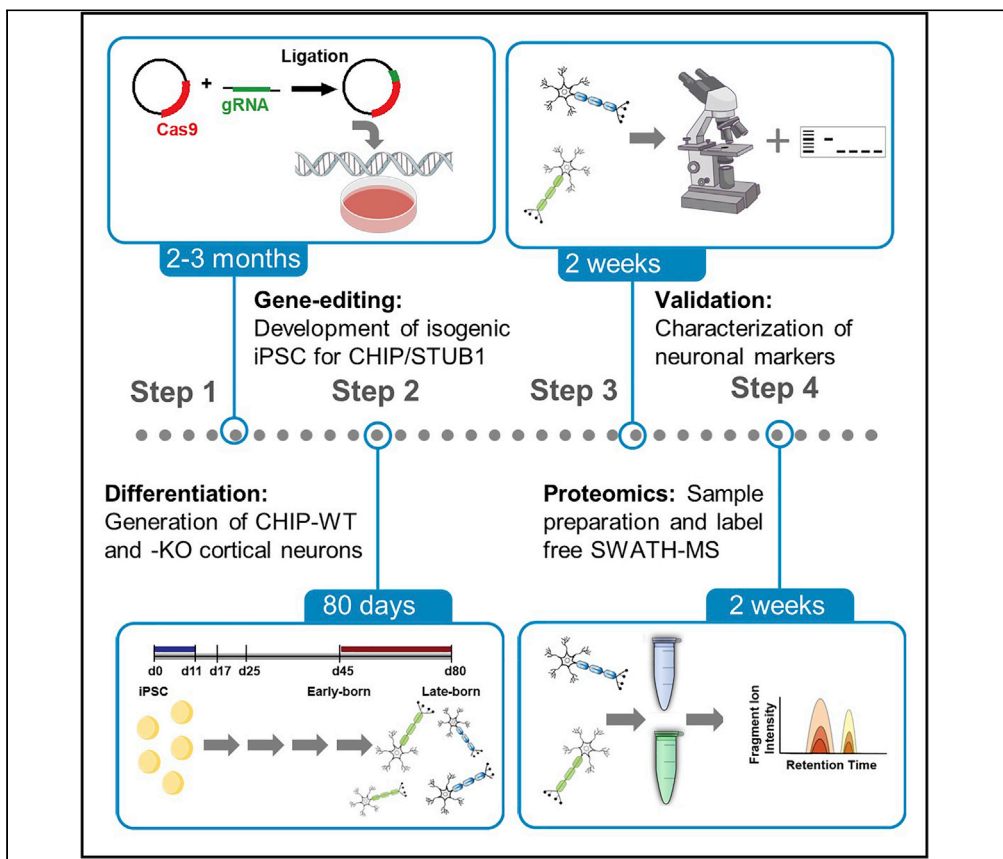


Protocol

Generation of a CHIP isogenic human iPSC-derived cortical neuron model for functional proteomics



The neuroprotective E3-ubiquitin ligase CHIP is linked to healthy aging. Here, we present a protocol using a patient-derived iPSC line with a triplication of the α -synuclein gene to produce gene-edited cells isogenic for CHIP. We describe iPSC differentiation into cortical neurons and their identity validation. We then detail mass spectrometry-based approaches (SWATH-MS) to identify dominant changes in the steady state proteome generated by loss of CHIP function. This protocol can be adapted to other proteins that impact proteostasis in neurons.

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Highlights

Generation of human iPSCs that are isogenic for the E3-ligase CHIP

Differentiation of iPSCs into cortical neurons and validation over 80-days

Preparation of cortical neurons samples suitable for label free proteomic analysis

SWATH-MS measurement of steady state protein levels to facilitate pathway analysis

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Protocol

Generation of a CHIP isogenic human iPSC-derived cortical neuron model for functional proteomics

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<https://doi.org/10.1016/j.xpro.2022.101247>

SUMMARY

The neuroprotective E3-ubiquitin ligase CHIP is linked to healthy aging. Here, we present a protocol using a patient-derived iPSC line with a triplication of the α -synuclein gene to produce gene-edited cells isogenic for CHIP. We describe iPSC differentiation into cortical neurons and their identity validation. We then detail mass spectrometry-based approaches (SWATH-MS) to identify dominant changes in the steady state proteome generated by loss of CHIP function. This protocol can be adapted to other proteins that impact proteostasis in neurons. For complete details on the use and execution of this protocol, please refer to Dias et al. (2021).

BEFORE YOU BEGIN

The protocol below describes steps that are specific to the iPSC AST23 cell line, derived from a patient carrying a triplication of the α -synuclein gene, however, the protocol is also suitable for all other iPSC lines (provided they meet the criteria that define iPSC with regards to self-renewal ability and pluripotency). Moreover, the iPSC lines used may be further edited by CRISPR/Cas9, for example, to investigate the function of a target protein. We developed this protocol to investigate the neuronal functions of the C-terminus of Hsc-70 interacting protein (CHIP), an E3-ubiquitin ligase and co-chaperone. The neuroprotective function(s) of CHIP have emerged following the generation of KO and mutant mice. However there remains a lack of mechanistic understanding into the pathways and individual proteins which mediate CHIPs functions in neuroprotection and healthy brain aging. Therefore, the AST23 line, a cellular model for the early stages of neurodegeneration, was subjected to CRISPR/Cas9 editing resulting in a set of iPSC lines that are isogenic for CHIP. This panel of iPSC lines was then differentiated into cortical neurons and the effect of CHIP loss on the steady state proteome was assessed. The ultimate aim is to identifying individual protein targets of CHIP and the pathways which are regulated in a CHIP-dependnet manner in order to delineate the neuroprotective activity of CHIP at a mechanistic level.

Generation of isogenic iPSC model for CHIP

© Timing: 2 months



When genetically editing iPSC for subsequent differentiation and proteomic analysis, maintenance of low stress levels and high pluripotency and self-renewal capacity are key. Although this protocol describes how to knock-out (KO) CHIP from iPSC using CRISPR/Cas9 technology, it can be applied to studies investigating other proteins.

1. Design the guide-RNA (gRNA):

- a. Target the gRNA downstream of ATG within the coding region of the gene of interest, as this increases the likelihood of an indel mutation ablating protein expression. To obtain CHIP KO lines, design the gRNA to target the coding region of *STUB1*. The following gRNA was successful: GGCCGTGTATTACACCAACC GGG (PAM sequence underlined). Its location within the *STUB1* gene is shown in [Figure 1](#).

Note: “Guides” is an Online tool that can suggest gRNA sequences for specific genes ([Zhang_Lab, n.d.](#)).

- b. Check its quality score and predicted off-target sites.

Note: “Guides” and “Off-Spotter” are Online tools ([Zhang_Lab, n.d.](#)) that can predict the efficiency and off-target sites of specific gRNA sequences, respectively.

Note: When this gRNA was created it had a quality score of 94%. This single-gRNA is predicted to favor precise genome editing, due to the specificity of its nucleotides at positions 4 and 5 before the PAM (associated with a median commonest indel frequency of 0.53) ([Chakrabarti et al., 2019](#)). Furthermore, such precision is associated with a strong bias towards single-nucleotide indels ([Chakrabarti et al., 2019](#)), as observed in our CHIP KO iPSC line. According to “Off-Spotter” ([Zhang_Lab, n.d.](#)), when considering a maximum of 4 mismatches, there are 6 potential off-target sites, although only 4 of these lie within protein-coding regions. These are HDAC4, ANK1, LRRC38, TCEANC2. However, none of the proteins were detected by unbiased mass spectrometry-based proteomics. When considering a maximum of 5 mismatches, there are 75 potential off-sites predicted (36 of which are in coding regions). CECR5 and UQCRC1 were the only ones identified in our proteomics analysis, although they were not of interest (failing to reach significant fold changes and, in some cases, statistical significance). Therefore, the off-targets associated with the gRNA are unlikely to interfere with the conclusions drawn.

Note: The existence of off-targets should be validated by transcriptomics and/or proteomics.

2. Engineer a plasmid containing the gRNA and Cas9 gene:

- a. Digest a total of 10 µg of LentiV2 CRISPR plasmid (Addgene, Plasmid 52961) containing Cas9 cDNA with the BsmB1 restriction enzyme in 10× CutSmart Buffer (B7204, NEB). Carry out the reaction at 55°C for 1 h.
 - b. Following plasmid digestion add 1 µL of phosphatase (M1821, Promega) and incubate at 37°C for 1 h.
 - c. Mix the forward and reverse gRNA strands in equal amounts and anneal in 10× T4 Ligation Buffer (B0202S, NEB). Incubate in a heat block at 95°C for 5 min.
 - d. Ligate the cut backbone plasmid and the annealed gRNAs in the presence of 1 µL of T4 ligase (M0202L, NEB) in 10× T4 Buffer (B0202S, NEB) for 1 h at room temperature (R.T; 20°C–21°C).
 - e. Sequence the plasmid using, for example, standard primers targeting the U6 promoter.
3. Culture iPSC (passaged at least 35 times) in iPSC-Brew XF media (130-104-368, StemMACS, Miltenyi Biotec Inc.) at 37°C and 5% (v/v) CO₂ in 6-well plates with wells pre-coated with laminin-521 diluted in phosphate-buffered saline (PBS) with calcium and magnesium (14040083, Gibco) according to the manufacturer’s instructions (<https://biolamina.com/wp-content/uploads/2020/12/instructions-bl001.pdf>). [Troubleshooting 1](#)

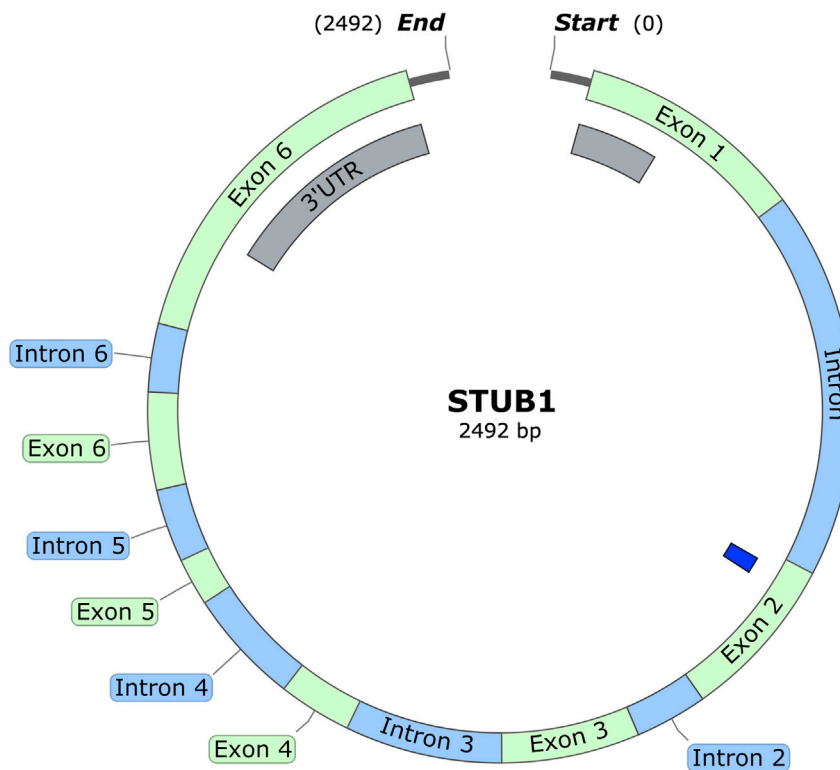


Figure 1. Strategy for guide RNA-targeted genetic editing

The guide RNA (gRNA) was designed to target the *STUB1* gene after the start codon located in the first coding exon, Exon 1. This increases the likelihood of obtaining an indel mutation, ablating CHIP expression.

4. Coat 10 cm plates and 96-well plates with laminin-521 as in step 3 (above). Coated plates can be kept with abundant PBS at 4°C for one week on a flat surface and sealed with parafilm (caution: coated wells must not dry). [Troubleshooting 1](#)
5. Dissociation from the well bottom as single cells:
 - a. Wash cells with PBS (D8537, Sigma) 3 times at R.T. using the recommended volume for each plate, e.g., 1 mL for wells in 6-well plates.
 - b. Incubate with Accutase solution (undiluted, A6964, Sigma) for about 10 min (until cells detach). Add the recommended volume for each dish, e.g., 1 mL for a well within a 6-well plate.
 - c. Transfer the cells in suspension to a centrifuge tube with culturing media that is 10× the volume of Accutase added.
 - d. Centrifuge for 3 min at 43 × *g*.
 - e. Discard supernatant.
 - f. Resuspend 8×10^5 cells in 1 mL of fresh culturing media supplemented with 10 μM Y27632 dichloride.
6. Transfection:
 - a. Use the Human Stem Cell Nucleofector (VPH-5012) according to the manufacturer's instructions (https://bioscience.lonza.com/lonza_bs/DK/en/download/product/asset/21587).
 - b. Transfect 8×10^5 cells in suspension with 3 μg of LentiV2 CRISPR plasmid.
 - c. Electroporate using the B-016 program in a Nucleofector™ 2b Device (AAB-1001, Lonza).
 - d. Seed cells at low density (14×10^3 cell/cm²) onto pre-coated 10 cm plates.

△ **CRITICAL:** Ensure cells are seeded as single cells and that the seeding density is low enough (i.e., cells are quite distant from each other, allowing small colonies to grow isolated from each other).

- e. Maintain cells in media supplemented with 10 μ M Y27632 dichloride for 2 days to promote cell survival.
7. Colony selection:
 - a. Wait for single cells to grow into small colonies.
 - b. Once small colonies grow, treat cells for 2 h with culturing media supplemented with 10 μ M Y27632 dichloride, to increase survival.
 - c. Wash cells once with pre-warmed (37°C) HBSS containing calcium and magnesium (14025, Gibco). Use 1 mL for a well within a 6-well plate.
 - d. Treat cells with 250 U/mL of Collagenase Type IV (17104019, Gibco) dissolved in HBSS containing calcium and magnesium (14025, Gibco) and incubate for 30 min to facilitate subsequent detachment from the matrix and improve survival. Use 1 mL for a well within a 6-well plate.
 - e. Remove the Collagenase Type IV solution.
 - f. Add culturing media supplemented with 0.5 U/mL Penicillin + 500 ng/mL Streptomycin (15140122, Gibco) and 10 μ M Y27632 dichloride.
 - g. Manually pick colonies by mechanical detachment (by aspiration and scraping) using a pipette with clipped tips, an inverted phase contrast microscope with a camera and auxiliary screen.
 - h. Transfer picked colonies to 96-well plates pre-coated with Laminin-521, and gently triturate by pipetting to partially dissociate the colony.
 - i. Grow the colonies in culturing media supplemented with 0.5 U/mL Penicillin + 500 ng/mL Streptomycin and 10 μ M Y27632 dichloride.
 - j. After two days, remove the media and add fresh iPS-Brew XF culturing media.

Note: Manual colony picking is preferred over single-cell sorting and selection methods as it maintains low stress levels and favors survival.

Note: Once cultures derived from single-colony isolation are growing robustly, take samples to validate the purity of the colonies using DNA sequencing and protein expression assays (as outlined in step 8).

8. Validate edited clones:

At a genetic level, knockout efficiency can be validated by PCR (Figure 2A), T7 endonuclease assay I (M0302, New England Biolabs) and TOPO-TA cloning assay (450641, Invitrogen) (<https://www.thermofisher.com/dk/en/home/brands/thermo-scientific/molecular-biology/molecular-biology-learning-center/molecular-biology-resource-library/thermo-scientific-web-tools/qpcr-efficiency-calculator.html> <https://www.thermofisher.com/dk/en/home/life-science/cloning/topo/topo-ta-cloning/topo-ta-for-subcloning.html>) followed by DNA sequencing. RT-qPCR analysis informs about *STUB1* expression at a transcriptional level (Figure 2B). Additionally, edited clones must be validated at a protein level by, for example, immunofluorescence assay and/or Western blotting (Figure 2C).

Note: An assessment of the purity of the cultures derived from single-cell colonies can be made in this Step. The existence of 'background noise' in the sequencing data from PCR products containing the edited *STUB1* gene can infer mixed populations. The TOPO-TA cloning assay assesses this further: a maximum of two sequences (derived from the two alleles of *STUB1*) is expected to be detected when PCR products are used for subcloning. The existence of other sequences infers that the edited culture was derived from a colony with low purity. Immunofluorescence assays are also useful to verify whether CHIP expression is uniform throughout the culture, which correlates with colony purity.

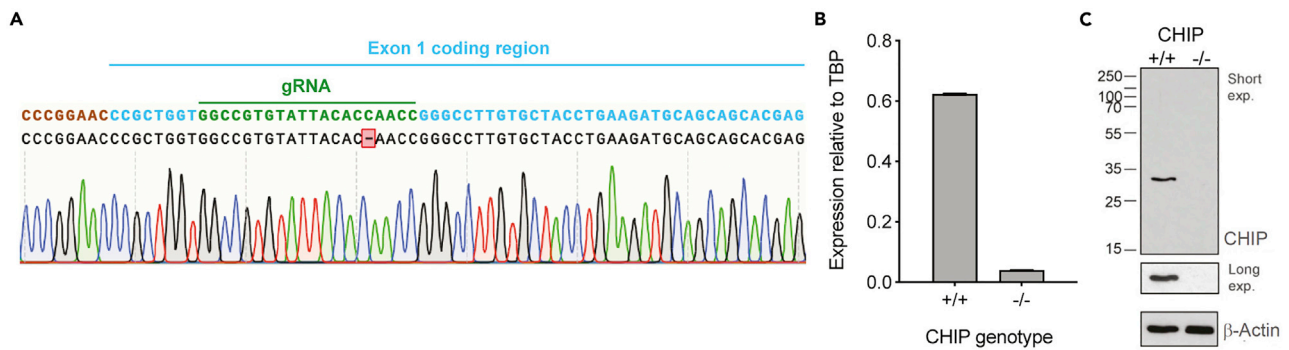


Figure 2. Validation of CRISPR/Cas9-edited CHIP KO clones

(A) Sequencing of PCR product derived from the homozygous CHIP KO iPSC clone.

(B) Analysis of STUB1 expression by RT-qPCR in iPSC clones. Error bars represent standard deviations of triplicates.

(C) Western blot analysis of CHIP expression in iPSC clones. Both long and short exposures are included. A portion of panels (A and B) is reproduced using data previously published by the authors (Dias et al., 2021).

Engineering of primers for quantitative reverse transcription polymerase chain reaction (qRT-PCR)

Ⓞ Timing: 2 weeks

For subsequent validation of cell identity by qRT-PCR, primers must be designed and validated.

9. Design primers against target genes (e.g., using the Roche UPL Assay design center or alternative software such as that provided on primers.neoformit.com). Give preference to intron-spanning primers and those with high predicted efficiency.
10. Purchase/synthesize the primers (all primers in the following protocol were purchased from Sigma).
11. Quantify the efficiency of each primers.
 - a. Set a dilution series of a positive control sample (cDNA) and include a negative control sample. For each sample have technical triplicates.
 - b. To each reaction add 2 μ L of 2 μ M primer mix (containing both forward and reverse), 0.1 μ L of 10 μ M UPL Probe (UPL61THRU70, Roche) and 5 μ L of 2 \times UPL Probe Master Mix (04707494001, Roche), in accordance with the manufacturer's instructions (<https://lifescience.roche.com/content/dam/RMS/lifescience/Documents/PDF/High-Throughput-and-Complex-Genes-Expression-Validation-Using-the-Universal-ProbeLibrary-and-the-LightCycler-480-System.pdf>).
 - c. Add to a single well of the 386-well plates, 7 μ L of this mix and 3 μ L of sample.
 - d. Seal the plate and centrifuge for 2 min at 67 \times g.
 - e. Analyze the plate using the Roche LightCycler[®] 480 System with the Universal Probe Library (UPL) (Roche).
 - f. For sample loaded per well calculate the crossing point (C_p) value (the cycle at which fluorescence achieved the threshold).
 - g. Calculate the average C_p value per sample.
 - h. Calculate the primer efficiency based on the relationship between the Log(dilution factor) and the C_p, where 2^{-(slope of the graph)} results in the amplification factor. Following this the qPCR Efficiency Calculator (<https://www.thermofisher.com/dk/en/home/brands/thermo-scientific/molecular-biology/molecular-biology-learning-center/molecular-biology-resource-library/thermo-scientific-web-tools/qpcr-efficiency-calculator.html> <https://www.thermofisher.com/dk/en/home/life-science/cloning/topo/topo-ta-cloning/topo-ta-for-subcloning.html>) can be used.

12. Select the more efficient primers to be used in subsequent experiments. Primers used must have an amplification factor close to 2 (i.e., close to 100% efficiency).

KEY RESOURCES TABLE

REAGENT or RESOURCE	SOURCE	IDENTIFIER
Antibodies^a		
Mouse monoclonal anti-CHIP (WB/IF 1:1000)	Vojtesek Lab (Narayan et al., 2011)	3.1
Mouse monoclonal anti- α -synuclein (IF 1:500)	BD Biosciences	Cat#610787
Mouse monoclonal anti- β -actin (WB 1:5000)	Sigma-Aldrich	Cat#A5441
Mouse (IgG2b) monoclonal anti- β (III)-tubulin (IF 1:1000)	Sigma-Aldrich	Cat#T8578
Rat monoclonal anti-CTIP2 (IF 1:500)	Abcam	Cat#ab18465
Rabbit polyclonal anti-TBR1 (IF 1:400)	Abcam	Cat#ab31940
Mouse monoclonal anti-Pax6 (IF 1:40)	DSHB	Cat#AB_528427
Goat polyclonal anti-Mouse (IgG1) A488 (IF 1:1000)	Invitrogen	Cat#A21121
Goat polyclonal anti-Mouse (IgG2b) A647 (IF 1:1000)	Invitrogen	Cat#A21242
Goat polyclonal anti-Mouse A555 (IF 1:1000)	Invitrogen	Cat#A21127
Donkey polyclonal anti-Rat A488 (IF 1:1000)	Invitrogen	Cat#A21208
Donkey polyclonal anti-Rabbit A555 (IF 1:1000)	Invitrogen	Cat#A31572
Rabbit polyclonal horseradish peroxidase-conjugated anti-Mouse (WB 1:2000)	Dako	Cat#PO260
Chemicals, peptides, and recombinant proteins		
LDN-193189	Miltenyi Biotec	Cat#130-103-925
SB431542	Merck Chemicals, Calbiochem	Cat#616461
Y27632 dichloride	R&D, Tocris Bioscience	Cat#1254
Sequencing grade modified trypsin	Promega	Cat#V5111
Laminin-111	Biolamina	Cat#LN111
Laminin-521	Biolamina	Cat#LN521
Hoechst-33342	Invitrogen	Cat#H3570
10 \times CutSmart Buffer	New England Biolabs	Cat#B7204
Phosphatase	Promega	Cat#M1821
10 \times T4 Ligation Buffer	New England Biolabs	Cat#B0202S
T4 ligase	New England Biolabs	Cat#M0202L
Taq Polymerase	Invitrogen	Cat#PCR-545-020T
RNaseOUT ribonuclease inhibitor	Invitrogen	Cat#10777019
dNTP mix	Invitrogen	Cat#10297018
5 \times First strand buffer	Invitrogen	Cat#28025013
Riboguard RNase inhibitor	Lucigen	Cat# RG90910K
DNase I	New England Biolabs	Cat# M0303
M-MLV reverse transcriptase (200 units/ μ L)	Invitrogen	Cat#28025013
iPS-Brew XF media	StemMACS, Miltenyi Biotec Inc.	Cat#130-104-368
PBS with calcium and magnesium	Gibco	Cat#14040083
PBS	Sigma-Aldrich	Cat#D8537
Accutase	Sigma-Aldrich	Cat#A6964
Collagenase Type IV	Gibco	Cat#17104019
HBSS containing calcium and magnesium	Gibco	Cat#14025
0.5 U/mL Penicillin + 500 ng/mL Streptomycin	Gibco	Cat#15140122
UltraPure 0.5 M EDTA, pH 8	Gibco	Cat#15575020
VECTASHIELD [®] Antifade Mounting Media	Vector Laboratories	Cat#H-1000
BDNF	PepruTech	Cat#450-02-100
GDNF	PepruTech	Cat#450-10-100
Bovine Albumin Fraction V, 7.5% solution	Gibco	Cat#15260037
DMEM/F12	Gibco	Cat#21331-020

(Continued on next page)

Continued

REAGENT or RESOURCE	SOURCE	IDENTIFIER
Neurobasal media	Gibco	Cat#21103-049
B27 supplement with retinoic acid (50x)	Gibco	Cat#17504044
N2 supplement (100x)	Gibco	Cat#17502-048
L-glutamine (200 mM)	Thermo Fisher Scientific	Cat#25030-024
Paraformaldehyde	Sigma-Aldrich	Cat#158127
Urea	Invitrogen	Cat#15505050
HEPES	Sigma-Aldrich	Cat#H3375-100G
KCl	Lach-Ner	Cat#30076-AP0-G1000-1
DTT	Invitrogen	Cat#28025013
n-Dodecyl-β-D-maltoside	Sigma-Aldrich	Cat#D4641-500 MG
Tris (HCL)	Bio-Rad	Cat#161-0716
Tris (2-carboxyethyl) phosphine hydrochloride	Sigma-Aldrich	Cat#C4706
Iodoacetamide	Sigma-Aldrich	Cat#16125
Ammonium bicarbonate	Merck	Cat#1066-33-7
Formic acid	Sigma-Aldrich	Cat#94318-250ML-F
Acetonitrile	VWR	Cat#34967
Trifluoroacetic acid (TFA)	VWR	Cat#84868.180
Critical commercial assays		
Human Stem Cell Nucleofector	Lonza	VPH-5012
Epicentre MasterPure™ Complete DNA and RNA Purification Kit	Epicentre	MC85200
LightCycler® 480 UPL Probe Master	Roche	Cat#04707494001
UPL Probe	Roche	Cat#UPL61THRU70
Quantitative Colorimetric Peptide Assay	Thermo Scientific	Cat#23275
Micro-BCA assay	Thermo Scientific	Cat#23235
T7 endonuclease assay I	New England Biolabs	Cat#M0302
TOPO-TA cloning assay	Invitrogen	Cat#450641
Experimental models: Cell lines		
AST23 iPSC: Parental line (with a triplication of SNCA)	Kunath Lab (Devine et al., 2011)	N/A
AST23 iPSC: CHIP KO	This paper	N/A
AST23 iPSC: CRISPR/Cas9 control (CHIP WT)	This paper	N/A
Oligonucleotides		
RT-qPCR primers	This paper	Table 1 in "materials and equipment"
Forward STUB1 primer	This paper	AGAACGAGGGTGCGATGC
Reverse STUB1 primer	This paper	GATGTCGTCGCCGAAGTTC
Recombinant DNA		
LentiV2	Zhang Lab	Addgene plasmid # 52961 ; http://n2t.net/addgene:52961 ; RRID:Addgene_52961
Software and algorithms		
Prism GraphPad	GraphPad	https://www.graphpad.com/scientific-software/prism/
SnapGene	GSL Biotech LLC	https://www.snapgene.com/
Micromanager Software (μManager)	Vale Lab	https://micro-manager.org/
ImageJ	(Schneider et al., 2012)	https://imagej.nih.gov/ij/download.html
Guides	Zhang Lab	http://guides.sanjanalab.org/#/
Off-Spotter	Zhang Lab	https://cm.jefferson.edu/Off-Spotter/
SWATH™ Acquisition MicroApp 1.0 plugin	PeakView® Software	https://sciex.com/content/dam/SCIEX/pdf/customer-docs/release-notes/SWATH_Processing_Release_Notes1.pdf
PeakView 1.2.0.3	AB-SCIEX	https://sciex.com/products/software/peakview-software
MarkerView 1.2.1.1	AB-SCIEX	https://sciex.com/content/dam/SCIEX/pdf/customer-docs/release-notes/MarkerView_1.2.1_relNotes.pdf
ProteinPilot 4.5	AB-SCIEX	https://sciex.com/content/dam/SCIEX/pdf/software/ProteinPilot_Software_501_Release_Notes.pdf

(Continued on next page)

Continued

REAGENT or RESOURCE	SOURCE	IDENTIFIER
Database for Annotation, Visualization and Integrated Discovery (DAVID)	(Huang et al., 2009a; 2009b)	https://david.ncifcrf.gov/tools.jsp
REduce + Visualize Gene Ontology (REVIGO)	(Supek et al., 2011)	http://revigo.irb.hr/
Ingenuity Pathway Analysis (IPA)	QIAGEN	https://digitalinsights.qiagen.com/products-overview/discovery-insights-portfolio/analysis-and-visualization/qiagen-ipa/?cmpid=QDI_GA_IPA&gclid=CjwKCAjw3pWDBhB3EiwAV1c5rHy2w0yPhQ699r3__ycWTHXrCPf3LBaTW4BUNfYAmpaSW2noKMgU4hoCRScQAvD_BwE
GORilla	(Eden et al., 2007, 2009)	http://cbl-gorilla.cs.technion.ac.il/
Other		
Nucleofector™ 2b Device	Lonza	Cat#AAB-1001
LightCycler® 480	Roche	N/A
Microcon centrifugal filter units (YM-10 membrane, NMWCO 10 kDa)	Merck	Cat#MRCPRT010
Micro Spin C-18 Columns	Harvard Apparatus	Cat#74-7242
C18 PepMap100 sorbent with 5 µm particle size	Thermo Scientific	Cat#160434
Capillary column (75 µm i.d. × 500 mm)	Thermo Scientific	Cat#164942
Thermo-shaker	Biosan	Cat#TS-100
SpeedVac	Savant	Cat#SPD210-115
Elmasonic S40H	Elma	Cat#100 2006
Centrifuge	Eppendorf	Cat#5424
Axygen® 1.5 mL MaxyClear Snaplock Microcentrifuge Tube	Corning	Cat#MCT-150-C
NanoDrop	Thermo Scientific	Cat#ND2000LAPTOP
Eksigent Ekspert nanoLC 400	AB-SCIEX	Cat#NanoLC 400
TripleTOF 5600+ mass spectrometer	AB-SCIEX	TripleTOF 5600+ System
10 cm plates	Greiner CELLSTAR	Cat#664160
6-well plates	Greiner CELLSTAR	Cat#657160
12-well plates	Greiner CELLSTAR	Cat#M9187-100EA
96-well plates	Greiner CELLSTAR	Cat#655180
4-well plates	Thermo Scientific	Cat#167063
386-well plates	Roche	Cat#04729749001
Hemocytometer	Merck	Cat#Z359629

^aIncluding dilutions for immunofluorescence (IF) and Western blot (WB).

MATERIALS AND EQUIPMENT

Differentiation media

Reagent	Final concentration	Amount
DMEM/F12	1×	24.5 mL
Neurobasal media	1×	24.5 mL
B27 supplement with retinoic acid (50×)	0.5×	500 µL
N2 supplement (100×)	0.5×	250 µL
L-glutamine (200 mM)	2 mM	500 µL
Total	n/a	50 mL

Store at 4°C for maximum of two weeks. Other supplements (not stated here) are only added immediately prior to use.

△ CRITICAL: Paraformaldehyde is harmful if swallowed or if inhaled, may cause skin irritation and an allergic reaction, as well as respiratory irritation and serious eye damage. Wear protective gloves, protective clothing, as well as eye protection and face protection. Use a fume hood when using paraformaldehyde.

Lysis buffer		
Reagent	Final concentration	Amount
Urea	8 M	2.4 g
HEPES, pH 8	50 mM	59.5 mg
KCl	100 mM	37.3 mg
DTT	1 mM	0.77 mg
n-Dodecyl- β -D-maltoside	0.5% (w/v)	25 mg
Total	n/a	5 mL

Use fresh.

Urea buffer		
Reagent	Final concentration	Amount
Urea	8 M	2.4 g
Tris-HCl, pH 8.5	100 mM	60.6 mg
Total	n/a	5 mL

Use fresh.

Table 1. List of primers for qRT-PCR

Target gene	UPL probe #	Forward sequence	Reverse sequence	Efficiency
<i>hCDK6</i>	85	gaactaggcaagacctactctga	ggtgggaatccaggtttct	2.161
<i>hNanog</i>	87	tctccaacatctgaacctca	ttgctattctcgccagtt	2.004
<i>hOct4</i>	78	tgccgtgaaactggagaag	gcttgcaaatgttcgagt	2.015
<i>hVimentin</i>	11	agatggcccttgacattgag	caggaggaaaagtttggaa	1.844
<i>hNestin</i>	65	acctgtgccagccttttta	gccaaaggtaggggtacgg	2.154
<i>hOtx2</i>	86	aacctccatgaggctgtaa	ggtggacaggttcagagtcc	2.007
<i>hEmx1</i>	25	ctctccgagacgcagggtg	ttcttctgctcggactcagg	2.015
<i>hPax6</i>	20	tcacatggcaataacctg	cagcatgcaggagtatgagg	1.980
<i>hFoxG1</i>	58	tactaccgcgagaacaagca	tcacgaagcactgttgagg	2.075
<i>hTbr2</i>	69	gagtcggcaggtgggtag	tcttccgaggggaaggtaa	1.953
<i>hTbr1</i>	31	ttcaataacaatggcagatg	gtctcagggaaaagtgaactct	2.156
<i>hCtip2</i>	82	ccgccagagatagggtttt	ctggtcatgcacaacctcag	2.032
<i>hCux1</i>	20	agaggccactgcctattct	ctggagatgatgaaagcagt	2.019
<i>hBrn2</i>	80	ctttgcaggcgagtaaccag	ttctagctatcacacactctctca	1.967
<i>hSNCA</i>	68	gagggagtgggtgatggt	tgctgtcacaccgtcac	(Devine et al., 2011)
<i>hStub1</i>	70	gttcgtggccgaaagta	ggccccgttggtgtaata	1.874
<i>hTBP</i>	87	gaacatcatggatcagaacaaca	atagggattccgggagtc	2.005

Note: These primers were designed specifically to be used for qRT-PCR, having an amplicon to which specific UPL probes bind to.

STEP-BY-STEP METHOD DETAILS

Neural induction

⌚ Timing: 11 days

Neural induction programs iPSC into the neuroectodermal lineage. Through dual inhibition of SMAD proteins (homologous to the gene products of the *Drosophila* gene “mothers against decapentaplegic” (*Mad*) and the *C. elegans* gene *sma*) this protocol results in a monolayer of neural progenitors with a robust expression profile that is critical for efficient cortical induction and subsequent

neuronal differentiation. Two SMAD inhibitors are used: SB431542 and LDN (selective BMP signaling inhibitor). The former is a potent and specific inhibitor of transforming growth factor- β superfamily type I activin receptor-like kinase (ALK) receptors (Inman et al., 2002), while LDN is a derivative of Drosomorphin and also inhibits BMP type I receptors, preventing activation of downstream SMADs (Boergemann et al., 2010).

By day 11 pluripotency and stem-renewal markers (*CDK6*, *NANOG* and *OCT4* genes) should decline dramatically, while markers of the primary cortical stem and progenitor cells (including *NES*, *OTX2* and *PAX6* genes) increase. Batches of differentiating cultures that achieve a homogenous layer of progenitor cells with these characteristics promote robust and reproducible differentiation experiments.

Note: This protocol is an optimization from a previous protocol (Chambers et al., 2009).

Note: Given the inherent variability of the differentiation, use an appropriate number of biological replications. Nonetheless, opportunities for expansion of differentiation cultures will be available in later steps.

1. Plate cells at a high density (80,000 cells/cm²; Days -1 to 2 of the differentiation)
 - a. On Day -1 of the differentiation: coat 12-well plates with laminin-111 according to the manufacturer's instructions (<https://biolamina.com/wp-content/uploads/2020/12/instructions-bl001.pdf>).

△ CRITICAL: Plates must be freshly coated and the matrix of laminin-111 formed must be even and properly hydrated. Plates must be kept at 4°C and sterile. [Troubleshooting 1](#)

Note: If coating glass coverslips or glass bottom plates these need to be pre-treated with poly-L-ornithine, according to the manufacturer's instructions (<https://www.sigmaldrich.com/DK/en/product/sigma/p4957>)

Note: Control wells should be included for subsequent assessment of the neural induction efficient. Some of these should include glass coverslips (pre-treated accordingly) to be used for immunofluorescence assay.

Optional: Other matrices may also be used but laminin-111 promotes stem cell survival and proliferation and drives differentiation.

- b. Incubate iPSC (routinely cultured in 6-well plates and passaged at least 35 times) with 0.5 mM EDTA (Thermo Fisher Scientific) diluted in PBS without calcium and magnesium (D8537, Sigma) for 15–20 min at 37°C (until cells detach). For each well in a 6-well plate add 1 mL of EDTA solution.
 - c. Count cells using a hemocytometer.

Optional: other cell counting methods may be used but the same method should be used throughout the protocol and for different runs of the differentiation experiments.

- d. Centrifuge the cells in suspension for 3 min at 180 g.
 - e. Resuspend cells in differentiation media (refer to "[materials and equipment](#)") supplemented with 10 μ M ROCK inhibitor Y-27632.
 - f. Plate cells at 80,000 cells/cm². Keep cells in this supplemented media for the two subsequent days. Culture cells at 37°C in 5% (v/v) CO₂.

△ **CRITICAL:** Cell density may vary across cell lines. Nonetheless, plates must be around 70% confluent after two days.

Note: ROCK inhibition promotes cell survival.

2. Start SMAD inhibition treatment (Day 2–11 of the differentiation)
 - a. Day 2: Remove the medium completely and incubate with cells with differentiation media (refer to “[materials and equipment](#)”) supplemented with 10 μM SB431542 (616461, Merck Chemicals, Calbiochem) and 100 nM LDN-193189 (130-103-925, Miltenyi Biotec). These must be previously reconstituted in DMSO, filter-sterilized and diluted to 1000 \times working stocks and aliquoted. Stocks must be kept protected from light and stored at -20°C . Once thawed, aliquots should be kept at room temperature for a maximum of 2 weeks. Culture cells at 37°C in 5% (v/v) CO_2 .
 - b. For each day until Day 11: Remove 80% of the media and incubate with cells with differentiation media (refer to “[materials and equipment](#)”) supplemented with 10 μM SB431542 and 100 nM LDN. Culture cells at 37°C in 5% (v/v) CO_2 .

△ **CRITICAL:** To prevent cell detachment, media should be removed and added with care.

△ **CRITICAL:** SB431542 and LDN must be added fresh to the differentiation media.

Note: By Day 11 of the differentiation, neural cultures should be characterized by a complete and homogenous neuroepithelial monolayer of neural progenitors.

Assessment of neural induction

⌚ **Timing:** Day 11 of the differentiation + three days of analysis.

Troubleshooting 2

3. Analysis of neural markers by immunofluorescence
 - a. Fixation of cells:
 - i. Gently wash control cultures three times with 4°C PBS. Use 0.5 mL for a well within a 24-well plate.
 - ii. Fix cells by incubating with 4% (v/v) paraformaldehyde for 15 min at room temperature and protected from light.
 - iii. Wash cells three times with 4°C PBS for 5 min at room temperature and shake gently.
 - b. Blocking of non-specific binding:

Incubate cells with blocking solution (either 2% (v/v) donkey or goat serum, in accordance to the species of origin of the secondary antibodies used, diluted in PBS with 0.1% (v/v) Tween-20) for 1 h at room temperature with gentle shaking.
 - c. Identifying target of interest with primary antibody:
 - i. Dilute the primary antibodies in blocking solution to the desired concentration.

△ **CRITICAL:** The expression of the key neural progenitor markers, Pax6, must be assessed ([Figure 3A](#)).

Optional: Although cultures are still immature for robust expression, the neural-specific marker, β (III)-tubulin, can also be analyzed.

- ii. Add mixture to cells and incubate overnight (16–20 h) at 4°C .
- iii. Wash cells three times with PBS with 0.1% (v/v) Tween-20 for 5 min at room temperature and shake gently.

- d. Secondary antibody:
 - i. Dilute fluorophore-conjugated secondary antibodies in the blocking solution.
 - ii. Add mixture to cells and incubate for 1 h at room temperature, protected from light.
 - iii. Wash cells three times with PBS with 0.1% (v/v) Tween-20 for 5 min at room temperature and shake gently.
- e. Marking the nuclei:
 - i. Dilute DAPI (62248, Invitrogen) in PBS (1:5000).
 - ii. Add mixture to cells and incubate for 5 min at room temperature, protected from light.
 - iii. Wash cells three times with PBS with 0.1% (v/v) Tween-20 for 5 min at room temperature and shake gently.
- f. Mounting and imaging:
 - i. Mount the coverslips onto microscope slides with mounting medium (VECTASHIELD® Anti-fade Mounting Media, H-1000, Vector Laboratories) and remove excess mounting media.
 - ii. Image cells after incubating for 24 h at 4°C.

Note: Control wells used for the immunofluorescence assay should include glass coverslips.

Optional: Other fixatives can be used instead of paraformaldehyde, although this may affect the protein epitopes that become exposed to the antibodies used.

4. Analysis of pluripotency and stem-renewal markers and markers of primary cortical stem and progenitor cells by qRT-PCR.
 - a. RNA extraction:
 - i. Use the Epicentre MasterPure™ Complete DNA and RNA Purification Kit (Epicentre, MC85200), according to the manufacturer's instructions (<https://www.lucigen.com/docs/manuals/MA110E-MasterPure-Complete.pdf>).
 - ii. Once resuspended, treat extracted RNA with riboguard RNase inhibitor to prevent decay.
 - iii. To ensure removal of contaminating DNA, treat with DNase I (New England Biolabs) according to manufacturer's instructions (<https://international.neb.com/protocols/0001/01/01/a-typical-dnase-i-reaction-protocol-m0303>).
 - iv. Assess the concentration and purity of the RNA.
 - b. cDNA synthesis (Table 2):
 - i. Incubate the purified RNA (1 µg and 12 µL) with 1 µL of 10 mM dNTP mix (10297018, Invitrogen) and 1 µL of random primers (50 ng/µL) (PCR-545-020T, Invitrogen) at 65°C for 5 min and then cool on ice.
 - ii. After a brief centrifugation, add 4 µL 5× First strand buffer (28025013, Invitrogen), 2 µL of 0.1 M DTT (28025013, Invitrogen) and 1 µL riboguard RNase inhibitor and incubate at 37°C for 2 min.
 - iii. Add 1 µL M-MLV reverse transcriptase (200 units/µL) (28025013, Invitrogen) or 1 µL RNase-free water (for the control sample) per sample and mix.
 - iv. Incubate the samples for 10 min at room temperature.
 - v. Incubate the samples for 60 min at 37°C.
 - vi. Incubate the samples for 10 min at 90°C to inactivate the reverse transcriptase.
 - vii. Cool the cDNA mix on ice.
 - viii. Add 180 µL of RNase-free water.
 - ix. Snap freeze the samples.

Note: When preparing multiple samples make a master mixtures.

- c. qRT-PCR:
 - i. Primers were designed using the Roche UPL Assay design center and validated (refer to "before you begin").

- ii. To each reaction add 2 μL of 2 μM primer mix (containing both forward and reverse) (Sigma), 0.1 μL of 10 μM UPL Probe (UPL61THRU70, Roche) and 5 μL of 2 \times UPL Probe Master Mix (04707494001, Roche), in accordance with the manufacturer's instructions (<https://lifescience.roche.com/content/dam/RMS/lifescience/Documents/PDF/High-Throughput-and-Complex-Gene-Expression-Validation-Using-the-Universal-ProbeLibrary-and-the-LightCycler-480-System.pdf>). Make a stock mix for all samples and replicates per gene analyzed.

Note: Multiple pluripotency and stem-renewal markers (*CDK6*, *NANOG* and *OCT4*) and neural progenitor markers (*VIM*, *NES*, *OTX2* and *EMX1*) should be assessed (Figures 4A and 4B).

- iii. Add to a single well of the 386-well plates, 7 μL of this mix and 3 μL of cDNA.
- iv. For each plate, include wells with primers designed to detect the housekeeping gene TATA-binding protein (TBP).
- v. Seal the plate and centrifuge for 2 min at 67 $\times g$.
- vi. Analyze the plate using the Roche LightCycler® 480 System with the Universal Probe Library (UPL; Roche).
- vii. For sample loaded per well calculate the crossing point (Cp) value (the cycle at which fluorescence achieved the threshold).
- viii. Calculate the average Cp of TBP expression per biological replicate.
- ix. To determine the level of relative expression, calculate the difference between the average Cp value of TBP and that obtained for each sample (i.e., each replicate from all primer sets used), i.e., $\Delta\text{Cp TBP} - \text{Cp Sample}$.
- x. Assuming a doubling of the target DNA during each cycle, calculate $2^{(\Delta\text{Cp TBP} - \text{Cp Sample})}$.
- xi. Calculate the average and standard deviations of the technical replicates per gene analyzed (for each primer set used).

Note: For each primer used, include three technical replicates for a single biological replicate.

△ CRITICAL: Only differentiating cultures with robust and homogenous expression of the neural progenitor marker *Pax6* should be taken further in the differentiation protocol. It is recommended that >90% of the neuroepithelial cells express *Pax6* and that the expression level is readily detectable by immunofluorescence assays and Western blotting. By qRT-PCR, *PAX6* expression relative to *TBP* expression should be about 6 at day 11 of differentiation.

Cortical neurogenesis

⌚ Timing: 69 days

Once cells are programmed towards a neural lineage, primary and secondary cortical stem and progenitor cells arise and populate the neural cultures (Shi et al., 2012b). The primary cortical progenitor cells (characterized by *Pax6*⁺/*OTX2*⁺/*Ki67*⁺, polarization and radial processes) generated from iPSC eventually form rosettes (Shi et al., 2012a; 2012b) (visible at Day 13 of the differentiation). These cells then give rise to secondary progenitor cells, which are composed of two subpopulations: basal/intermediate progenitor cells (characterized by *TBR2*⁺/*Ki67*⁺/*Pax6*⁻ and the lack of basal processes) and radial glial cells (marked by *Pax6*⁺/*TBR2*⁻ and with lengthy processes) (Shi et al., 2012a; 2012b). Secondary progenitor cells are present at the periphery of each rosette (Shi et al., 2012a; 2012b) (seen more clearly at Day 18).

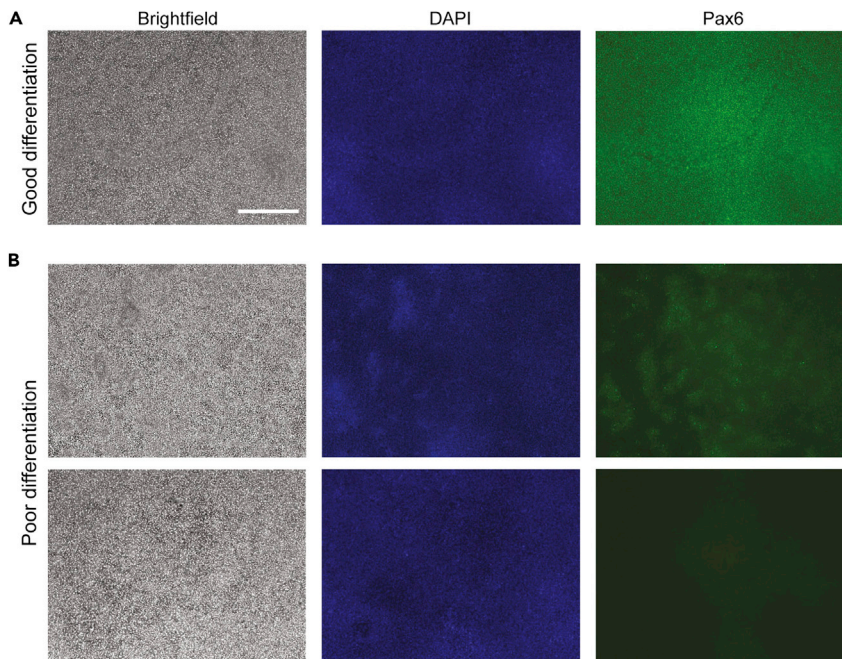


Figure 3. Neural induction

(A and B) At day 11 of the differentiation a confluent monolayer of neuroepithelial cells with high expression levels of Pax6 is expected (A). Examples of poor differentiation (B) are when the expression of Pax6 is uneven (top row) and/or low (bottom row). Scale bar, 200 μ m.

Following the establishment of cortical neural rosettes, fundamental principles of neural development govern the ability of these three critical classes of progenitors to generate complex populations of cortical projection neurons, in a cell-intrinsic manner. Around 2–3 weeks into differentiation, early-born deep-layer neurons (TBR1⁺ and/or CTIP2⁺) form. Late-born upper-layer neurons (Stab2⁺/Cux1⁺/Brn2⁺), on the other hand, can only be detected later (after around 70 days of differentiation) (Shi et al., 2012a; 2012b).

5. Passage neuroepithelial cells (Day 11 of the differentiation).
 - a. At Day 10 of the differentiation: coat wells within 4-well plates with laminin-111 according to the manufacturer's instructions (<https://biolamina.com/wp-content/uploads/2020/12/instructions-bl001.pdf>). [Troubleshooting 1](#)
 - b. Incubate cells with 250 U/mL Collagenase Type IV (17104019, Gibco) diluted in HBSS containing calcium and magnesium (14025, Gibco) for 30 min at 37°C. [Troubleshooting 3](#)

Optional: Other passaging reagents can be used (e.g., Dispase), although cells should be lifted as clumps rather than dissociated as single cells. Note that some enzymes are not inactivated by the media and must therefore be thoroughly removed from the media to prevent degradation of the cultures (this is the case of, for example, Dispase).

- c. Triturate the cells by pipetting several times (with 1 mL pipette tips) to get small aggregates of cells. [Troubleshooting 4](#)

Note: While titrating, a point is reached where clumps of cells do not dissociate to smaller fragments. At this point titration should be halted to prevent further cell stress. Clumps should consist of 300–500 cell aggregates.

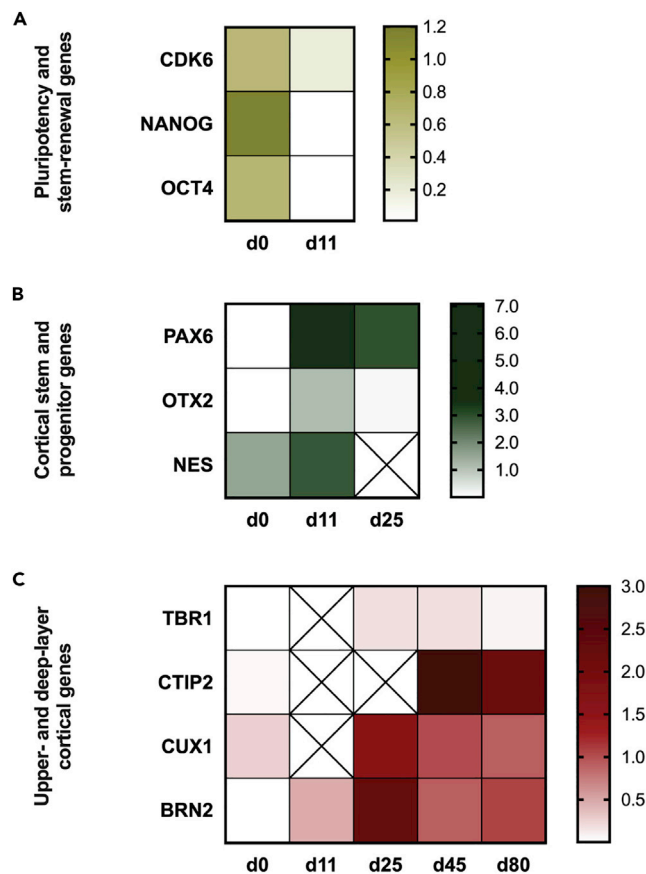


Figure 4. Transcriptomic profile through cortical differentiation

(A) The expression of pluripotency and stem-renewal genes declines dramatically from the start of the differentiation process (day 0) to day 11, when stem cells are expected to have transformed into primary stem and progenitor cells. (B) Markers of cortical stem and progenitor genes peak at day 11. (C) Markers of deep-layer (TBR1 and CTIP2) and upper-layer (CUX1 and BRN2) cortical neurons increase throughout the differentiation process. Heatmaps show mean expression levels derived from qRT-PCR and normalized to the TATA binding box protein (TBP) gene. Abbreviation: days (d).

- d. Wash cells in wash media (1:1 DMEM/F12 and neurobasal media supplemented with 10 μ M Y27632 dichloride).
 - e. Centrifuge the cell suspension for 3 min at 180 g.
 - f. Resuspend the pellet in wash media.
 - g. Centrifuge the cell suspension for 3min at 180 g.
 - h. Resuspend the pellet in differentiation media supplemented with 10 μ M Y27632 dichloride, 100 nM LDN and 10 μ M SB431542.
 - i. Plate at a 1:1.5 ratio.
6. After 24 h, remove all the media and incubate cells with differentiation media only (Day 12 of the differentiation).
 7. Renew the differentiation media daily or every 2 days, depending on the need. [Troubleshooting 5](#)

Note: Small rosettes become visible by Day 13 of the differentiation, particularly when imaged at low magnification in brightfield. [Troubleshooting 6](#)

8. Passage the neuroepithelial cells (Day 17 of the differentiation).

Table 2. Steps for cDNA synthesis

Steps	Reagent	Volume per sample
1. Mix the following and incubate at 65°C for 5 min and then cool on ice.	Purified RNA	1 µg and 12 µL
	10 mM dNTP mix (10297018, Invitrogen)	1 µL
	Random primers (50 ng/µL) (PCR-545-020T, Invitrogen)	1 µL
2. Add the following and incubate at 37°C for 2 min.	5× First strand buffer (28025013, Invitrogen)	4 µL
	0.1 M DTT (28025013, Invitrogen)	2 µL
	Riboguard RNase inhibitor	1 µL
3. Add the following and mix.	M-MLV reverse transcriptase (200 units/µL) (28025013, Invitrogen)	1 µL
	Control: RNase-free water	
4. Incubate the samples for 10 min at room temperature		
5. Incubate the samples for 60 min at 37°C.		
6. Incubate the samples for 10 min at 90°C to inactivate the reverse transcriptase.		
7. Cool the cDNA mix on ice		
8. Add 180 µL of RNase-free water		
9. Snap freeze the samples.		

- a. At Day 16 of the differentiation: coat 4-well plates with laminin-111 according to the manufacturer's instructions (<https://biolamina.com/wp-content/uploads/2020/12/instructions-bl001.pdf>). [Troubleshooting 1](#)
 - b. Incubate cells with 250 U/mL Collagenase Type IV (17104019, Gibco) diluted in HBSS containing calcium and magnesium (14025, Gibco) for 30 min at 37°C. [Troubleshooting 3](#)
 - c. Triturate the cells by pipetting several times (with 1 mL pipette tips). [Troubleshooting 4](#)
 - d. Wash cells in wash media (1:1 DMEM/F12 and neurobasal media supplemented with 10 µM Y27632 dichloride).
 - e. Centrifuge the cell suspension for 3 min at 180 g.
 - f. Resuspend the pellet in wash media.
 - g. Centrifuge the cell suspension for 3 min at 180 g.
 - h. Resuspend the pellet in differentiation media supplemented with 10 µM Y27632 dichloride.
 - i. Plate at a 1:2 ratio.
9. After 24 h, remove all the media and incubate cells with differentiation media only (Day 18 of the differentiation).

Note: Larger rosettes become visible by Day 18 of the differentiation and progenitor cells with length processes can be seen at the periphery of the rosettes. [Troubleshooting 6](#)

10. Renew the differentiation media daily or every 2 days, depending on the need. [Troubleshooting 5](#)
11. Passage the neural stem cells (Day 25 of the differentiation).
 - a. At Day 23 of the differentiation: pre-coat 4-well plates with poly-L-ornithine, according to the manufacturer's instructions (<https://www.sigmaaldrich.com/DK/en/product/sigma/p4957>). These will be used for immunofluorescence assay.
 - b. At Day 24 of the differentiation: coat the poly-L-ornithine pre-coated 4-well plates with laminin-111 according to the manufacturer's instructions (<https://biolamina.com/wp-content/uploads/2020/12/instructions-bl001.pdf>). [Troubleshooting 1](#)
 - c. Incubate cells with Accutase (A6964, Sigma) for 10–15 min at 37°C.
 - d. Triturate the cells by pipetting several times (with 1 mL pipette tips) until dissociated into single cells.
 - e. Add wash media.
 - f. Filter the cells in suspension using a 40 µm cell strainer to ensure that cell aggregates are removed.

- g. Count cells using a hemocytometer.
- h. Centrifuge the cell suspension for 3 min at 180 g.
- i. Resuspend cells in differentiation media supplemented with 10 μM Y27632 dichloride.
- j. Plate cells at a low density (35,000 cells/cm²) or high density (80,000 cells/cm²), depending on intended use (e.g., for immunofluorescence assay or mass spectrometry, respectively).

▣ **Pause point:** According to Shi and colleagues, at this point neural stem cells can be cryo-stored (Shi et al., 2012a).

12. After 24 h, remove the media completely and incubate the cells with differentiation media supplemented with 20 ng/mL BDNF (450-02-100, Peprotech) and 20 ng/mL GDNF (450-10-100, Peprotech) that were previously reconstituted in PBS (D8537, Sigma) with 0.1% BSA (Bovine Albumin Fraction V, 7.5% solution, 15260037, Gibco).

Note: These neurotrophic factors promote neuronal survival.

Note: BDNF and GDNF are both reconstitute with 0.1% BSA in PBS to a 20 $\mu\text{g/ml}$ working stock.

13. From Day 26–80 of the differentiation protocol carefully replace half of the media with fresh differentiation media supplemented with 20 ng/mL BDNF and 20 ng/mL GDNF.

△ **CRITICAL:** As neuronal cultures develop, their attachment to the plate becomes weaker so care is critical when manipulating the cultures. [Troubleshooting 5](#)

△ **CRITICAL:** Observe cultures routinely to detect early the appearance of any proliferating contaminating cells. [Troubleshooting 7](#)

Validation of cortical identity

⌚ **Timing:** ~2 week

Given the inherent variability of the differentiation process and the need to characterize the identity of our neural cultures, a thorough investigation of the expression of different neuronal markers is key. This should be done not only at the end of the differentiation process, but also throughout its course to ensure reproducibility of the protocol and assess whether the neuronal cultures are maturing with similar dynamics and have similar differentiation potentials. Throughout cortical neurogenesis, a rise in markers of secondary progenitors (*FOXP1* and *TBR2*) is expected. This is followed by an increase in markers of early-born deep-layer neurons (*TBR1* and *CTIP2*) and subsequently, late-born upper-layer neurons (*CUX1* and *BRN2*).

14. Analysis of early-born upper-layer neurons by immunofluorescence at Day 50 and 80 of the differentiation. Please refer to major step 3 for experimental details.

△ **CRITICAL:** The expression of the key early-born deep-layer neuronal markers, *TBR1* and *CTIP2*, must be assessed.

Note: Quantify the percentage of neurons that are *TBR1*⁻/*CTIP2*⁺, *TBR1*⁺/*CTIP2*⁻ and *TBR1*⁺/*CTIP2*⁺.

Note: Assess the expression of late-born upper-layer neuronal markers (e.g., *CUX1*) at Day 80 of the differentiation.

Note: Conduct an immunofluorescence assay targeting the expression of the neuron-specific marker, β (III)-tubulin, to assess the complexity of the neuronal networks.

15. Analysis of markers of secondary progenitor cells, early-born upper-layer neuron and late-born deep-layer neurons by qRT-PCR. Please refer to step 4 for experimental details.

Note: To assess changes in expression through the course of the differentiation and identify peaks in expression profiles with temporal-specificity, we recommend conducting qRT-PCR at Days 0, 11, 25, 45 and 80 of the differentiation.

△ CRITICAL: Primers targeting *FOXC1*, *TBR1* and *CTIP2*, and *CUX1* and *BRN2* are key to monitor the development of secondary progenitors, early-born deep-layer neurons and late-born upper-layer neurons, respectively, in the cortical cultures.

Optional: The expression of pluripotency and stem-renewal markers (including *CDK6*, *NANOG* and *OCT4*) and primary cortical stem and progenitor cells markers (including the *VIM*, *NES*, *OTX2*, *EMX1* and *PAX6* genes) can also be assessed. Expression of pluripotency and stem-renewal markers is expected to remain low during cortical neurogenesis. Expression of most primary cortical stem and progenitor cells markers is expected to peak during neural induction and then decrease during cortical neurogenesis. Of note, *PAX6* expression may remain higher because a sub-population of secondary progenitors are also marked by Pax6.

Sample preparation for SWATH-MS

⌚ **Timing:** ~1 week

Cell lines intended for proteomic comparison are lysed in 8 M Urea buffer to generate whole cell protein extracts. Next, extracted proteins are proteolytically digested into (tryptic) peptides compatible with bottom-up mass spectrometry. Protein trypsinization is carried-out on 10 kDa filters for Filter-Aided Sample Preparation (FASP) (Wiśniewski et al., 2009). The filter aided digestion comprises of first reducing any disulfide bridges of the lysate, to disrupt protein tertiary structure, followed by alkylation of free sulfhydryl groups. Proteins prepared this way can be efficiently digested to provide peptides as the cleavage sites are easily accessible by the protease. Resultant peptides are desalted on C18 columns and dried by evaporation.

16. Harvesting of mature cortical neurons
 - a. From day 25–80 of the differentiation culture neurons in 4-well plates (plated at 80,000 cells/cm² at day 25).
 - b. At day 80 of the differentiation, gently wash twice the neuronal cultures with 4°C PBS (500 μ L per well).
 - c. Add 100 μ L of lysis buffer to each well and incubate for 30 min on a shaking table at room temperature or, if possible, 8°C.
 - d. Transfer the lysate to an Eppendorf tube.
 - e. Centrifuge the samples at 14,000 \times g for 20 min, 8°C.
 - f. Transfer the supernatant to new labeled tubes (Axygen® 1.5 mL MaxyClear Snaplock Microcentrifuge Tube, MCT-150-C, Corning).
 - g. Measure the protein concentration using micro-BCA (Thermo Fisher 23235) following the manufacturer's instructions (<https://www.thermofisher.com/order/catalog/product/23235>).

⏸ **Pause point:** Snap freeze the samples in liquid nitrogen and store at -80°C .

17. FASP
 - a. Use Microcon centrifugal filter units (YM-10 membrane, NMWCO 10 kDa, Merck).

- b. Mix 100 μg of protein extract (in up to 30 μL volume) with 200 μL urea buffer on the filter. [Troubleshooting 8](#)
- c. Centrifuge the Microcon unit at 14,000 $\times g$ for 15 min at 20°C. [Troubleshooting 9](#)
- d. Add 200 μL of urea buffer to the filter and repeat the centrifugation.
- e. Discard the flow-through.
- f. Add 100 μL urea buffer with 100 mM Tris (2-carboxyethyl) phosphine hydrochloride (C4706, Sigma) pH 8.5 (for protein reduction).
- g. Incubate the unit in a thermomixer at 600 rpm for 30 min at 37°C.
- h. Centrifuge the filter unit at 14,000 $\times g$ for 15 min at 20°C.
- i. Add 100 μL urea buffer with 50 mM iodoacetamide (I6125, Sigma) pH 8.5 (for alkylation of free sulfhydryl groups).
- j. Incubate the unit without shaking in the dark for 20 min at room temperature.
- k. Centrifuge the filter unit at 14,000 $\times g$ for 15 min at 20°C.
- l. Discard the flow-through.
- m. Add 100 μL 100 mM ammonium bicarbonate pH 7.8.
- n. Centrifuge the filter unit at 14,000 $\times g$ for 15 min at 20°C, repeat this step twice.
- o. Transfer the filter unit to a new collection tube.
- p. Add 100 μL 50 mM ammonium bicarbonate pH 7.8 with sequencing grade modified trypsin (V5111, Promega) at an enzyme-to-protein ratio of 1 : 50 (w/w).
- q. Incubate the unit in a thermomixer (Thermo-shaker TS-100, Biosan) at 600 rpm for 60 min at 37°C followed by an overnight incubation (16–20 h) in a humid chamber at 37°C.
- r. Centrifuge the filter unit at 14,000 $\times g$ for 15 min at 20°C until the solution completely passed the filter membrane (for peptide elution).
- s. Measure the peptide concentration using the Quantitative Colorimetric Peptide Assay (23275, Pierce, Thermo Scientific), following the manufacturer's instructions (https://www.thermofisher.com/document-connect/document-connect.html?url=https%3A%2F%2Fassets.thermofisher.com%2Fassets%2Fmanuals%2F23275_quantpeptide_color_UG.pdf).
- t. Dry the sample in SpeedVac (Savant) with thermostat set to 37°C until the liquid has completely evaporated.

Pause point: The samples can be stored at -80°C .

18. Peptide desalting

- a. Dissolve the evaporated sample in 100 μL 0.1% (v/v) formic acid (aqueous) using a bath sonicator (Elmasonic S40H, Elma, DE) for 5 min at room temperature.
- b. Use Micro Spin C-18 Columns (Harvard Apparatus, USA).
- c. Wash the C-18 columns with 200 μL 100% (v/v) acetonitrile with 0.1% (v/v) formic acid (aqueous)
- d. Centrifuge at 100 $\times g$ for 3 min at room temperature (Centrifuge 5424, Eppendorf)
- e. Repeat the wash steps three times, discard the flow-through from the collection tube.
- f. Wash the columns with 200 μL 0.1% (v/v) formic acid (aqueous).
- g. Centrifuge at 300 $\times g$ for 3 min at room temperature.
- h. Add 200 μL 0.1% (v/v) formic acid and hydrate the column for 15 min at room temperature.
- i. Centrifuge at 300 $\times g$ for 3 min at room temperature and discard the flow-through from the collection tube.
- j. Check the pH of the sample.

Note: The sample must be acidic.

- k. Load the sample onto the column and centrifuge at 500 $\times g$ for 3 min at room temperature

- l. Wash the column three times with 200 μ L 0.1% (v/v) formic acid (aqueous) followed by centrifugation at 500 \times g for 3 min at room temperature.
- m. Transfer the column to a new collection tube prior to peptide elution.
- n. Add 200 μ L 20% (v/v) acetonitrile with 0.1% (v/v) formic acid and centrifuge at 500 \times g for 3 min at room temperature.
- o. Add 200 μ L 40% (v/v) acetonitrile with 0.1% (v/v) formic acid and centrifuge at 500 \times g for 3 min at room temperature.
- p. Add 200 μ L 60% (v/v) acetonitrile with 0.1% (v/v) formic acid and centrifuge at 500 \times g for 3 min at room temperature.
- q. Transfer all fractions containing the eluted peptides to new eppendorf tubes (Axygen).
- r. Dry the samples by evaporation in a SpeedVac (Savant) with thermostat set to 37°C until the liquid has completely evaporated.
- s. Store the sample at -20°C (short-time period to analysis) or at -80°C (long time period to analysis).

Note: All solvents should be HPLC grade. Never use plastic pipette tips when transferring acids >2% concentration, use glass pipettes or Hamilton syringes. Prepare solutions fresh in glass bottles.

SWATH-MS

⌚ Timing: ~1 week

The label-free quantitative comparison of proteomes is performed by a method termed Sequential Windowed Acquisition of all Theoretical Spectra (SWATH) (Gillet et al., 2012). The SWATH method has been described elsewhere (Huang et al., 2015; Rosenberger et al., 2014). Using SWATH, the mass spectrometer cycles through 400–1100 Th precursor mass ranges, following a scheme of precursor isolation windows. In this data-independent mode, all product ion spectra are obtained in a systemic and unbiased fashion (Ludwig et al., 2018). Following, SWATH mass spectrometry, data are extracted to provide quantitative information on proteins expressed in two different states – in this case, neurons expressing and lacking CHIP protein. In order to extract such data, we first prepared a spectral library by measurement of the same sample set but in a data-dependent manner (DDA/shotgun proteomics) using the same liquid chromatography conditions.

19. Peptide sample dissolution (evaporated samples). Process up to 100 samples per batch
 - a. Add 100 μ L of 5% (v/v) acetonitrile (ACN) and 0.05% (v/v) trifluoroacetic acid (TFA) (to de-salted tryptic peptides).
 - b. Vortex for 30 s and sonicate using a sonicating waterbath (Electrosonic, S40H) for 5 min.
 - c. Centrifuge for 10 min at 10,000 g.
 - d. Transfer the supernatant into the clear vial (Axygen® 1.5 mL MaxyClear Snaplock Microcentrifuge Tube, MCT-150-C, Corning).
20. Sample quality control
 - a. Determine the absorbance of the dissolved peptide samples at 220 nm and 280 nm using a Nano-drop (Thermo, MA, USA).
 - b. Inject 1 μ L of the most concentrated and the least concentrated samples into the LC-MS/MS system and measure in a data-dependent (DDA) mode.
 - c. Intensities and profiles of total ion currents are checked if they resemble profiles of the cell line peptide digest and if any contaminant is present. Additionally the number of identifications is determined (should be at least 1,000 proteins identified FDR<1%).
 - d. Sample injection volumes are set to reach the maximum allowed full mass spectrometric intensity (1.0e9 AU) utilizing absorbance values at 220 nm and 280 nm measured by NanoDrop (2000) (Thermo, MA, USA). Next, use these absorbance values to dilute the samples in order to load approximately 2 μ g of peptides in a column injection volume of 3.7 μ L.

21. Liquid chromatography separation and data dependent acquisition to generate an in-house spectral library
 - a. Pool replicates from knockout and wild-type cell lines equimolarly to prepare a spectral library of pooled samples.
 - b. Inject approximately 2 μg of pooled peptides onto a Eksigent Ekspert nanoLC 400 (AB-SCIEX, Canada) connected to a TripleTOF 5600+ mass spectrometer (AB-SCIEX, Canada).
 - c. Pre-concentrate the sample on a cartridge trap column (300 μm i.d. \times 5 mm) packed with C18 PepMap100 sorbent with 5 μm particle size (Thermo Scientific, MA, USA) using a mobile phase composed of 0.05% trifluoroacetic acid (TFA) in 2% (v/v) ACN.
 - d. Separate pre-concentrated peptides analytically on a capillary column (75 μm i.d. \times 500 mm) packed with C18 PepMap100 sorbent, 2 μm particle size (Thermo Fisher Scientific, MA, USA).
 - i. Mobile phase A is composed of 0.1% (v/v) formic acid (FA) in water while mobile phase B is composed of 0.1% (v/v) FA in ACN.
 - ii. The analytical gradient starts from 2% B, the proportion of mobile phase B increases linearly up to 40% B in 120 min, with a flow rate of 300 nl/min.
 - iii. The analytes are ionized by a nano-electrospray ion source, where the temperature and flow rate of the drying gas is set to 150°C and 12 psi.
 - iv. Voltage at the capillary emitter is 2.65 kV.
 - e. Measure the spectral library by the DDA method with a precursor range from 400 Th up to 1250 Th and a product ion range from 200 Th up to 1600 Th. Set cycle time to 2.3 s and during each cycle the top 20 most intensive precursor ions are fragmented. Set the precursor exclusion time to 12 s.
 - f. Search data derived from DDA against a Homo sapiens reference database (Database: Uniprot+TrEmbl, 2016_02, 70005 entries + decoys) in ProteinPilot 4.5 (AB-SCIEX, Canada). Set trypsin as the protease and carbamidomethyl moiety must be defined as a fixed modification. Set the tolerated mass error in MS1 and MS2 to predefined TripleTOF 5600 settings. No emphasis to a particular variable modification should be given. Perform the FDR calculation by searching the DDA MS/MS data against the decoy entries.

Note: SWATH data could be used to generate a spectral library or spectral libraries could be downloaded from public repositories.

22. SWATH data acquisition
 - a. Acquire SWATH data by injecting approximately 2 μg of three biological replicates of peptide samples from knockout and wild type cell line each measured in technical triplicate.
 - b. Perform liquid chromatography under completely identical chromatography conditions as described for the spectral library above.
 - c. Acquire SWATH data in high sensitivity positive mode. The precursor range is set from 400 Th up to 1200 Th and it is divided into 67 precursor SWATH windows with a 12 Th width and 1 Th overlap. Accumulation per SWATH window is set to 50.9 ms resulting in a 3.5 s cycle time. Product ions are scanned in a range from 360 Th to 1360 Th. Rolling collision energy setting with 15 mV collision energy spread is used.

Note: Biological duplicates are considered a minimal for each condition (and controls) studied if the results are followed up by validation. Immunoblot analysis, 2D-gel electrophoresis, activity assays or quantitative immunofluorescence approaches can all be used to confirm changes in steady state protein levels, post-translational status or catalytic activity, for example, as appropriate. Biological Triplicates are generally thought to be minimal if no validation is performed.

Analysis of proteomics

© Timing: ~2 weeks

Quantitative proteomic data are extracted from the SWATH-derived spectral data. This allows protein quantities to be quantified and statistically compared between conditions (CHIP-expressing cortical neurons with the knockout cells) resulting in protein fold-changes and their corresponding p-values. Bioinformatic analysis are undertaken to identify significantly associated Gene Ontology (GO) terms (biological processes, molecular functions and cellular components) and these are graphically represented using Database for Annotation, Visualization and Integrate Discovery (DAVID). Ingenuity Pathway Analysis (IPA) is used for pathway analysis. Together these tools contribute to the identification of pathways predicted to be altered that may be driven by proteomic differences between the CHIP isogenic cortical neurons. This aids in the identification of putative targets that may be directly or indirectly affected by the expression of functional CHIP, shaping subsequent investigation and validation screens.

23. SWATH quantitation of proteins in the spectral library

- a. Include a maximum of 4 peptides per protein (if detectable) and 6 product ions per peptide in the spectral library and were analyzed using the SWATH™ Acquisition MicroApp 1.0 plugin applied to PeakView 1.2.0.3 (AB-SCIEX, Canada).
- b. Determine the retention time window based on retention time variability in the dataset. Set retention time extraction window width to ± 4 min around the expected retention time.
- c. Set product ion chromatogram extraction mass accuracy as ± 0.05 Da around the expected product ion mass.
- d. Use extracted product ion chromatograms to determine peak areas for each transition in MarkerView 1.2.1.1. (AB-SCIEX, Canada) software.
- e. Use extracted transition intensities to calculate protein and peptide levels in MarkerView software.
- f. Perform a t-test on quantitative data from wild type and knockout cell lines to determine significantly altered steady-state protein levels ($p > 0.01$). Use a Benjamini-Hochberg test to adjust p-values.

Note: A wide palette of SWATH data extraction and analysis software is currently available. Protein quantitation could be performed by an alternative approach using a powerful combination of Skyline or DIANN SWATH data extraction followed by MSSStats statistics.

Bioinformatic analysis of quantitative proteomic data

⌚ Timing: 1 month

24. GO term analysis using GOrilla (<http://cbl-gorilla.cs.technion.ac.il/>):

- a. Convert protein identifiers to gene symbols.
- b. Choose Homo sapiens as the organism of interest.
- c. For the target set, include all proteins with fold changes ≤ 0.67 and ≥ 1.5 .

Note: We selected 'mid-range' stringency criteria in order to optimize the 'capture' of potential components of CHIP regulated processes (e.g., enzymes or other regulatory factors) to aid in the pathway mapping. If we had been interested only in individual CHIP-targets (rather than CHIP regulated processes/pathways) we may have used more stringent criteria i.e., fold change of < 0.5 and > 2.0 .

- d. For the background set, include all proteins with fold changes > 0.67 and < 1.5 .
- e. Search for enriched GO terms related to process, function and cellular component.
- f. For each GO term the following parameters are calculated:
 - i. Enrichment score: $(b/n)/(B/N)$, where N is the total number of genes, B is the number of genes associated with a specific GO term, n is the number of entries in the target set and b is the number of entries in the target set that are associated with a specific GO term.

- ii. Enrichment *p*-value: describes the probability of observing that *b* or more entries in the target set are associated with a given GO term, under the null assumption that all GO term occurrence in a ranked list are equiprobable (Eden et al., 2009).
 - iii. Adjusted *p*-value (*q*-value): this uses the FDR approach and a threshold of 0.05 is considered significant.
25. GO term analysis using DAVID (<https://david.ncifcrf.gov/tools.jsp>):
- a. Convert protein identifiers to gene symbols.
 - b. For the gene list, include all proteins with fold changes ≤ 0.67 and ≥ 1.5 along with their exact fold changes. Select Homo sapiens and "Official_Gene_Symbol" as the identifier.
 - c. Different categories of GO terms (including molecular function and biological process) and pathways (including KEGG) can be derived.
 - d. For each GO term/pathway the following is given:
 - i. Number of entries from the input list that are involved in a term ("count").
 - ii. Gene-enrichment analysis presented as a Modified Fisher Exact *p*-value that ranges from 0 (corresponding to a perfect enrichment) to 1 ("p-value").
 - iii. An adjusted *p*-value using Benjamini ("Benjamini").
26. Graphical representation of GO terms using REduce + Visualize Gene Ontology (REVIGO) software (<http://revigo.irb.hr/>):
- a. Add the GO terms previously identified from the GOrilla software, along with their *p*-values.
 - b. GO terms plotted are color-coded depending on their $\text{Log}_{10}(\textit{p}\text{-value})$. Only significantly enrich GO terms (*q* val > 0.05) are plotted.
27. Pathway analysis prediction using Ingenuity Pathway Analysis (IPA) software (Quiagen)
- Use IPA to predict changes in canonical pathways and potential upstream regulators.
- a. Upload the protein data with fold changes and *p*-values.

Note: When focusing on membrane proteins, for example, these can be sorted using the IPA software.

- b. Use the following cut off: $-\log(\textit{p}\text{-value}) > 1.3$ (which corresponds to *p*-values of <0.05).
- c. For each GO term/pathway a Z-score is calculated which reflects the predicted activation state of the pathway or potential upstream regulator (a positive/negative z-score indicates activation/inhibition). This is calculated based on comparison of the protein data with literature-based molecular networks and regulatory dynamics.

Note: The directionality parameter (i.e., the z-score) and literature-based pathway prediction make the IPA tool unique compared to others that just focus on statistical enrichment in an overlapping set of genes (Krämer et al., 2014).

EXPECTED OUTCOMES

Expected outcomes from cortical differentiation

Differentiation of cortical neurons from iPSC is a highly dynamic process, where cells are subjected to multiple gene expression and morphological changes. During neural induction it is expected that iPSC transform into primary cortical stem and progenitor cells, forming a monolayer of neuroepithelial cells by Day 11 that show a robust and homogenous Pax6 expression (Figure 3A). In parallel, the expression of pluripotency and stem-renewal markers (CDK6, NANOG and OCT4 genes) should decline dramatically by Day 11 and remain low throughout the differentiation (Figure 4A). The expression of other markers of primary cortical stem and progenitor cells (including NES and OTX2) is also expected to increase by Day 11 (Figure 4B).

Later, these primary cortical stem and progenitor cells form rosettes (Figures 5B, 5C, and 5E), from where secondary cortical stem and progenitor cells derive. This has been thoroughly described

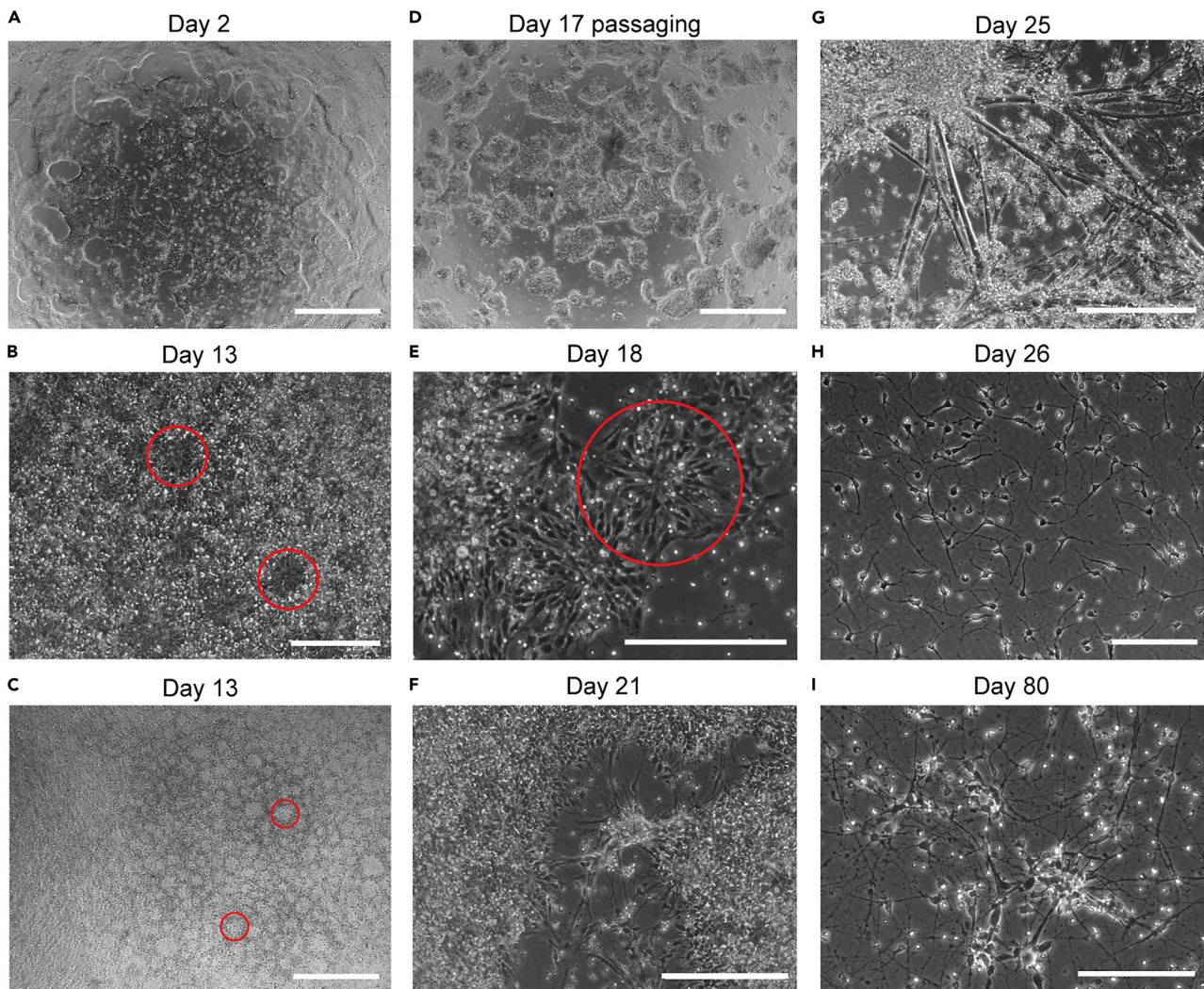


Figure 5. Morphological changes throughout neural induction and cortical neurogenesis

(A) Stem cells are plated at a high plating density.

(B and C) By day 13 of the differentiation neural rosettes are visible (a couple are indicated by the red circles) and consist of primary cortical stem and progenitor cells.

(D) At day 17 cortical progenitor cells are passaged as clumps. The image shows floating cell aggregates subsequent to dissociation from the matrix. (E) Neural rosettes (indicated by the red circle) give rise to secondary progenitor cells. At the center of the rosette these are cycling, while at the periphery some become post-mitotic.

(F and G) These newly-born post-mitotic neurons mature from neural rosettes and form lengthy processes.

(H and I) After passaging neurons as single-cells, neurons increase in complexity in terms of processes and connections with neighboring neurons, forming neural networks. Scale bars, 200 μm (except C and E), 100 μm (E) and 80 μm (C).

elsewhere (Shi et al., 2012a; 2012b). These progenitors are essential to generate functional, post-mitotic neurons of both deep and upper layers. Their expression profile is complex, expressing multiple genes (Figures 4B and 4C, *d11* and *d25*), such as Pax6 and TBR1 (Figure 6).

The expression of markers of early-born deep-layer neurons (*TBR1* and *CTIP2*) is expected to be readily detected around Day 50. Accordingly, an increase in *CTIP2* expression is detected at Day 45 by qRT-PCR (Figure 2C), while monitoring *TBR1* expression using this assay is more challenging. By immunofluorescence imaging, expression of TBR1 and CTIP2 is expected to be

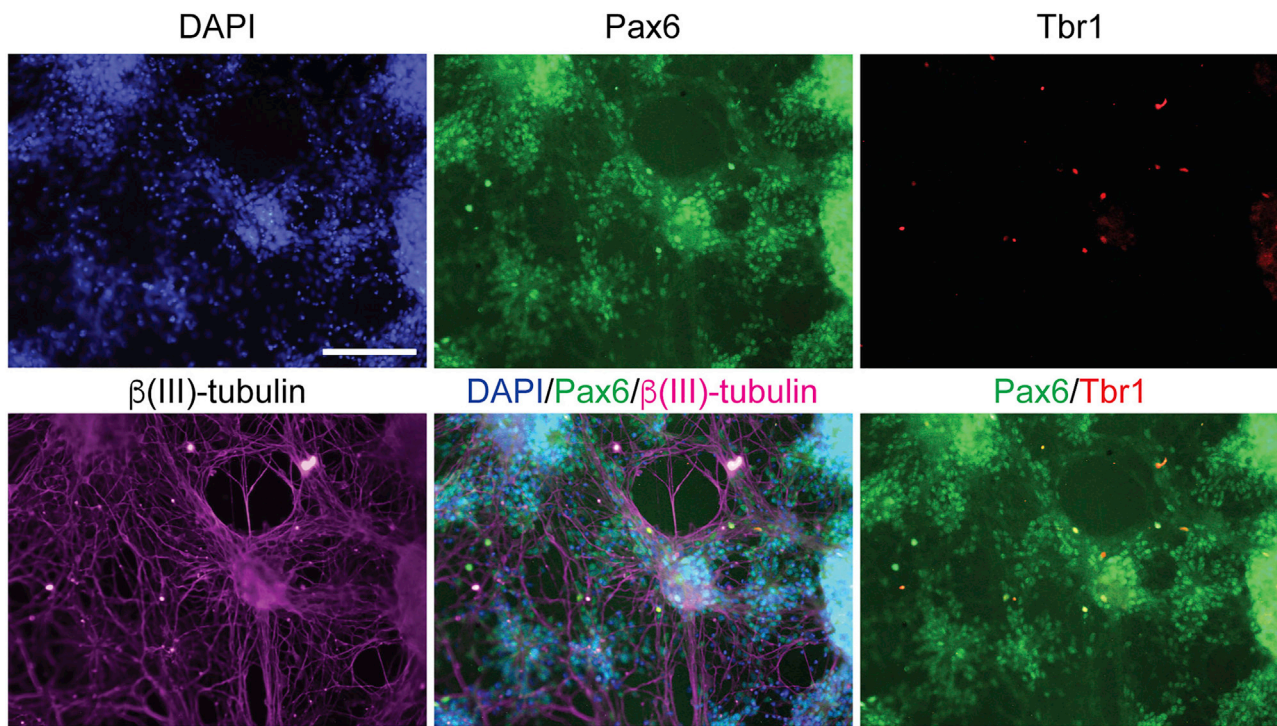


Figure 6. Progenitor cells express markers of cortical neurogenesis

At day 25 of differentiation, progenitor cells are expected to show a heterogeneous but strong pattern of Pax6 staining and some TBR1 staining. β (III)-tubulin is a neuron-specific marker. The projection of processes and the three-dimensional architecture of neuronal networks is evident. Progenitor cells are both found in clumps and as single cells. Scale bar, 100 μ m.

detectable from Day 50 onwards (Figure 7). About 22% and 8% of neurons within a culture are expected to express only TBR1 or CTIP2, respectively, while approximately 38% express both markers (Figure 7C).

With regards to late-born upper-layer neurons, these are expected to be formed in the cultures because throughout the differentiation key markers of this neuron type (*BRN2* and *CUX1*) begin to be expressed (Figure 7A). By qRT-PCR, *BRN2* expression is expected to increase from Day 11 and peak at Day 25 (Figure 2C). *CUX1* expression is expected to be higher between Days 25–80 (Figure 2C). By Day 80 neurons are expected to express *Cux1* and this should be detectable by immunofluorescence imaging.

Therefore, by the end of the differentiation protocol (Day 80), co-cultures of mature deep-layer and upper-layer neurons are expected to be achieved, adequately modeling cerebral neural networks (Figures 7A and 5I). Furthermore, this protocol is expected to result in reproducible differentiation experiments and almost pure cultures.

Expected outcomes from SWATH-MS data in general

SWATH-MS analysis provides a set of identified proteins, each with a relative ratio between two conditions (in this case CHIP WT vs KO), described as fold changes, and a p-value that is derived from a statistical t-test. This aids in the investigation of protein targets with significantly altered steady-state levels across biological conditions, although statistical significance does not necessarily translate into biological significance and further experimental validations are required. Furthermore, proteomic analysis contributes to validating the genetic editing of the cell lines under investigation (please refer to (Dias et al., 2021)).

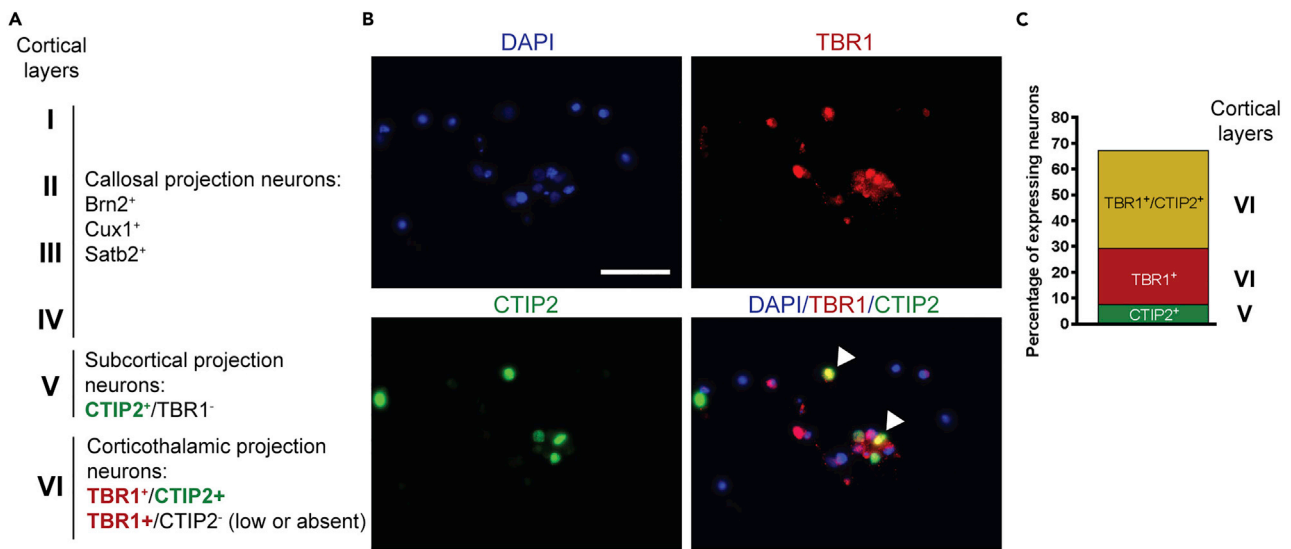


Figure 7. Deep-layer cortical neurons

(A) The cortex is composed of 6 layers, each characterized by a specific expression profile.

(B) At day 50 of the cortical differentiation protocol, CTIP2⁺/TBR1⁻, CTIP2⁺/TBR1⁺ and CTIP2⁻/TBR1⁺ neurons can be identified in the neural cultures (white arrowheads mark the latter population). Scale bar, 100 μ m.

(C) Quantification of these deep-layer neuronal markers demonstrates that layers V and VI are present at day 50.

P-values are computed based on the assumption that there is no difference in relative abundance of a particular protein between biological conditions (the null hypothesis). Thus, it represents the probability of observing this protein data if no real difference existed (Yu et al., 2016). A small p-value indicates that the difference in relative abundance is unlikely to be due to random sampling (Nonlinear_Dynamics, n.d.). Furthermore, when the p-value is below the significance threshold ($p < 0.01$) there is statistical evidence to reject the null hypothesis and, instead, argue that the difference is significant (simplistically, the difference is “statistically real”) (Yu et al., 2016). Next, depending on the size difference of relative abundance (the fold change), the protein may be of interest.

The p-value is influenced by the variance in the group abundance (i.e., the range of the differences in protein abundance between groups) (Nonlinear_Dynamics, n.d.). The technical replicates (that reflect the accuracy of measurement) and biological replicates contribute to this variance. A small variance “facilitates” small differences in relative abundance to have significance ($p < 0.01$); statistical significance may not be reached if the variance is big (Kammers et al., 2015; Nonlinear_Dynamics, n.d.). Therefore, protein data is more robust when intra- and inter-group variability is limited, increasing the likelihood of identifying proteins of interest. However, proteins with p-values that have not reached statistical significant should not be discarded prior to further consideration heavily driven by a biological questioning. For example, comparisons where in one biological condition a known protein is of low abundance (such as CHIP in the CHIP KO neurons when compared to CHIP WT neurons), if CHIP is detected in some replicates but not others this might increase the variance, making statistically significant p-value less likely (refer to (Dias et al., 2021)). This becomes more pronounced when the size of the difference (the fold change) is smaller. Nonetheless, biologically, this variance favors a difference in relative abundance of CHIP between CHIP KO and WT neurons.

When the fold change of a particular protein is < 0.67 and > 1.5 , this under- or over-representation of the protein in one of the conditions being compared may be of biological interest. Changes in a family of proteins or proteins that belong to the same signaling cascade are of particular interest, as these are associated with an increased likelihood of pathway changes and a cellular phenotype. Thus, GO term and pathway analyses aid in the search for potential biologically relevant targets.

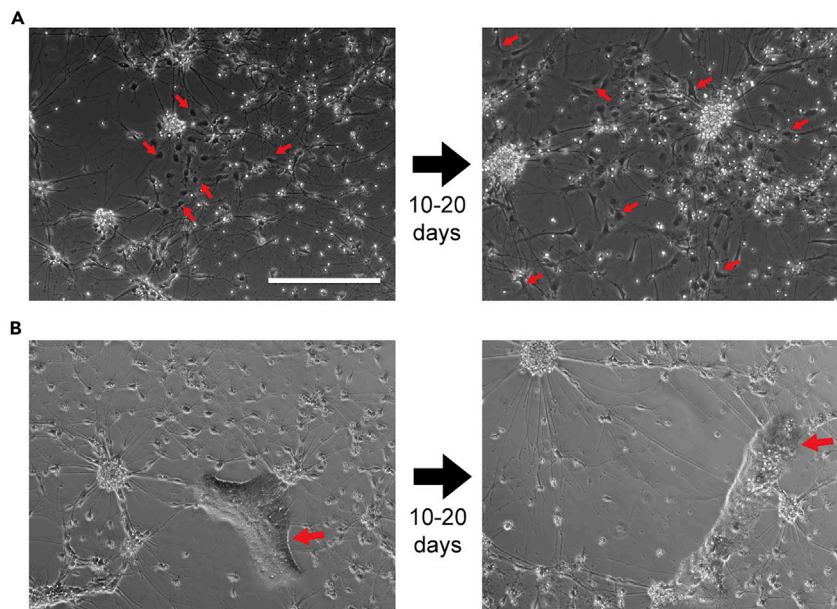


Figure 8. Contaminants of neural cultures

(A and B) In a minority of neural cultures, cells with a characteristic flat morphology (indicated by the red arrows) can arise throughout the differentiation process (typically around day 50) and proliferate rapidly (becoming more numerous and widespread, as in example A, or occupying a greater area, as in B, over time). Scale bar, 200 μm .

Expected outcomes from proteomic analysis of cortical neurons with differential CHIP expression

Given that the isogenic set of cell lines under investigation are expected to be predominantly cortical neurons, it is assumed that a substantial fraction of the proteome identified and quantified will be similar. This would include proteins involved in the structure and function of neurons. Widespread changes in these proteins would argue against a homogenous set of cortical cultures, compromising the study.

Furthermore, when modeling early disease, stress proteins (e.g., heat shock proteins) should not be enhanced. This low basal stress level is also found in CHIP KO cells, despite the role of CHIP in proteotoxic stress – a function that has been extensively documented but poorly studied in earlier publications (discussed in (Dias et al., 2021) and (Ball et al., 2016)). As demonstrated in (Dias et al., 2021), in a model of early disease (AST23 iPSC line) CHIP is expected to perform functions associated with maintaining health (i.e., regulation of a focused cohort of proteins) rather than its chaperone-dependent functions (regulating multiple client proteins based on their state of ‘foldedness’), which are hypothesized to be secondary and come into play in the context of proteotoxic stress. Therefore, CHIP KO cells under basal conditions are expected to produce a small subset of under- and over-represented proteins, rather than global changes in the proteome. Within this subset of CHIP-mediated changes were proteins involved in cytoskeletal and membrane-related processes (Dias et al., 2021). One might also find other established protein targets of CHIP to be altered from the SWATH-MS data (e.g., IRF-1 (Narayan et al., 2011), Smad1/5 (Wang et al., 2011), the insulin receptor (Tawo et al., 2017) and Dax (McDonough et al., 2009) Cystic fibrosis transmembrane conductance regulator (Meacham et al., 2001) and the glucocorticoid receptor (Connell et al., 2001)) although this may be challenging due to their rapid turnover and, in some cases, K63-mediated ubiquitination (that does not cause changes in steady-state protein levels).

We anticipate that, despite its inherent limitations, depletion of CHIP expression is likely to influence the cellular proteome in a more subtle manner than if CHIP were to be overexpressed – a common

strategy when investigating the protein targets of an enzyme E3-ligase. Overexpression of CHIP is expected to interfere with tightly regulated enzymatic housekeeping pathways (including ubiquitination and protein quality control mechanisms) most likely through inappropriate interactions with other TPR-containing proteins.

LIMITATIONS

Limitation of cortical differentiation

Although this cortical differentiation protocol is successful in the generation of early-born deep-layer and late-born upper-layer excitatory neurons, it is unclear whether all classes of cortical stem and progenitor cells are generated during the course of differentiation though this is key to achieve the full spectrum of neurons (Shi et al., 2012a). While this protocol generates glutamatergic projection neurons, it is not expected to result in GABAergic interneurons (because these are generated outside of the cortex and migrate in during development, rather than being derived from cortical stem and progenitor cells) (Shi et al., 2012b). This could be a limitation when modeling the cerebral cortex because the cortex mainly consists of excitatory projection neurons (making up about 80%) and GABAergic interneurons. The protocol is therefore unsuitable when GABAergic interneurons are the focus of the experiment.

Clonal variability is another possible drawback of the cortical differentiation protocol. Similar to other differentiation protocols using iPSC as the starting material, clonal variation may affect the outcome. It can arise from possible epigenetic-driven bias that reprogrammed iPSC have towards their source cell lineage. However, the iPSC used to optimize our differentiation protocol were reprogrammed from fibroblasts and others have also been able to differentiate iPSC to neuronal lineages (Shi et al., 2012a, 2012b; Chambers et al., 2009), arguing against the biological significance of this possible limitation. Furthermore, seeing that the differentiation protocol *per se* drives pronounced and constantly-changing epigenetic-changes in cultured cells, clonal variability may result in slight differences in phenotypic outcome during or in the aftermath of differentiation, particularly across different cell lines (Devine et al., 2011; Vitale et al., 2012; Liang and Zhang, 2013). This may be a challenge when looking to differentiate an isogenic set of iPSC lines, where comparable differentiation potential is a prerequisite (Vitale et al., 2012); despite being potentially challenging, this is possible, as demonstrated in (Dias et al., 2021)). Likewise, others have reported reproducible results in neural induction and cortical neurogenesis across different cell lines (Chambers et al., 2009; Shi et al., 2012a, 2012b), arguing that the robustness of the protocol surpasses clonal variability.

Technical limitations of SWATH-MS

Due to technical limitations of mass spectrometer and requirements for proper quantitation (at least 10 datapoints per chromatographic peak) the SWATH method cycle time must be slightly longer than usual (up to 3.5 s). Therefore, a compromise between the method sensitivity and cycle time must be optimized by a mass spectrometrists, at the expense of a limited precursor mass range investigated ($m/z = 400\text{--}1100$ Th). Narrowing the precursor mass range decreases the number of scan events that take place during each cycle. Precursor ions not covered by the selected mass range are not analyzed. This can be counteracted by increasing the SWATH isolation window width. In our case we used a 12 Th window width, producing a method with 67 SWATH precursor windows (scan events) with sufficient sensitivity within the 3.5 s cycle time. However, wider isolation windows may lead to co-isolation of multiple precursors, complicating the interpretation of MS/MS spectra during subsequent data analysis. Moreover, in a relatively rare number of cases some of the co-isolated precursor ions might provide a portion of overlapping product ions, this usually has a fatal impact on the accuracy of the quantitation. In addition, some precursor ions may be missed by SWATH-MS because their concentration in the sample is under the limit of detection (LOD) and limit of quantification (LOQ) of the mass spectrometer. Another inherent limitation of proteomic analysis arises from sample preparation. During digestion, complete generation of tryptic peptides may be achieved and some miscleaved peptides may result. Both semi-tryptic and miscleaved peptides could be lost during a DDA database search when the search engine is set to look for tryptic peptides

(when trypsin was used for sample preparation). Also, miscleaved peptides might be retained on the 10 KDa filter of the Microcon, resulting in them being lost from the sample prior to analysis by SWATH-MS.

Limitations of MS-based proteomics data

Processing of SWATH-MS data results in a series of under- and over-represented proteins. However, caution is required during the interpretation, as protein data reflect differences in the abundance of ionized peptides and not necessarily differential protein steady-state levels. The abundance of ionized peptides can be influenced by a combination of factors, including post-translational modifications, intermolecular interactions and compatibility with MS detection (ionization and fragmentation potentials) (Bassani-Sternberg et al., 2015). Therefore, fold changes tend to be an underestimation of the real value, particularly for ions present at high concentrations (Huang et al., 2015). Quantification accuracy is greater when the fold changes are close to 1. It decreases substantially when the protein abundances of the samples to be compared differs greatly (e.g., by more than 4-fold) (Huang et al., 2015; Liao et al., 2011), although large changes can be detected (Burande et al., 2009). All these reasons highlight the need for MS-derived proteomics data to be validated using other methods. However, for most biological studies, the exact fold change is not necessary to determine its biological significance (Huang et al., 2015). For example, if chaperones or E3-ligases have a fold change of 2 this may still result in pronounced cellular effects due to their wide interactome.

TROUBLESHOOTING

Problem 1

Cells dying or forming big aggregates at the periphery of the well (“neural induction”, major step 2 and “cortical neurogenesis”, major steps 5–13).

Potential solution

Poor well coating will result in this phenomenon. Increase the volume of coating solution that is added when coating and ensure that the plates are sealed with parafilm, to reduce evaporation, and are incubated on a flat surface.

Problem 2

Poor neural induction (“neural induction”, major step 3) – illustrated in Figure 3B.

Potential solution

Achieving a uniform monolayer of neuroepithelial cells with strong Pax6 expression is dependent on a high cell density at the early stages of neural induction and potent SMAD inhibition. Adjust the plating density accordingly to achieve 70% confluency after two days. Check the concentration and activity of the SMAD signaling inhibitors used (SB431542 and LDN). Once resuspended and aliquots are made, stocks stored at -20°C should only be used for up to 6 months, should be protected from light and not subjected to freeze-thaw cycles. Only add the inhibitors to the differentiation media prior to use and protect from light.

Problem 3

Struggling to lift cells with Collagenase Type IV (“cortical neurogenesis”, major steps 5 and 8).

Potential solution

Collagenase Type IV is a mild dissociation reagent that favors passaging as clumps rather than single cells. If cells are not lifting after 30 min at 250 U/mL, increase the incubation time and/or concentration of collagenase or supplement with extra calcium (add 3 mM CaCl_2). The Collagenase Type IV stock should not undergo freeze-thaw cycles and should be protected from light. Alternatively, another type of collagenase or dissociating enzyme (e.g., dispase) can be used.

Problem 4

Difficulties in obtaining the correct size of cell aggregates (“[cortical neurogenesis](#)”, major steps 5 and 8).

Potential solution

Aggregates of 300–500 cells are ideal although some heterogeneity in clump size is expected and does not negatively affect the outcome of the differentiation ([Figure 5D](#)). Smaller clumps are associated with less neurogenesis (yielding fewer cortical stem and progenitor cells), while bigger clumps result in spontaneous differentiation ([Shi et al., 2012a](#)).

Problem 5

Neurons are dissociating as a monolayer or as single-cells (“[cortical neurogenesis](#)”, major steps 5–13)

Potential solution

During the course of the differentiation, both the formation of a tight monolayer of neurons with multiple projections connecting cells and the frequent exchanges of media can cause the dissociation of the neuronal monolayer from the well bottom. To prevent this lifting, keep more residual volume in the well when exchanging media, take care when adding and removing media and dispense media to the wall of the wells. Keep mechanical stress to a minimum. In addition, wells can be pre-treated with poly-L-ornithine to promote a greater attachment of cells with the well bottom.

To prevent the dissociation of single-cells, other have suggested to use fresh B27 supplement and differentiation media ([Shi et al., 2012a](#)).

Problem 6

No neural rosettes are formed (“[neural induction](#)”, major steps 2 and 3 and “[cortical neurogenesis](#)”, major steps 5–10)

Potential solution

Although this was not experienced during the optimization of our cortical differentiation protocol, one of the protocols on which it is based ([Shi et al., 2012a](#)) suggested that keeping neuroepithelial clumps above 300 cells and dissociating the neuroepithelium less harshly are potential solutions. This could be done by triturating less times in major steps 5 and 8 (under “[cortical neurogenesis](#)”).

Problem 7

Existence of contaminating cells with a distinctive flat morphology with the neuronal cultures ([Figure 8](#)). These can be observed in about 10% of the differentiating cultures and they tend to emerge late in the differentiation process (being observed around day 45). They do not express neuron-specific markers such as β (III)-tubulin. (“[cortical neurogenesis](#)”, major steps 5–14)

Potential solution

These cells failed to differentiate properly and are described as fibroblast-like cells ([Park et al., 2012](#)). Due to their proliferative nature, when present in cultures they will eventually dominate, compromising the purity and success of the cortical differentiation. Although anti-proliferative treatments can be tested to prevent the spread of these proliferating cells, these tend to be unsuccessful and compromise the use of the cultures for proteomics (due to possible side-effects of the treatment). The best solution is to halt the differentiation of these cultures and use them for single-cell focused assay (e.g., immunofluorescence assay, where the area with contaminating cells can be discarded). Alternatively, these cultures should be discarded.

Problem 8

During FASP, the sample containing tryptic peptides becomes contaminated with uncleaved peptides due to disruption of the filter (“[sample preparation for SWATH-MS](#)”, major step 17).

Potential solution

Avoid touching the filter with the pipette tip.

Problem 9

Solution remains on the filter unit after the centrifugation step ("sample preparation for SWATH-MS", major step 17).

Potential solution

Repeat the centrifugation step to ensure all the solution has passed the filter unit.

RESOURCE AVAILABILITY

Lead contact

Further information and requests for resources and reagents should be directed to and will be fulfilled by the lead contact, Kathryn Ball (Kathryn.ball@ed.ac.uk).

Materials availability

The isogenic CHIP^{-/-} and CHIP^{+/+} iPSC lines are available.

Data and code availability

Please refer to ([Dias et al., 2021](#)) for datasets generated from the application of this protocol to the study of CHIP function.

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AUTHOR CONTRIBUTIONS

K.L.B, T.K., and C.D. designed the study. C.D. developed the protocol and carried out the experiments. E.N. designed the CRISPR/Cas9-editing construct. J.F. and L.H. developed the proteomics analysis protocol based on mass spectrometry and performed experiments. C.D., J.F., L.H., and K.L.B. wrote the manuscript.

DECLARATION OF INTERESTS

The authors declare no competing interests.

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