play a critical role in the pathogenesis of DS. On the one hand, our current analyses provide candidates for identifying therapeutic targets and screening drugs for the treatment of DS. Moreover, DSCAM and PAK1 are potential therapeutic targets to reverse abnormal neurodevelopment and improve postnatal cognitive function in DS.

Methods

iPSC culturing and generation of organoids. Trisomy 21 iPSC lines (DS1, 2DS3, and DSP) and euploid iPSC lines (IMR90-4, H9, DS2U and ihtc-03) were used in this study (a detailed list is provided in Supplementary Table 1). DS iPSC lines were generated from 2 patients with DS as reported by Weick et al. (11) or from an individual with mosaic DS. The euploid iPSC lines DS2U (the euploid control was from the same patient with DS1), wild-type IMR90-4 (WiCell agreement no. 17-W0063), ihtc-03 (established in our laboratory), and the hESC line H9 (WiCell agreement no. 16-W0060) served as euploid controls. iPSC lines were maintained under feeder-free conditions by coating the culture plates with vitronectin (Thermo Fisher Scientific) as described in our previous studies (35). After 5–7 days of culturing in EB medium (Thermo Fisher Scientific), hPSCs were dissociated with EDTA (Lonza) for 1–2 minutes at 37°C and seeded in a 6-well plate at a density of 1 x 10^5 cells per well. Detachment of iPSCs to obtain embryoid bodies (EBs) required dispase (Thermo Fisher Scientific) to begin the process of neural differentiation; the bodies were then cultured in neural induction medium containing N2 supplement (Thermo Fisher Scientific), nonessential amino acids (MEM-NEAA, Thermo Fisher Scientific), and DMEM/F12 (Thermo Fisher Scientific) for 7 days. On day 7, EBs were resuspended in Matrigel (Corning), which was pipetted cold in 3 mm droplets on a sheet of Parafilm that was sterilized by UV light for 30 minutes. These droplets solidified at 37°C and
were subsequently removed from the Parafilm and grown in differentiation medium that was changed every 5 days.

**Genome editing.** DS iPSC lines with DSCAM KD were generated by CRISPR/Cas9. Exon 1 of DSCAM was selected for the guide RNA (gRNA) design according to the CRISPR online design tool at http://crispr.mit.edu/. The DSCAM gRNA sequences of a pair of oligonucleotides for the targeting site were as follows: forward, 5′-CAGGCGATGAAAGACGTGAAATGT-3′; reverse, 5′-AACCATGAGAGGCAATGTTG-3′. After

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**Figure 7.** Knocking down DSCAM rescues abnormal neurogenesis in DS-derived cortical cultures. (A) Representative images of day-30 organoids stained with Hoechst, which show the quantitation of the different parameters. Scale bar: 50 μm. (B and C) Quantitation of the basal membrane length and loop tissue area in the neuroepithelial loops of trisomy 21 and euploid organoids after 30 days of differentiation. Organoids (n ≥ 15) from 3 independent biologic replicate experiments were analyzed for each cell line. Data represent the mean ± SEM. *P < 0.05 and **P < 0.01, by 1-way ANOVA followed by Dunnett’s multiple-comparison test. (D) Representative images of day-30 trisomy 21 and DSCAM-KD organoids for Ki67, SOX2, PAX6, MAP2, CTIP2, and TUJ1 expression. Scale bar, 50 μm. (E) Quantification of the proportion of Ki67+, SOX2+, and PAX6+ cells in day-30 trisomy 21 and DSCAM-KD organoids. n = 17–33 VZ-like regions in at least 10 organoids per cell line. Data represent the mean ± SEM. **P < 0.01 and ***P < 0.001, by 1-way ANOVA followed by Dunnett’s multiple-comparison test. (F and G) Immunocytochemical staining and quantification of CTIP2+ cells in both trisomy 21 and DSCAM-KD organoids after 50 days of differentiation. n = 13–15 neural tube–like regions in at least 7 organoids per cell line. Data represent the mean ± SEM. ***P < 0.001, by 1-way ANOVA followed by Dunnett’s multiple-comparison test. Scale bar: 35 μm. (H and I) Immunocytochemical staining and quantification of the proportion of SATB2+ cells in both trisomy and DSCAM-KD organoids on day 70. n = 11 neural tube–like regions in at least 6 organoids per cell line. Data represent the mean ± SEM. ***P < 0.001, by Student’s t test. Scale bar: 35 μm.
oids were maintained in 0.5% Triton for 20 minutes at 20°C, after
organoids were fixed with 4% paraformaldehyde for 2 hours at room
2 hours. Then, the neural induction medium was discarded before the
M, Life Technologies, Thermo Fisher Scientific) for
um with EdU (10
perfused. Coverslips were mounted for fluorescence imaging. Images
Supplemental Table 2) diluted in 5% donkey serum. After treatment with
incubated for 1 hour at 20°C in secondary antibodies (see detailed list in
sections were then used for immunostaining. For immunohistochemi-
were embedded in OCT compound and cryosectioned at 10
sections were washed with PBS 3 times and then blocked
overnight at 4°C. After the organoids sank to the bottom of the tube, the
Drylling solution was replaced by 30% sucrose in PBS at 4°C. Organoids
minutes 3 times, the organoids were submerged in 20% sucrose in PBS
and permeabilized in 1% Triton (Bio-Link) and 5% donkey serum (Mil-
cal analysis, sections were washed with PBS 3 times and then blocked
were electroporated with gRNA-containing
and rescued organoids. n = 13–14 neural tube–like regions in at least 7
organoids per cell line. Data represent the mean ± SEM. ***P < 0.001, by Student’s t test. (J and K) Immunocytochemical
staining and quantification of the proportion of CTIP2+ cells in day-50
trisomy 21 and rescued organoids. n = 9–14 neural tube–like regions in at least 6
organoids per cell line. Data represent the mean ± SEM. **P < 0.01, by Student’s t test. Scale bar: 35 μm.
transformation and extraction of the plasmid, 15 µg gRNA-containing
plasmid was transferred to 50,000 DS1 cells using an electroporation
apparatus (Lonza). After 24 hours of electroporation, puromycin was added
to the cell cultures for 2 days. Three to 4 days after electroporation, sin-
gle clones were picked and reseeded in a 24-well plate. KD of the DSCAM
gene was verified by sequencing, and 2 iPSC lines — DSCAM-KD2-1-12 and
DSCAM-KD2-1-6 — were obtained. As KD groups, DSCAM-KD 2-1-12 and
DSCAM-KD2-1-6 were used to verify the molecular mechanism related to
the DSCAM/PAK1 pathway during cortical development.

Immunostaining. Organoids were fixed with 4% paraformaldehyde
for 2 hours in an Eppendorf tube. After they were washed with PBS for 10
minutes 3 times, the organoids were submerged in 20% sucrose in PBS
overnight at 4°C. After the organoids sank to the bottom of the tube, the
soaking solution was replaced by 30% sucrose in PBS at 4°C. Organoids
were embedded in OCT compound and cryosectioned at 10 μm. Tissue
sections were then used for immunostaining. For immunohistochemical
analysis, sections were washed with PBS 3 times and then blocked and
permeabilized in 1% Triton (Bio-Link) and 5% donkey serum (Mil-
liporeSigma) in PBS. Organoids were incubated at 4°C overnight in pri-
mary antibodies (see detailed list in Supplemental Table 2) diluted in
0.2% Triton and 5% donkey serum. On the second day, organoids were
incubated for 1 hour at 20°C in secondary antibodies (see detailed list in
Supplemental Table 2) diluted in 5% donkey serum. After treatment with
primary and secondary antibodies, three 10-minute washes in PBS were
performed. Coverslips were mounted for fluorescence imaging. Images
were acquired using an Eclipse 80i fluorescence microscope (Nikon).

EdU Click-it assay. Organoids were kept in neural induction medium
with EdU (10 μM, Life Technologies, Thermo Fisher Scientific) for
2 hours. Then, the neural induction medium was discarded before the
organoids were fixed with 4% paraformaldehyde for 2 hours at room
temperature. After they were washed twice with 3% BSA, the organ-
oids were maintained in 0.5% Triton for 20 minutes at 20°C, after
which an EdU Click-IT assay was performed according to the manu-
facturer’s instructions (Invitrogen, Thermo Fisher Scientific, C10338),
followed by immunostaining.

Western blot analysis. Organoids were lysed in RIPA buffer containing
protease and a protease inhibitor cocktail (Roche). Proteins were loaded
onto gels (SurePage) and separated by SDS-PAGE with 100 V electropho-
resis. Then, proteins were transferred onto polyvinylidene fluoride mem-
branes at 300 mA for 2 hours and blocked in 5% skim milk for 2 hours
at room temperature. Primary antibodies (listed in Supplemental Table
2) were incubated overnight at 4°C before the membranes were washed
with 8× PBST solution 5 times for 8 minutes the next day. The secondary
antibodies were incubated with the membranes on a shaker for 2 hours
at room temperature. After the incubation was completed, the secondary
antibody was decanted, and the membranes were again washed 5 times
with 1× PBST for 8 minutes. Anti-GAPDH was used as an internal refer-
ence and loading control. HRP-conjugated IgG was used as the second-
ary antibody, and the ECL system was used for detection of the protein
bands. The luminol substrate solutions A and B were mixed at a volume
of 1:1 and then added to the surface of the membrane in the dark. After 1
minute, the protein bands were exposed over a time gradient.

Bulk RNA-Seq, ATAC-Seq, and bioinformatics analysis. Following a
standard protocol, total RNA from day-30 trisomy 21 and euploid organ-
oids was extracted using a TRIzol reagent kit (Invitrogen, Thermo Fisher
Scientific). RNA integrity was checked on an Agilent 2100 BioAnalyzer to
count quality control (Agilent Technologies). Library construction and
high-throughput RNA-Seq were performed with the HiSeq 4000 sequenc-
ing platform (Illumina). RNA-Seq reads were aligned to the human refer-
ence genome (GRCh37/hg19) using HISAT2 software (version 2.1.0) (54).

The gene abundances were calculated and normalized as trans-
cripts per million (TPM). We determined DEGs between the trisomy
21 and euploid groups using the DESeq2 (55) package (version 1.30.0).
Enriched GO terms were identified with MGI Gene Ontology Term
Finder (http://www.informatics.jax.org/gotools/MGI_Term_Finder.
html). A log fold change of 1 or higher and a P value of less than 0.05
were used as thresholds. Protein-protein interaction (PPI) network
analysis of the differentially expressed proteins was performed using
STRING version 10.0a software.

For ATAC-Seq, organoids were dissociated into a single–cell sus-
pension. Approximately 50,000 single cells from each group were used
for nuclei preparation. First, cells were spun for 5 minutes at 500g and
4°C and then washed once with 50 µL cold 1× PBS buffer before another
centrifugation step for 5 minutes at 500g and 4°C. Next, cells were lysed
in 50 µL cold lysis buffer (10 mM Tris-HCl, pH 7.4; 10 mM NaCl; 3 mM
MgCl2 and 0.1% IGEPAL CA-630) on ice, and nuclei were pelleted by
centrifugation at 500g for 10 minutes. Nuclei were collected and resus-
pended in 50 µL transposase reaction mix from a Nextera DNA Sample
Preparation Kit (illumina), followed by incubation at 37°C for 30 minutes
to carry out the Tn-5 transposition reaction. Next, DNA fragments were
purified using a MinElute PCR Purification Kit (Qiagen). Transposed
DNA fragments were then amplified using the following PCR condi-
tions: 1 cycle at 72°C for 5 minutes and 98°C for 30 seconds, followed
by 10 cycles at 98°C for 10 seconds, 63°C for 30 seconds, and 2°C for 2
minutes. The resulting ATAC-Seq libraries were purified (MinElute Kit,
Qiagen) and 150 bp paired-end sequenced on the Illumina Nova 6000
platform to a depth of 4.0 × 107 reads.

ATAC-Seq data were then processed after minor modifications
(including quality control, trimming, filtering, aligning, and peak calling).
In brief, FastQC (version 0.11.7, Babraham Bioinformatics, http://www.bioinformatics.bbsrc.ac.uk/projects/fastqc) was used to evaluate the quality of the sequencing data, and reads with a Phred quality score greater than 30 were used for downstream analysis. For the purpose of obtaining clean data with minimal background noise, we removed and trimmed the adaptor sequences using Trimmomatic. Subsequently, the remaining clean reads were aligned to the hg38 reference genome using Burrows-Wheeler Aligner (BWA) software. SAMtools (version 1.3.1) was applied to filter out multiple mapped reads, and BED tools were used to filter out mitochondrial reads.

We applied Homer software (version 4.6; findPeaks-style dnanse) and macs2 software with a Q value cutoff of less than 0.05 to identify peaks, determine peak position and distribution on the genome, identify peak-associated genes, and discover de novo binding motifs.

Differential peaks between trisomy 21 organoids and euploid organoids were identified by DESeq2 (55), with the thresholds of a log fold change of greater than 1 and a P value of less than 0.05. Genome-wide normalized signal coverage tracks were created by bamCoverage in deepTools (version 3.3.0) and were visualized in the Integrative Genomics Viewer (IGV version 2.5.0). GO enrichment analysis of the genes associated with ATAC-Seq peaks was performed using clusterProfiler (version 3.10.1; ref. 56). Fisher’s exact test was applied to identify the significant GO categories, and the FDR was adopted to correct the P values.

Dissociation of brain organoids and scRNA-Seq. Day-30 and day-70 organoids derived from trisomy 21, euploid, and DSCAM-KD iPSC lines were prepared for scRNA-Seq (sample information is provided in Supplemental Table 3). Briefly, 5-7 cerebral organoids were dissociated into a single-cell suspension via incubation with 1 mL tryp-LE (Life Technologies, Thermo Fisher Scientific) for 35 minutes at 37°C with gentle agitation every 5–8 minutes, followed by 3 washes with 2% FBS in DPBS and gentle titration using a P200 pipette. A single-cell suspension was subsequently collected into 1.5 mL microtubes at a cell density of 1000 cells/μL, and approximately 12,000 cells in each channel were loaded onto a Chromium Single Cell 3’ Chip (10x Genomics, PN-120236) and processed through a Chromium controller to generate single-cell gel beads in emulsions (GEMs) according to the manufacturer’s instructions. Captured cells were lysed, and the released RNA was barcoded through reverse transcription in individual GEMs. Reverse transcription was performed on a S1000 Touch Thermal Cycler (Bio-Rad) at 53°C for 45 minutes, followed by 85°C for 5 minutes and a final hold at 4°C. cDNA was generated and then amplified, and quality was assessed using an Agilent 4200 (Agilent Technologies). scRNA-Seq libraries were prepared with the Chromium Single Cell 3’ Library & Gel Bead Kit V3 (10x Genomics, 1000075) and then sequenced on an Illumina NovaSeq 6000, with a sequencing depth of at least 750,000 reads per cell and a paired-end 150 bp (PE150) reading strategy.

Data analysis for scRNA-Seq. Reads were aligned to the hg38 human reference genome, and gene-level unique molecular identifiers (UMI) counts were obtained using Cell Ranger (version 3.1.0). The expression matrix was processed with Seurat (version 3.1.5; ref. 57). The criteria to select cells for subsequent analysis were as follows: 547 < UMI per cell < 18040, 398 < detected genes < 4849, and a mitochondrial transcript proportion < 0.1731563. SCTransform normalization was applied to each Seurat object to control confounding sources of variations such as sequencing depth and mitochondrial fraction (58). In addition, the integration of single-cell data was done to correct the batch effect. Expression matrices were summarized by the top 14 principal components. Visualization of the transcriptomic profiles was conducted by UMAP. The Louvian modularity optimization algorithm was applied to iteratively group cells into clusters. Cell clusters were annotated to known biological cell types using canonical cell marker genes. Identification of DEGs in trisomy 21 organoids compared with euploid organoids in each cluster was performed using the MAST (version 1.14.0) package (59). The following model was fit with MAST: zlm (~ group + time, sca, ebayes = TRUE).

Multiple hypothesis testing corrections were performed using Bonferroni and Holm corrections (60). GO enrichment and Kyoto Encyclopedia of Genes and Genomes (KEGG) enrichment of DEGs were performed using ToppGene (https://toppgene.cchmc.org/) (61), and the results were visualized with R. Developmental trajectory analysis was performed using slingshot (version 1.4.0, Bioconductor) with default parameters (62). Unbiased spatial mapping of all clusters was performed using BrainSpan, the largest data set containing the postmortem developmental human brain atlas (63).

Cell lines. The H9 and IMR90-4 cell lines were obtained via a WiCell agreement (H9: NO.16-W0060; IMR90-4: NO.17-W0063); DSI, 2DS, DSP, and DS2U cell lines were gifts of the Bhattacharyya laboratory (Department of Cell and Regenerative Biology, School of Medicine and Public Health, University of Wisconsin, Madison, Wisconsin, USA). The httc-03 cell line was established in our laboratory (55).

Generation of DSCAM-KD iPSC lines using CRISPRi. The CRISPRi dual vector was packaged in lentivirus to establish the CRISPRi-based DSCAM-KD human iPSC line. First, the lentiviral vector containing dCas9-KRAB was used for this study was modified from the lentCRISPR vector (no. 61425) obtained from Addgene, with the dCas9-VP64 cassette replaced by dCas9-KRAB. We then designed sgRNAs targeting the DNA region from ~50 to 300 bp near the transcription start site (TSS) of the DSCAM gene and used a CRISPRi web tool (http://crispr.mit.edu/) to minimize off-target effects of the sgRNAs. The location of the TSS was determined using NCBI’s GenBank database (https://www.ncbi.nlm.nih.gov/nuccore/NM_001271534.3). Three gRNAs targeting DSCAM as well as a nontargeting negative control gRNA were selected (Supplemental Table 4) using the online CRISPRi design tool at BioinfoGP (https://bioinfo-fgp.cnbc.es/tools/breakingcas/), these sequences were cloned into the hi/h6-sgRNA-SV40-EGFP vector (GeneChem Technologies, GV37).

For the dual-vector multiplex experiments, human iPSCs from DSI were first infected with lentivirus expressing dCas9-KRAB three days after mechanical passage for 24 hours. Selection was applied 7 days after infection with blastocidin (1 μg/mL) in E8 medium in the presence of the ROCK inhibitor Y27632 (2 μM) (STEMCELL Technologies) and then persisted in the presence of 0.2 μg/mL blastocidin 13 days after infection to maintain the selection, which was continued for 5 weeks until stable colonies appeared. A second transduction in clones was performed using lentivirus encoding either targeting or scrambled sgRNA. Twenty-four hours after lentiviral infection, the cell culture medium was replaced with E8 medium supplemented with ROCK inhibitor (STEMCELL Technologies). Fourteen days after infection, cells were dissociated with Accutase treatment for 10 minutes at 37°C to create a single-cell suspension; this suspension was then transferred to a 5 mL flow cytometry tube with a strainer cap before FACS purification could be completed on a BD FACS Aria Fusion instrument. Clones were then expanded into larger vessel formats and used for further experiments, including functional those for CRISPRi activity and organoid differentiation.
qPCR. All RNA samples were extracted using a TRIzol kit (Thermo Fisher Scientific). One microgram of total RNA from each sample was reverse transcribed into cDNA and then subjected to qPCR using the SuperScript III First-Strand Synthesis System (Thermo Fisher Scientific). The primers used for qPCR were as follows: DSCAM forward primer, CCAGGCTCAGGTAATCTGA and reverse primer, AGCATGCTGTTGTCCGA; PAK1 forward primer, CAGCCCCCTCCGAGAATA, and reverse primer, CAAAACCGACATGAATTGTGTGT.

Cyto genetic analysis. Cerebral organoids were collected for cell culturing, followed by karyotyping. GTG banding was performed according to a standard protocol. Karyotypes were determined from G-banding analysis using a standard protocol according to the International System for Human Cytogenomic Nomenclature (ISCN) 2016 nomenclature.

Quantification of neural tubes in organoids and statistical analysis. Organized portions of the organoids around the VZ-like structure were the target area for quantification of neural tubes in organoids. The image was rotated such that the VZ-like region was horizontal. Then, a box was defined with a specific width but flexible height to cover the entire stratified region from the apical surface of the VZ to the top surface of the organoid. All Hoechst-stained cells and cells expressing the target markers within that box were counted for statistical analysis. Organoids from the same experiment and from the same clone, but from independent experiments, were used as technical replicates. The data were averaged to obtain a single value. Average data from different biological replicates from different individuals were used to determine the average and standard error.

Data availability. The raw data of scRNA-Seq data, bulk RNA-Seq data, and ATAC-Seq data used in this study have been deposited in the NCBI’s Sequence Read Achieve (SRA) (accession number SRR14243996–SRR14244067).

Statistics. All data represent the mean ± SEM. Statistical analyses and graphing were done using GraphPad Prism, version 8 (GraphPad Software). The statistical methods relevant to each figure are described in the figure legends. Statistical comparisons between 2 groups were performed using the Student’s t test. Other statistical analyses were performed using 1-way ANOVA with Dunnett’s multiple-comparison test. P values of less than 0.05 were considered to indicate a significant difference between groups.

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Address correspondence to: Yan Liu, Department of Stem Cell and Neural Regeneration, State Key Laboratory of Reproductive Medicine, School of Pharmacy, Nanjing Medical University, 101 Longmian Avenue, Nanjing 211166, China. Phone: 86.25.8686.8478; Email: yanliu@njmu.edu.cn (YL). Or to: Mingyan Lin, Department of Neurobiology, School of Basic Medical Sciences, Nanjing Medical University, 101 Longmian Avenue, Jiangning District, Nanjing 211166, China. Phone: 86.25.8686.9323; E-mail: linmingyan@njmu.edu.cn (ML). Or to: Xing Guo, Department of Neurobiology, School of Basic Medical Sciences, Nanjing Medical University, 101 Longmian Avenue, Jiangning District, Nanjing 211166, China. Phone: 86.25.8686.9385; Email: guox@njmu.edu.cn (XG).

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