Supplemental Information

Suppressing the DSCAM/PAK1 pathway reverses neurogenesis deficits in Down Syndrome patient iPSC-derived cerebral organoids.

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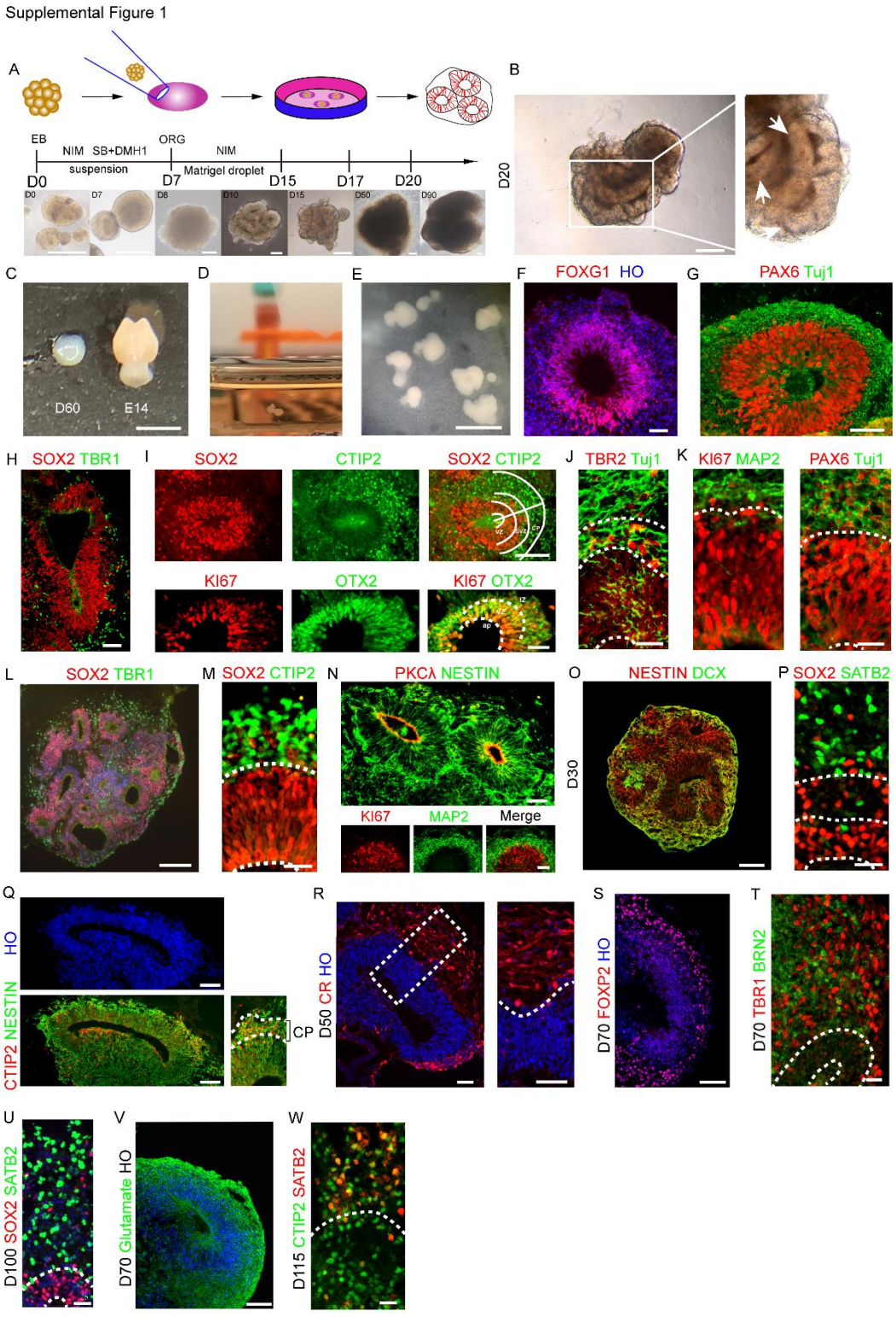
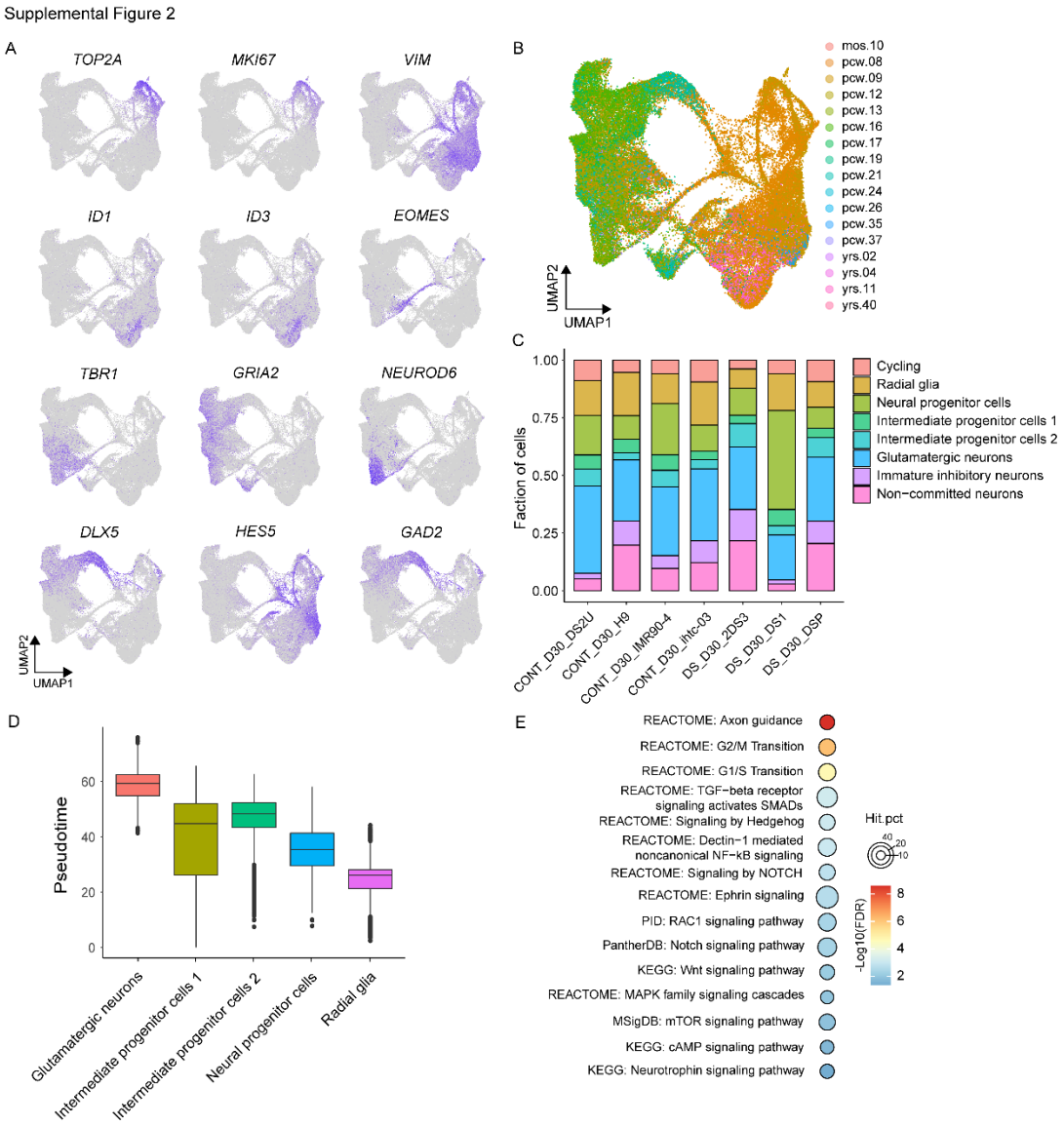
Supplemental Figures and Figure legends

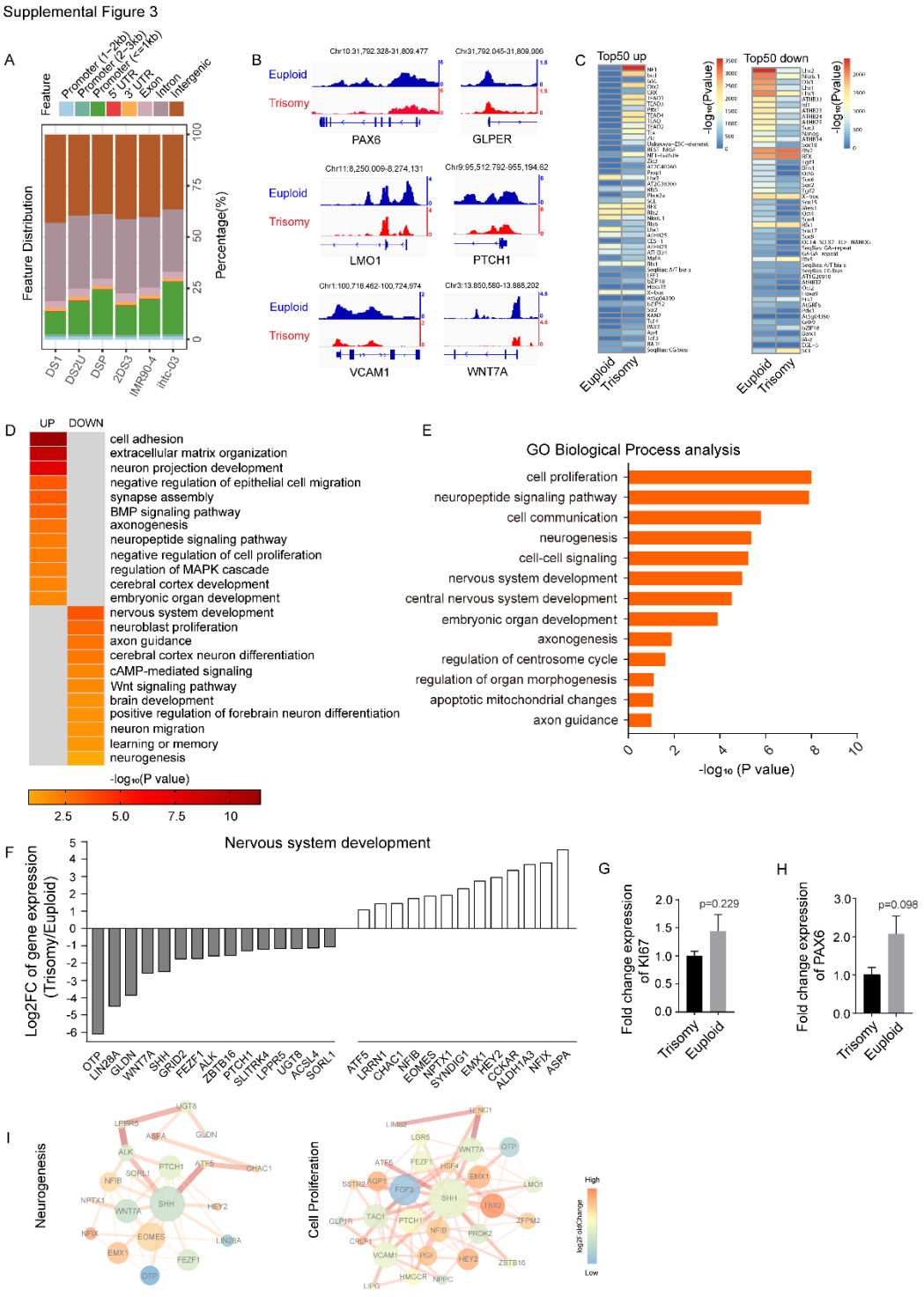


Supplemental Figure 1. Generation of human cerebral organoids (hCOs). (**A**) Schematic diagram of the organoid generated from hPSCs and representative images at each stage. Scale bars, 250 μm. (**B**) Bright-field images of forebrain organoids at 20 days after initiation of differentiation showing several optically clear neuroepithelial bud outgrowths (white arrows) surrounding a lumen.

Scale bar, 250 μm. (**C**) Comparison size of a day 60 cerebral organoid and a dissected E14 mouse brain. Scale bar, 5 mm. (**D**) Representative image of forebrain organoids after 60 days of differentiation. (**E**) Representative image of forebrain organoids captured by stereoscopy after 60 days of differentiation. Scale bar, 2 mm. (**F**) Immunostaining for the forebrain marker FOXG1 after 30 days of differentiation. Scale bar, 50 μm. (**G**) Immunofluorescence of the dorsal forebrain marker PAX6 and the neuronal marker TUJ1 after 30 days of differentiation. Scale bar, 100 μm. (**H**) Immunostaining for the neural progenitor marker SOX2 (red) and the layer VI neuron marker TBR1, showing ventricle-like structures. Scale bar, 100 μm. (**I**) Sample images of immunostaining for the progenitor marker SOX2, the deep-layer neuron marker CTIP2, proliferative marker Ki67 and forebrain progenitor marker OTX2 after 30 days of differentiation. Scale bar, 50 μm. (J) Immunofluorescence for the intermediate progenitor marker TBR2 and TUJ1 after 30 days of differentiation. Scale bar, 100 μm. (**K**) Sample images of immunostaining for Ki67, PAX6 and neuronal marker MAP2/TUJ1. Scale bar, 25 μm. (**L**) Representative sections of whole organoids stained for SOX2 (red) and TBR1 after 30 days of differentiation. Scale bar, 100 μm. (**M**) Staining for SOX2 and CTIP2 expression after 30 days of differentiation. Scale bar, 50 μm. (**N**) Immunohistochemical staining for the adherent junction marker PKCλ (red, above), neural stem cell marker NESTIN (green, above), proliferation marker Ki67 (red, below) and neuronal marker MAP2 (green, below) after 30 days of differentiation. Scale bars, 50 μm. (**O**) A representative full-sized organoid (30 days) that labelled with NESTIN (red) and DCX. Scale bar, 100 μm. (**P**) Staining for SOX2 and the upper-layer neuron marker SATB2 after 50 days of differentiation, revealing typical VZ-, SVZ- and CP-like regions. Scale bar, 50 μm. (**Q**) Sample images of immunostaining for early-born neuron marker CTIP2 (red) and NESTIN (green) at 40 days. Scale bars, 100μm. (**R**) Calretinin (CR, red) staining revealing CP-like cells along the basal surface of cortical tissue. Scale bar, 50 μm. (**S**) Immunofluorescence of the early-born neuron marker FOXP2. Scale bar, 100 μm. (**T**) Immunofluorescence of TBR1 (red) and the late-born neuron marker BRN2 at 70 days. Scale bar, 25 μm. (**U**) Immunohistochemical staining for SOX2 and SATB2 after 100 days of differentiation, revealing a decreased VZ-like region. Scale bar, 25 μm. (**V**) Immunofluorescence of the glutamatergic neuron marker glutamate after 70 days of differentiation. Scale bar, 100 μm. (**W**) Representative images of the CP-like region in forebrain organoids with immunofluorescence identifying CTIP2 and the superficial layer neuron marker SATB2 (red). Scale bar, 25 μm.

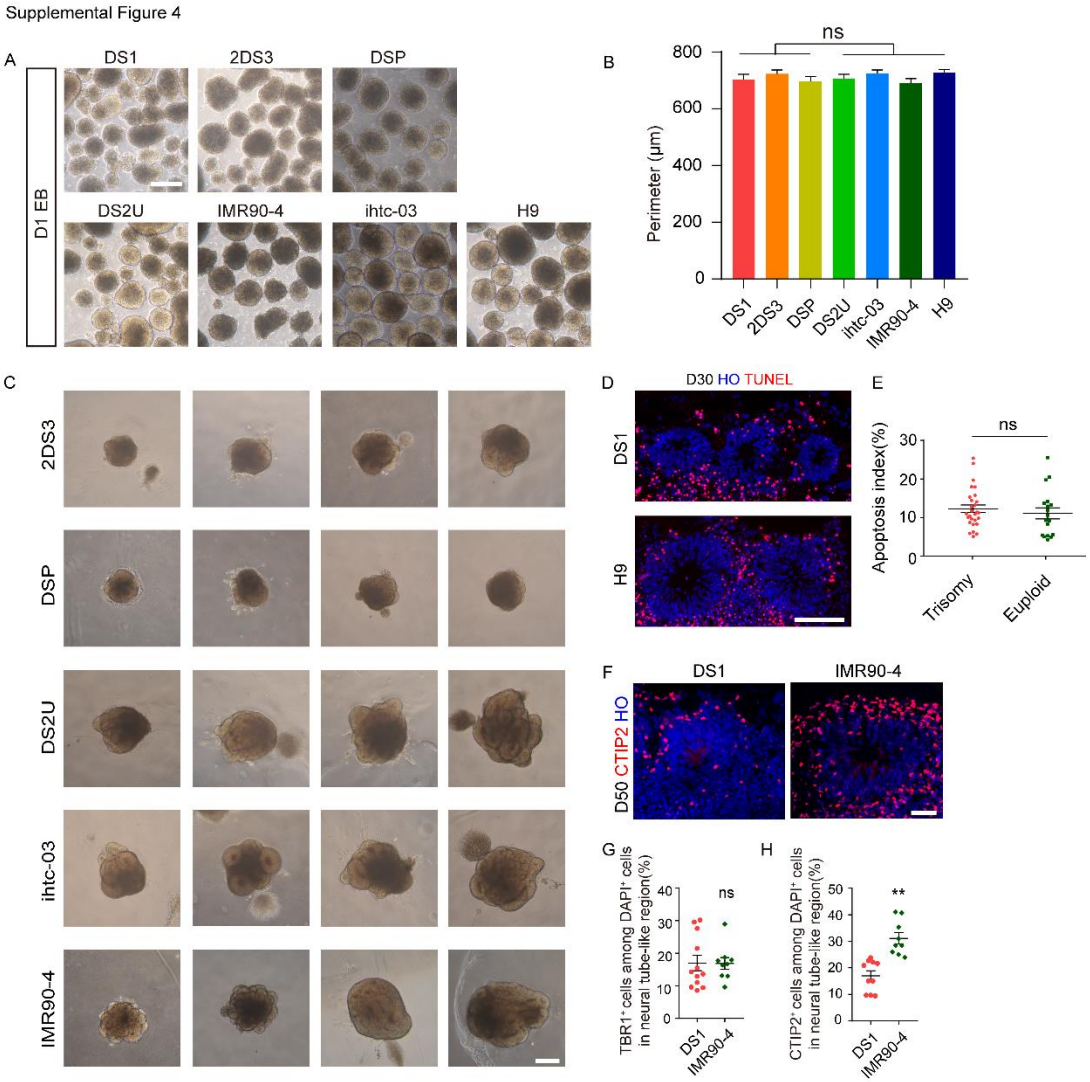


Supplemental Figure 2. Single-cell RNA-seq analysis of trisomy 21 and euploid cerebral organoids. (**A**) UMAP visualization of gene expression of canonical marker genes in the cerebral organoids at days 30 and 70 of in vitro differentiation. Cycling: TOP2A, MKi67; radial glial cells: VIM; NPCs: ID1, ID3; intermediate progenitor cells: EOMES; glutamatergic neurons: NEUROD6, TBR1; immature inhibitory neurons: DLX5, GAD2; neuronal growth cone: GRIA2; and neurogenesis and gliogenesis: HES5. The expression is depicted from grey (low) to blue (high). (**B**) Temporal mapping of cell types in trisomy 21 and euploid organoids to BrainSpan. Most neural stem cells and NPCs are coloured orange (pcw 08-09), while most neurons are coloured green (pcw 12 -pcw 13). (**C**) Cell compositions in each group. (**D**) Boxplot showing the distribution of pseudotime within each cell type. (**E**) Pathway enrichment analysis of DEGs between trisomy 21 and euploid organoids. Scale indicates the FDR, and circle size indicates the percentage of all genes enriched in selected pathways.



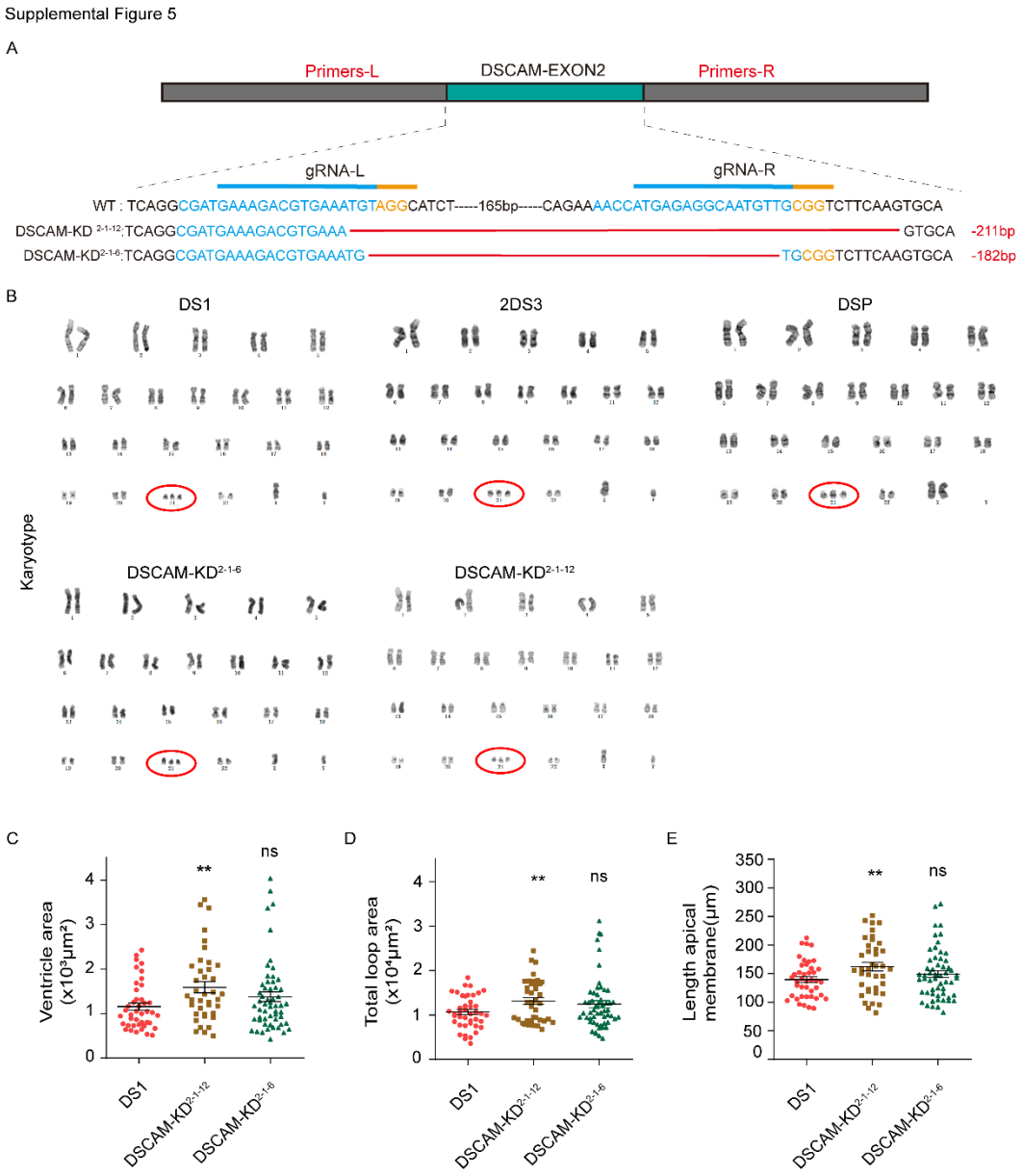
Supplemental Figure 3. Genome-wide analysis of chromatin accessibility and gene expression in trisomy 21 and euploid cerebral organoids. (**A**) Genome annotation of all the peaks in the trisomy 21 and euploid organoids. (**B**) ATAC-seq read distribution around the transcription start site (TSS) in the trisomy 21 and euploid organoids. (**C**) Heatmap showing the significance of 100 transcription factor motifs enriched in trisomy 21- and euploid-accessible loci. Motifs are sorted from the loci with increased/decreased accessibility in trisomy 21 organoids compared to those in euploid organoids. (**D**) Heatmap of GO biological processes enriched in dOCRs between trisomy 21 organoids and euploid organoids. (**E**) Seventeen enriched terms for dysregulated

genes during trisomy 21 organoid differentiation were identified using GO analysis. (**F**) Representative genes associated with nervous system development were differentially expressed in trisomy 21 organoids. (**G-H**) RNA-seq analysis of Ki67 and PAX6 expression. (**I**) The top 20 gene interaction networks were involved in neurogenesis, and 29 gene interaction networks were involved in cell proliferation.

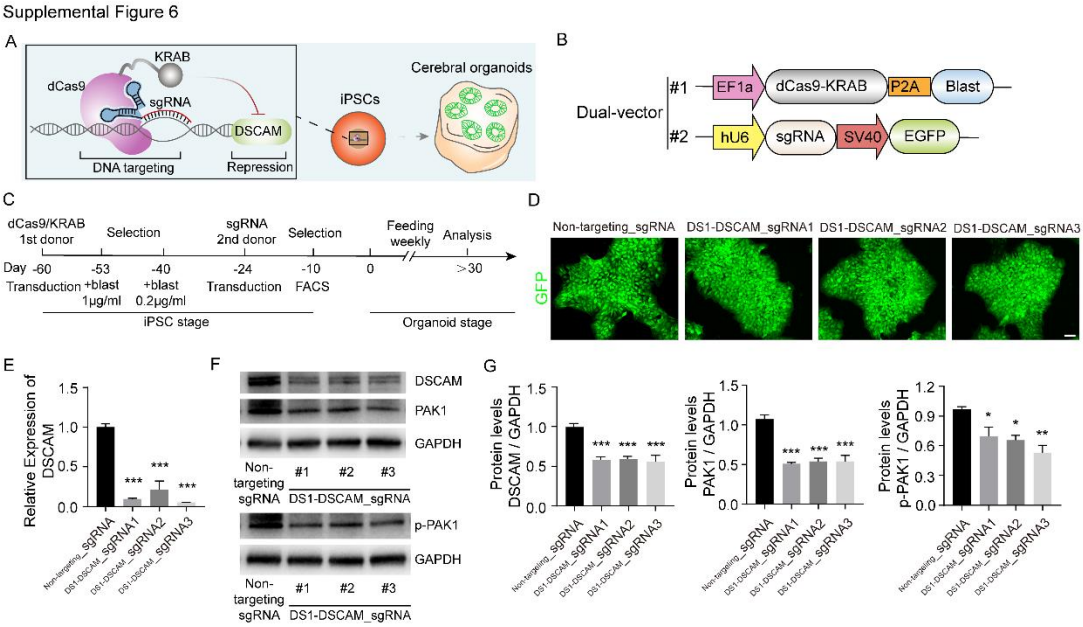


Supplemental Figure 4. Characterization of organoids from trisomy and euploid organoids, related to Figure 3. (**A**) Bright-field microscopy images of trisomy 21 and euploid EBs at day 1. Scale bar, 250 μm. (**B**) Quantification of perimeter at day 1. At least 50 EBs were analysed for each cell line, n ≥3 for independent experiments. Error bars, ± SEM; one-way ANOVA followed by Dunnett's multiple comparisons test. (**C**) Bright-field images of trisomy 21 and euploid organoids at different developmental time points. Scale bar, 250 μm. (**D**) Representative imaging of TUNEL staining in day 30 organoid sections. Scale bar, 100 μm. (**E**) Comparison of the apoptosis index in the trisomy 21 and euploid groups. n=18 to 28 neural tube-like regions in at least 6 organoids per cell line were counted. Error bars, ± SEM; Student's t test**.** (**F**) Decreased mature CTIP2^{+} neurons at 50 days in trisomy 21 organoids compared to euploid organoids. Scale bar, 50 μm. (**G-H**) Quantification of the proportion of TBR1+ and CTIP2+ cells in trisomy 21 and euploid organoids

after 50 days of differentiation. n=9 to 12 neural tube-like regions in at least 5 organoids per cell line were counted**.** Error bars, ±SEM; Student's t test.

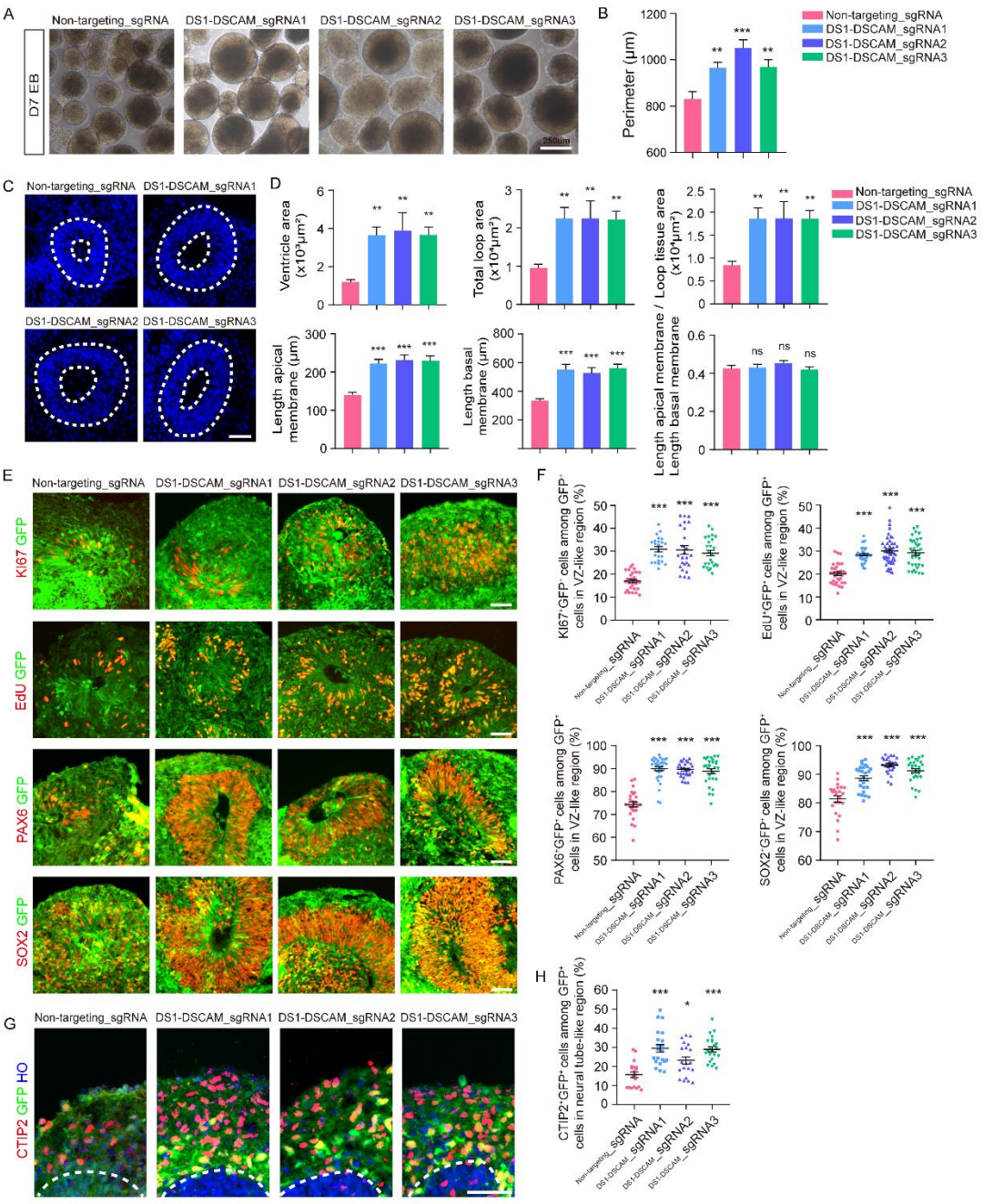


Supplemental Figure 5. Deletion of DSCAM using CRISPR/Cas9 genome editing in DS related to Figure 5 and Figure 6. (**A**) Characterization of the deletion break regions by sequencing. (**B**) Karyotype analysis of trisomy 21 and DSCAM-KD organoids. (**C-E**) Quantitative assessment of multiple parameters in neuroepithelial loops of trisomy- (DS1 n=15) and DSCAM-KD- (DSCAM-KD2-1-12 n=16; DSCAM-KD2-1-6 n=18) organoids after day 30 of differentiation. Shown are the ventricle-like area (b), total loop area (c), and length apical membrane (d).



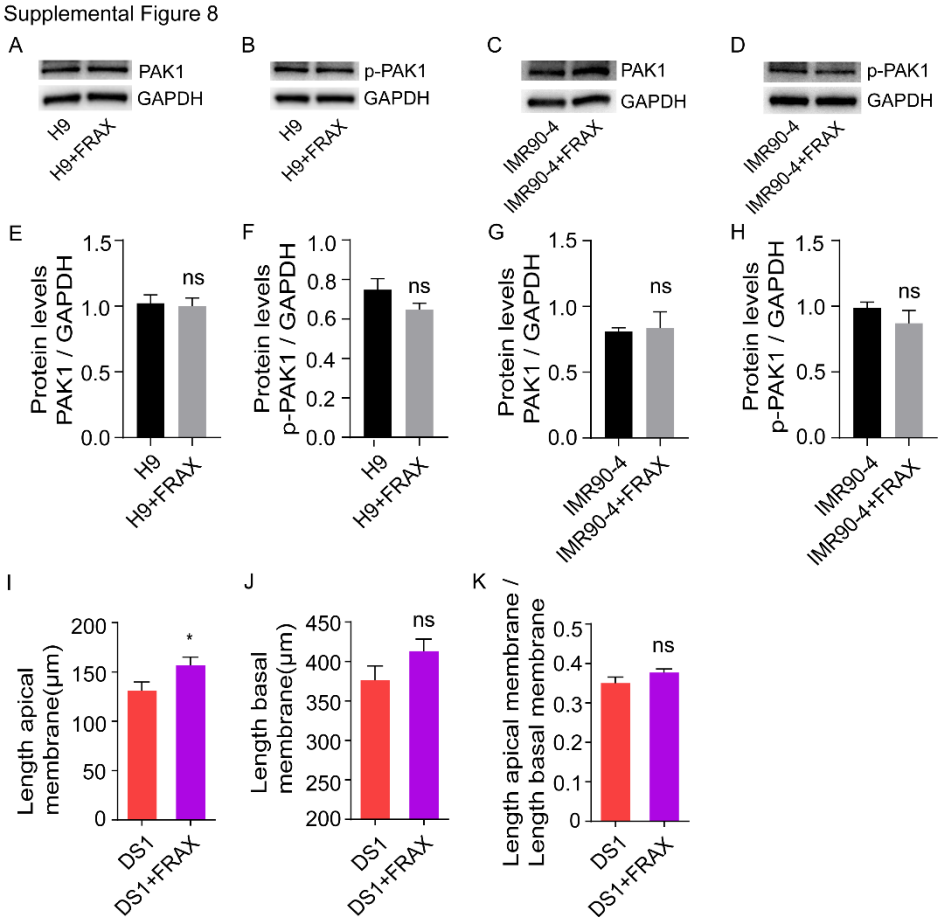
Supplemental Figure 6. CRISPRi-based inactivation of DSCAM in trisomy 21 organoids. (**A**) Diagram of the CRISPR interference (CRISPRi) system in trisomy 21 cerebral organoids. (**B**) Schematic representation of the dual-vector strategies for DSCAM-targeting CRISPRi. (**C**) Timeline for sgRNA transduction, selection and functional analysis of CRISPRi-DSCAM iPSCs. (**D**) CRISPRi experiments in human iPSCs. Lentiviruses generated from the CRISPRi dual-vector were used for DSCAM knockdown in human iPSCs. Scale bar, 50 μm**.** (**E**) qPCR analysis of mRNA in organoids infected with DSCAM targeting CRISPRi lentivirus. The DSCAM expression in the control group was normalized to 1. n ≥ 3 for independent experiments. Error bars, ± SEM; ***P < 0.001; one-way ANOVA followed by Dunnett's multiple comparisons test. (**F-G**) Effects of DSCAM knockdown on the DSCAM-PAK1 pathway in trisomy 21 organoids. Shown are immunoblot analysis (F) and quantification (G) of DSCAM, PAK1 and p-PAK1 expression in trisomy 21 organoids infected with DSCAM sgRNA and non-specific control sgRNA at 30 days. (n ≥ 3 for independent experiments; error bars, ± SEM; *P < 0.05, **P< 0.01, ***P < 0.001; one-way ANOVA followed by Dunnett's multiple comparisons test).

Supplemental Figure 7



Supplemental Figure 7. CRISPRi-based inactivation of DSCAM ameliorates neurogenesis impairments in trisomy 21 organoids. (**A**) Bright-field images of embryonic bodies infected with DSCAM sgRNA and non-specific control sgRNA at day 7. (**B**) Quantification of the perimeter length of the EBs at day 7. At least 45 EBs were analysed for each cell line, n ≥3 for independent experiments; error bars. ± SEM. *P < 0.05, **P< 0.01, ***P < 0.001; one-way ANOVA followed by Dunnett's multiple comparisons test. (**C**) Representative Hoechst staining in neuroepithelial loops of trisomy 21 organoids infected with DSCAM sgRNA and non-specific control sgRNA, which displayed the method of quantitation of the different parameters. (**D**) Quantification of a series of parameters in the neuroepithelial loops of trisomy 21 organoids infected with DSCAM sgRNA and non-specific control sgRNA. n ≥16 organoids from three independent biological replicate

experiments were analysed for each cell line. Error bars, ± SEM. *P < 0.05, **P< 0.01, ***P < 0.001; one-way ANOVA followed by Dunnett's multiple comparisons test. (**E**) Effects of DSCAM knockdown on cell proliferation in trisomy 21 organoids. Representative images of trisomy 21 organoids infected with DSCAM sgRNA or non-specific control sgRNA at 30 days are shown. Ki67, EdU, PAX6, SOX2 are shown in red. Scale bar, 50 µm. (**F**) Quantification of the proportion of GFP^+ cells expressing Ki67, EdU, PAX6 and SOX2 in trisomy 21 organoids infected with DSCAM sgRNA or non-specific control sgRNA at 30 days. (n=23 to 45 VZ-like regions in at least 6 organoids per cell line were counted; error bars, ± SEM. *P < 0.05, **P< 0.01, ***P < 0.001; one-way ANOVA followed by Dunnett's multiple comparisons test). (**G**) Immunostaining for the deep-layer neuron marker CTIP2 along the neural tube-like region of 30-day trisomy 21 organoids infected with DSCAM sgRNA or non-specific control sgRNA. Scale bar, 50 µm. (**H**) Quantification of the percentage of GFP^+ cells expressing CTIP2 in trisomy 21 organoids infected with DSCAM sgRNA or non-specific control sgRNA at 50 days after differentiation. n=20 to 24 neural tube-like regions in at least 5 organoids per cell line were counted. Error bars, ± SEM. *P < 0.05, **P< 0.01, ***P < 0.001; one-way ANOVA followed by Dunnett's multiple comparisons test.



Supplemental Figure 8. Analysis of the effects of a PAK1 inhibitor on euploid organoids and multiple parameters of neuroepithelial loops in trisomy 21 and rescued organoids (related to Figure 8). (**A-D**) Detection of PAK1 and p-PAK1 levels in euploid organoids at 30 days after treatment with FRAX486 as measured by western blot. (**E-H**) Western blot analysis of PAK1 and p-PAK1 in euploid organoids at 30 days after treatment with FRAX486. n ≥ 3 for independent

experiments. Error bars, ± SEM; Student's t test. (**I-K**) Quantitation of length apical membrane (I), length basal membrane (J) and length apical membrane/length basal membrane (K) in neuroepithelial loops of trisomy 21 and rescued organoids. n≥ 10 organoids from three independent biological replicate experiments were analysed for each cell line. Error bars, ± SEM; *P < 0.05; Student's t test.

Supplemental Table 1

Basic information of different iPSC lines in this study

hPSC line name	Karyotype	Source, gender, age
DS1	Trisomy 21	AG05397, male, 1 year
2DS3	Trisomy 21	GM02504, male, 1 month
DSP	Trisomy 21	WC-24-02, female, 25 years
DS2U	Euploid	AG05397, male, 1 year
IMR90-4	Euploid	CCL-186, female
H9	Euploid	WA09, female
ihtc-03	Euploid	Female, 28 years
DSCAM-KD$^{2\text{-}1\text{-}12}$	Trisomy 21 KD	AG05397, male, 1 year
DSCAM-KD$^{2\text{-}1\text{-}6}$	Trisomy 21 KD	AG05397, male, 1 year

Supplemental Table 2

Antibodies used in this study including suppliers and working dilutions.

Antibody	Isotype	Dilution	Source	Cat.NO.
BRN2	Mouse IgG	1:100	Santa Cruz	SC-393324
Calretinin	Rabbit IgG	1:200	Epitomics	2624-1
CTIP2	Rat IgG	1:300	abcam	ab18465
DCX	Rabbit IgG	1:400	Cell Signaling	4604
DSCAM	Rabbit IgG	1:500	abcam	Ab85362
DSCAM	Rabbit IgG	1:500	This paper, by ABclonal	N/A
FOXG1	Rabbit IgG	1:1000	abcam	ab18259
FOXP2	Rabbit IgG	1:1000	abcam	ab16046
GAPDH	Mouse IgG	1:5000	affinity	T0004
Glutamate	Rabbit IgG	1:1000	sigma	G6624
KI67	Rabbit IgG	1:200	ZYMED	18-0191
MAP2	Mouse IgG	1:1000	sigma	M1406
NESTIN	Goat IgG	1:1000	Santa Cruz	SC-21247
PAK1	Mouse IgG	1:500	Santa Cruz	SC-166887
P-PAK1	Rabbit IgG	1:500	Santa Cruz	2605s
PAX6	Rabbit IgG	1:500	convance	PRB-278P
PKCλ	Mouse IgG	1:1000	BD	610207

SATB2	Mouse IgG	1:100	abcam	ab51502
SOX2	Goat IgG	1:500	R&D Systems	AF2018
SOX1	Goat IgG	1:500	R&D Systems	AF3369
PHH3	Mouse IgG	1:1000	Cell Signaling Technology	9706s
pVimentin	Mouse IgG	1:1000	MBL	D076-3
TBR1	Rabbit IgG	1:1000	abcam	ab31940
TBR2	Rabbit IgG	1:200	abcam	Ab23345
TUJ1	Rabbit IgG	1:2000	Covance	PRB-435P
OTX2	Rabbit IgG	1:1000	R&D Systems	AF1979
GFP	Rabbit IgG	1:1000	Chemicon	AB3080

Supplemental Table 3

Information of single cell RNA sequencing in organoids

	Trisomy	Euploid
Day 30 cerebral organoids	DS1, 2DS3, DSP, DSCAM-KD$^{2\text{-}1\text{-}12}$, DSCAM-KD$^{2\text{-}1\text{-}6}$	DS2U, IMR90-4, ihtc-03, H9
Day 70 cerebral organoids	DS1	IMR90-4

Supplemental Table 4

Sequences of sgRNAs targeting *DSCAM* in CRISPRi experiment

sgRNA	Sequence (5’ to 3’)
DS1-DSCAM_sgRNA1	GTGAGCTCATCCCGGGCACT
DS1-DSCAM_sgRNA2	TGAGCTCACGCCCGCGTCTG
DS1-DSCAM_sgRNA3	GGGATCCATGTGACTGAGGC
Non-targeting sgRNA	TTCTCCGAACGTGTCACGT