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Supplemental Information

Human Cerebral Organoids Reveal Early Spatiotemporal Dynamics and Pharmacological Responses of UBE3A

Dilara Sen, Alexis Voulgaropoulos, Zuzana Drobna, and Albert J. Keung

SUPPLEMENTAL FIGURES AND LEGENDS

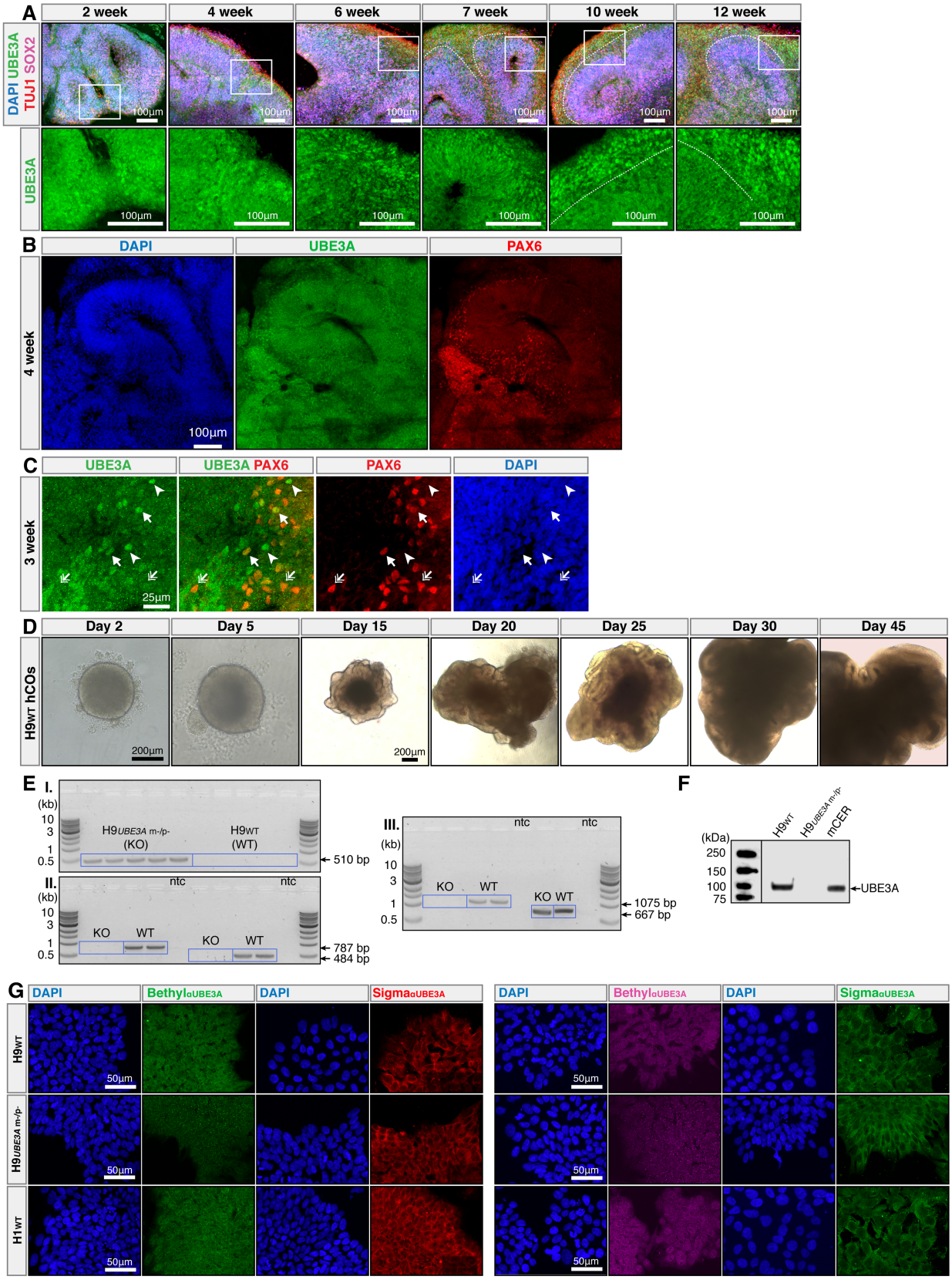

Figure S1. Additional time-points in hCO development revealing the weak to strong nuclear UBE3A transition in neurons and antibody validation experiments, Related to Figure1.

(A) Immunostaining time course of neurotypical H9-derived hCO neurodevelopment. Dotted white lines delineate boundaries between TUJ1+ and SOX2+ cells. (B) Low magnification images showing PAX6 progenitor organization (C) Strong (arrows) and weak (double arrows) nuclear UBE3A in PAX6+ cells. Strong nuclear UBE3A in PAX6-/weak cells (arrow heads). (D) Macroscopic brightfield images showing overall H9 hCO development. (F-G) Validation of the H9$_{UBE3A\text{m-/p-}}$ cell line. (E) PCR using genomic DNA as template. Primers targeting the junction at the deletion site (I), primers targeting intronic regions in the deleted region (II, III-left), primers targeting intronic *TBP* (III-right). (F) Immunoblot analysis of UBE3A in whole cell lysates of H9$_{WT}$, H9$_{UBE3A\text{m-/p-}}$ hESCs and cerebellum of C57BL/6 mouse (mCER). (G) Immunostaining of H9$_{WT}$, H9$_{UBE3A\text{m-/p-}}$, H1 hESCs and hiPSCs with two different UBE3A antibodies (Bethyl Laboratories A300-351A and Sigma Aldrich SAB1404508) and with two different secondary antibody sets (Right panel: rabbit488 and mouse 546. Left panel: rabbit647 and mouse488). SAB1404508 showed higher background staining in human pluripotent cells compared to mouse sections (data not shown).

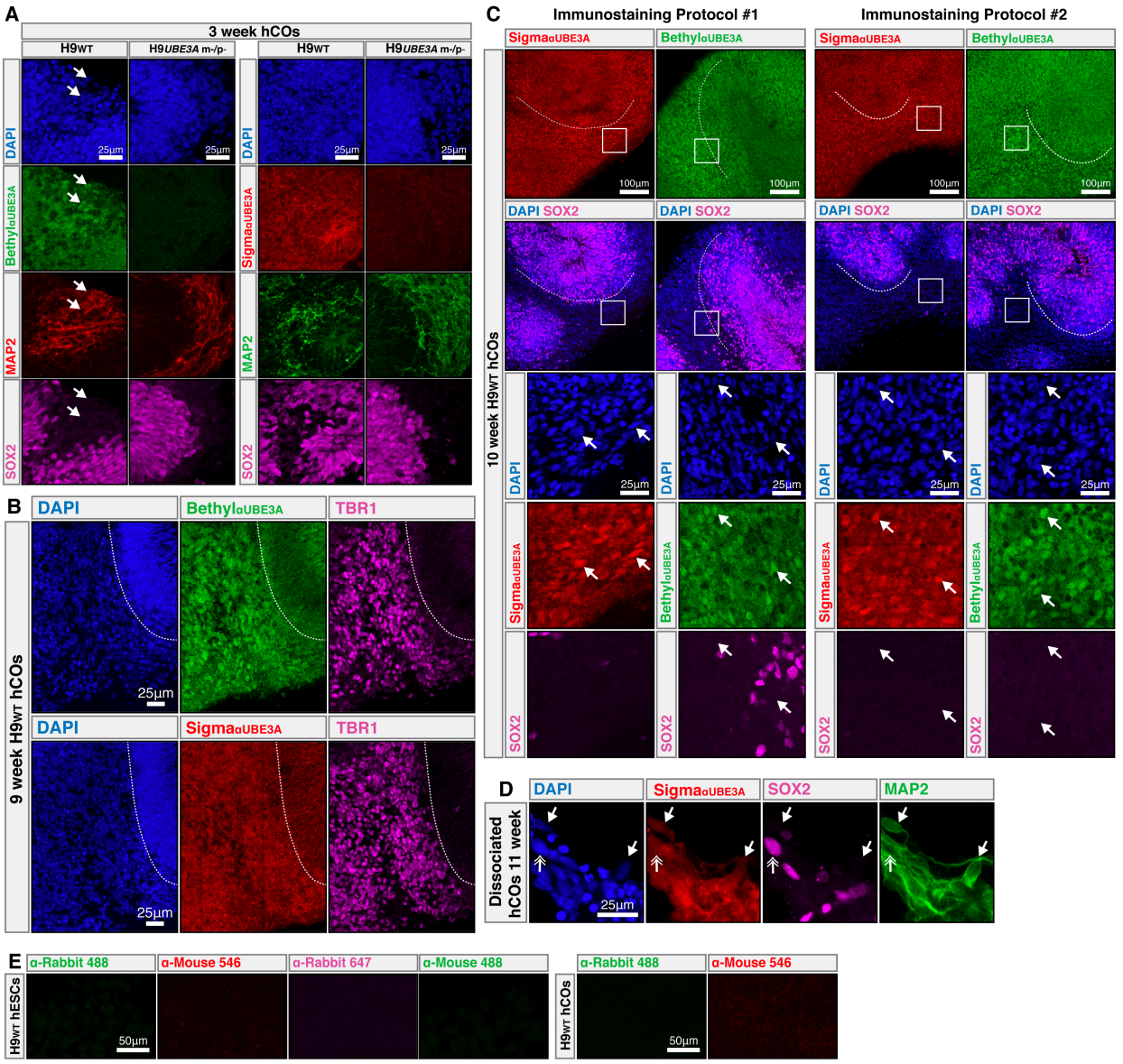

Figure S2. Additional UBE3A antibody and immunostaining protocol validation, Related to Figure 1.

(A) H9WT and H9*UBE3A*m-/p- derived organoids immunostained with two different UBE3A antibodies (Bethyl Laboratories A300-351A and Sigma Aldrich SAB1404508). Nuclear UBE3A in neurons (arrows). (B) Immunostaining images of 9 week H9WT hCOs comparing Sigma and Bethyl UBE3A antibody staining patterns. Dotted white lines delineate the boundaries of TBR1+ neuronal regions. (C) Comparison of two distinct but similar immunostaining protocols. Protocol 1: Keung Lab protocol described in experimental procedures. Protocol 2: Described previously by Judson and colleagues (Judson et al., 2014). Dotted white lines delineate the boundaries of SOX2+ progenitor regions. Nuclear UBE3A in potentially neuronal regions (arrows). (D) 2D immunostaining of dissociated H9 hCOs with Sigma UBE3A antibody (SAB1404508). Nuclear UBE3A in neurons (arrows), weaker diffuse staining in progenitors (double arrows). (E) H9 hESCs and H9 hCOs immunostained with secondary antibodies only.

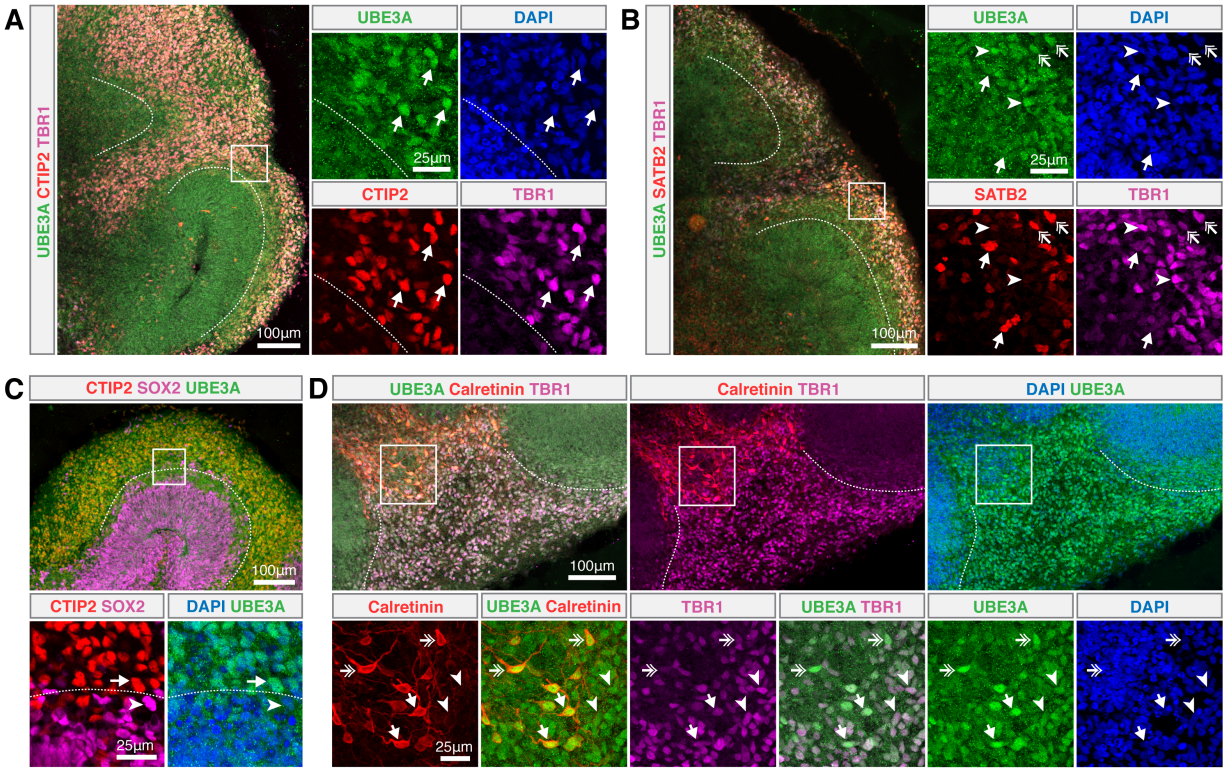

Figure S3. UBE3A in cerebral cortex-like regions and progenitor zones, Related to Figure2.

(A-D) UBE3A in cortical cells in neurotypical hCOs. (A, arrows) CTIP2, TBR1 and strong nuclear UBE3A co-localize. (B) Strong nuclear UBE3A in TBR1+/SATB2+ (double arrows) and TBR+/SATB2- (arrow heads) cells. The signal from UBE3A in the nuclei is weaker in TBR1-/SATB2+ (arrows) cells. (C) Strong nuclear UBE3A in CTIP2+ neurons (arrow). Weaker nuclear UBE3A in SOX2+ progenitors (arrow head). (D) Strong nuclear UBE3A in TBR1+/Calretinin+ (arrows), TBR1weak/Calretinin+ (double arrows) and TBR+/Calretinin- (arrow heads) cells.

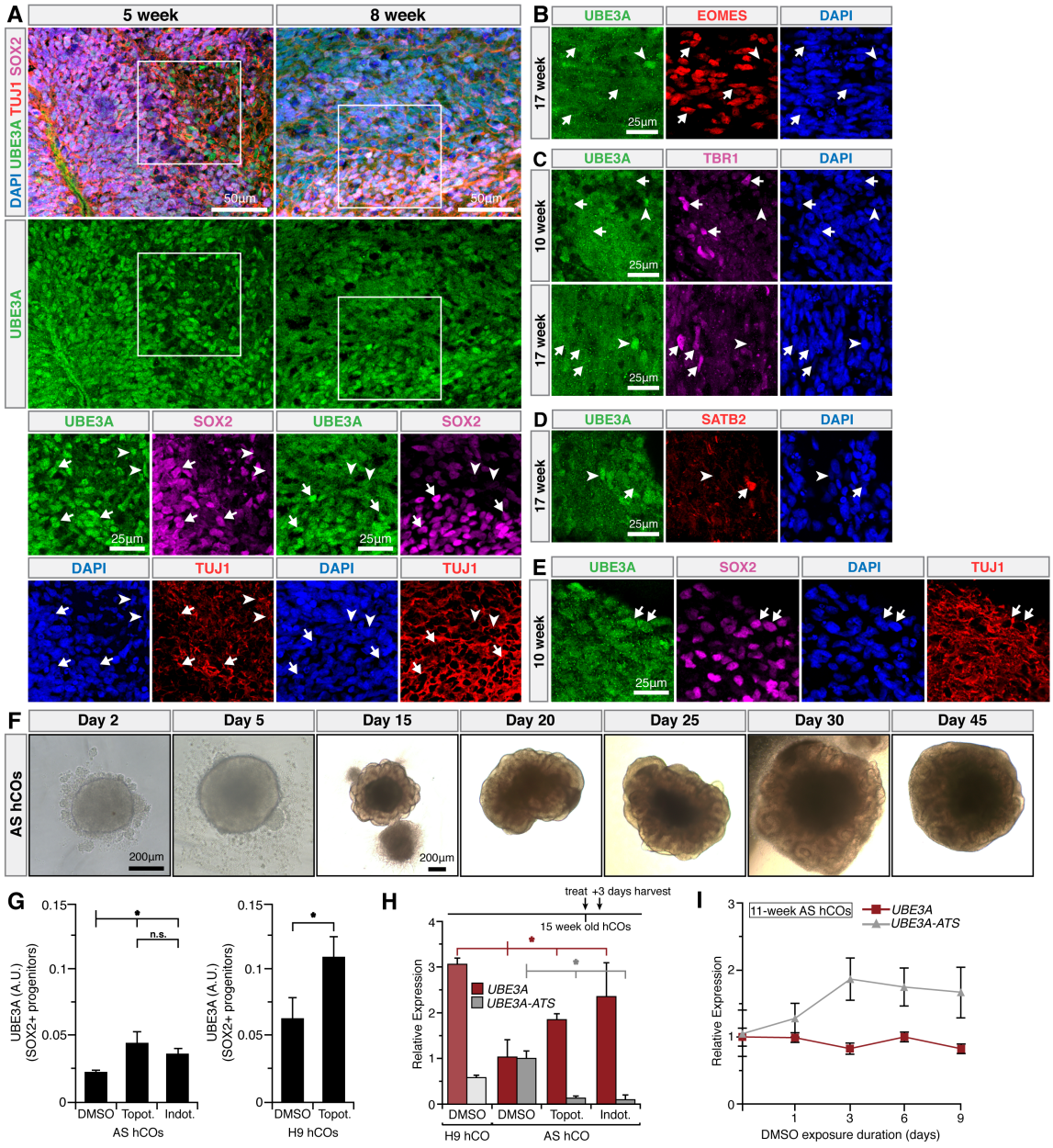

Figure S4. Paternal UBE3A expression dynamics during AS hCO development and transcriptional and functional responses to topoisomerase inhibitors, Related to Figures 3 and 4.

(A) Strong nuclear UBE3A in 5-8 week SOX2+ progenitors (arrows). Strong nuclear UBE3A in TUJ1+/SOX2- neurons at 5 weeks becomes weaker at 8 weeks (arrowheads). (B) Weak nuclear UBE3A in EOMES+ cells (arrows). Strong nuclear UBE3A in some EOMES- cells (arrowhead). (C) Weak nuclear UBE3A in TBR1+ cells (arrows). Strong nuclear UBE3A in some TBR1- cells (arrow heads). (D) Weak nuclear UBE3A in SATB2+ cells (arrow). Strong nuclear UBE3A in some SATB2- cells (arrowhead). (E) Strong nuclear UBE3A in SOX2+/TUJ1+ immature neurons (arrows). (F) Macroscopic brightfield images showing overall AS hCO development. (G) Immunostaining quantification of UBE3A in SOX2+ cells after treatment with topoisomerase inhibitors. (H) *UBE3A* and *UBE3A-ATS* expression in 15 week AS hCOs 3 days after a single drug treatment. (I) *UBE3A* and *UBE3A-ATS* expression after 1-9-days of continuous vehicle (DMSO) treatment in 11 week AS hCOs. Statistics; for (G-I) *P<0.05, n.s. not significant, full tick marks compared to half tick marks by one-way ANOVA with Tukey-Kramer post hoc, n=3 independent experiments with 3-5 organoids in each replicate, error bars = 95% confidence intervals. A.U. arbitrary fluorescence units.

SUPPLEMENTAL EXPERIMENTAL METHODS AND MATERIALS

Histology and Immunofluorescence

Tissues were fixed in 4% paraformaldehyde for 15min at 4 °C followed by 3 x 10 minute PBS washes. Tissues were allowed to sink in 30 % sucrose overnight and then embedded in 10 % gelatin/7.5 % sucrose. Embedded tissues were frozen in an isopentane bath between −50 and −30 °C and stored at -80 °C. Frozen blocks were cryosectioned to 30-µm. For immunohistochemistry, sections were blocked and permeabilized in 0.3 % Triton X-100 and 5 % normal donkey serum in PBS. Sections were incubated with primary antibodies in 0.3 % Triton X-100, 5 % normal donkey serum in PBS overnight at 4 °C in a humidity chamber. Sections were then incubated with secondary antibodies in 0.3 % Triton X-100, 5 % normal donkey serum in PBS for 2h at RT, and nuclei were stained with DAPI (Invitrogen). Slides were mounted using ProLong Antifade Diamond (Thermo Fisher Scientific). Secondary antibodies used were donkey Alexa Fluor 488, 546 and 647 conjugates (Invitrogen, 1:500).

Antibody validation experiments were conducted using *UBE3A* double knock-out H9 cells ($H9_{UBE3A\ m-/p-}$) and hCOs (Figure S1 and S2). Two different commercially available UBE3A antibodies were tested for immunostaining (Sigma-Aldrich, mouse monoclonal, SAB1404508, referred to as Sigma, and Bethyl Laboratories, rabbit polyclonal, A300-351A, referred to as Bethyl). Our results showed that the Sigma antibody had a stronger background signal in the pluripotent stage compared to the Bethyl antibody (Figure S1G). However, once the cells passed the pluripotent stage, both antibodies performed similarly in terms of capturing important localization changes in the cell types of interest (Figure S2A-D). In addition, a very similar, previously described protocol for UBE3A immunostaining with minimal differences was also tested (Judson et al., 2014). The two protocols did not show significant differences (Figure S2C). Immunostaining experiments in the main figures of this work were carried out using the Bethyl antibody.

Images were taken using a Nikon A1R confocal microscope (Nikon Instruments). High magnification images captured using thin (1.5 µm) optical sectioning. All samples within experiments were processed at the same time, imaged using the same microscope settings, and adjusted identically for quantification purposes. Quantifications were performed manually except for Figures 4C and S4G, where a CellProfiler pipeline automated the identification first of nuclei using DAPI, then DAPI that were positive for each marker protein, and finally the mean background intensity of UBE3A was subtracted from the nuclear UBE3A intensity. For all quantifications, intensities of all channels were maintained equally across all images. For displayed images, individual channels were balanced equally across the entire image. In manual image quantifications, up to 50 cells from 5 different regions in each hCO were analyzed.

Primary antibodies used in immunostaining experiments are listed below.

Antigen	Host	Supplier	Cat. No.	RRID	Dilution
SOX2	Goat	R&D systems	AF2018	AB_355110	1:20
TUJ1	Mouse	Sigma Aldrich	T8578	AB_1841228	1:100
TUJ1	Rabbit	Sigma Aldrich	T2200	AB_262133	1:100
UBE3A	Rabbit	Bethyl Laboratories	A300-351A	AB_185563	1:250
UBE3A	Mouse	Sigma-Aldrich	SAB1404508	AB_10740376	1:1000
MAP2	Mouse	Millipore Sigma	M1406	AB_477171	1:250
MAP2	Rabbit	Millipore Sigma	AB5622	AB_91939	1:500
TBR1	Chicken	Sigma Aldrich	WH0010716M1	AB_1843877	1:100
EOMES	Mouse	R&D systems	MAB6166	AB_10919889	1:25
CTIP2	Rat	Abcam	ab18465	AB_2064130	1:100
SATB2	Mouse	Abcam	ab51502	AB_882455	1:100
Calretinin	Mouse	Millipore Sigma	MAB1568	AB_94259	1:100
Calretinin	Rabbit	Abcam	Ab702	AB_305702	1:100
GFP	Chicken	Abcam	ab13970	AB_300798	1:500

Preparation of whole cell lysate, nuclear and cytosolic extracts and Immunoblot analysis

The whole cell lysates from H9 (wild type cells), H9$_{UBE3A\ m-/p-}$ (*UBE3A* double KO cells) and mouse cerebellum (mCER) derived from C57BB/6 mouse were prepared as previously described (Drobná et al., 2010). Briefly, tissue of mCER was homogenized and H9 cells lysed in an ice-cold RIPA buffer (50 mM Tris.HCl (pH 7.4), 1% NP-40, 150 mM NaCl, 0.25% Na-deoxycholate, 1 mM EDTA, 1 mM PMSF, 1 mM NaF, and 1X HALT, a cocktail of proteases inhibitors). Lysates were cleared by centrifugation at 10,000 x g. Protein concentrations were determine using BCA assay (ThermoFisher). Equal amount of proteins (35 µg in total) were mixed with 10X Laemmli sample buffer (0.5 M Tris.HCl (pH 6.8), 20% Glycerol, 20% SDS, 0.02% Bromphenol blue, 10% 2-Mercaptoethanol) and incubated at 95 °C for 5 min before separated on 4-15% TGX gel (BioRad). Proteins were transferred onto PVDF membrane (BioRad) and membrane was blocked with 5% non-fat milk (BioRad) in TBS buffer.

The membranes were treated with the following UBE3A antibodies: mouse monoclonal antibody from Sigma (E8655) and rabbit polyclonal antibody from Bethyl Laboratories (A300-351A). β-actin and Lamin B1 were used as a loading control. The antigen-antibody complexes were visualized after incubation with the corresponding HRP-conjugated antibodies and by enhanced chemiluminescence detection (BioRad, Clarity Western ECL Substrate) using Licor Odyssey Fc imaging system.

Nuclear and cytoplasmic extracts were prepared as previously described (Drobná et al., 2003). Organoids in different stages of the development (2-, 6-, and 9-weeks old) were first treated with Accutase (Stem Cell Technologies) to dissociate them into single cells. Samples were collected in duplicate and each replicate comprised 15-25 organoids. Cell suspensions were washed twice with ice-cold DPBS containing 1 mM DTT and 1X HALT inhibitors (Fisher Scientific). Washed organoids were lysed on ice using cytoplasmic extraction buffer (10 mM Tris.HCl (pH 7.4), 60 mM KCl, 1 mM EDTA, 1 mM DTT, 1X HALT) and incubated on ice for 10 min. Igepal CA-630 (Sigma) was added to cell lysate to a final concentration of 1%, vortexed, and centrifuged at 12,000 x g for 5 min at 4 °C. Soluble cytoplasmic extracts (CE) were separated and gently washed with cytoplasmic extraction buffer without Igepal CA-630. Each nuclear fraction was then resuspended in nuclear extraction buffer (20 mM Tris.HCl (pH 8.0), 400 mM NaCl, 1.5 mM $MgCl_2$, 25% glycerol, 1 mM DTT, 1X HALT). The nuclear extracts (NE) were kept on ice for 1 hour with occasional vortexing before clarification by centrifugation at 12,000 x g for 5 min at 4 °C. Cytoplasmic and nuclear extracts were stored at -80 °C. The concentration of proteins in extracts were determined by BCA assay (ThermoFisher). 15 µg of protein from CE and NE were combined with Laemmli sample buffer and, after heat denaturation, separated on a 4-15% TGX gel (BioRad). Proteins were transferred onto a PVDF membrane (BioRad) and the membrane was blocked with 5% non-fat milk (BioRad) in TBS buffer.

The membranes were treated with the UBE3A antibody. GAPDH and H3 were used as a loading controls for cytoplasmic and nuclear extracts, respectively. The membranes were visualized by enhanced chemiluminescence detection (BioRad, Clarity Western ECL Substrate) using a LiCor Odyssey Fc imaging system. Calculation of the UBE3A CE/NE ratio was based on the total yield of proteins in each fraction and adjusted to the amount of proteins loaded on the gel. The following formulas were used for evaluation of UBE3A in each fraction and for the UBE3A CE/NE ratio:

$$\Sigma\text{CE (or NE)} = \text{Signal Intensity} \times \frac{\text{Total yield of proteins for CE (or NE)}(\mu g)}{\text{Total proteins loaded to the gel}\ (15 \mu g)}$$

$$\text{Ratio} = \frac{\Sigma\text{CE}}{\Sigma\text{NE}}$$

Immunoblot analysis in this work was completed with the Sigma (E8655) UBE3A antibody due to its better labeling efficiency in this particular analysis (comparison data not shown). Primary and secondary antibody details used in immunoblot analysis are listed below.

Antigen	Host	Supplier	Cat. No.	RRID	Dilution/Working concentration
UBE3A	Mouse	Sigma-Aldrich	E8655	AB_261956	2 µg/ml
GAPDH	Mouse	Calbiochem	CB1001	AB_2107426	2 µg/ml
H3	Rabbit	Abcam	ab1791	AB_302613	1 µg/ml

β-actin	Mouse	Santa Cruz Biotechnology	sc47778	AB_2714189	1:2500
Lamin B1	Rabbit	Abcam	ab16048	AB_10107828	1 μg/ml
m-IgGκ BP-HRP	Mouse	Santa Cruz Biotechnology	sc516102	AB_2687626	1:2500
anti-rabbit IgG-HRP	Mouse	Santa Cruz Biotechnology	sc2357	AB_628497	1:2500

Transfection of HEK 293FT cells, lentiviral particle production and transduction of human cerebral organoids

To generate lentiviral particles, HEK 293FT cells (ThermoFisher) were seeded on a 6-well plate at a density of 5.0 x 10^6 cells/ml in complete cultured media (DMEM with high glucose containing 10% FBS and non-essential amino acids; Corning). When cells reached 80% confluence, the medium was replaced with Opti-MEM reduced serum medium containing GlutaMax (ThermoFisher) and exposed to 25 μM chloroquine diphosphate (Sigma-Aldrich) for 5 hours before being transfected with the plasmid mixture. The plasmid mixture consisted of pLenti-CamKIIa-GFP (Addgene, Plasmid #96941), pCMVR8.74 (packaging plasmid, Addgene, Plasmid #22036), pCMV-VSV-G (envelope plasmid, Addgene, Plasmid #8454), and pAdVAntage vector (Promega, E1711) each at 300 fmol. Polyethyleneimine (PEI, Sigma) was used as the transfection reagent at a ratio of 3:1 (PEI:DNA). PEI was combined with the plasmid mixture, incubated for 20 minutes at room temperature, and spread drop-wise over the culture. A fresh media change was performed after 18 hours. Media containing lentiviral particles were harvested 48 and 72 hours post transfection, spun down at 500 g for 5 min and filtered using 0.45 μm PES syringe filters (VWR). The particles were concentrated by centrifugation at 2,500 g for 15 min using Amicon Ultra-15 centrifugal filter units (EMDMillipore, UFC910008). Concentrated lentivirus was aliquoted and stored at -80°C. hCOs were transduced by incubating 100μL pLenti-CamKIIa-GFP virus in 1mL cerebral organoid differentiation media for 12 hours.

RNA extraction and qPCR

hCOs were washed 3 times in cold PBS. Matrigel was dissolved by incubating the hCOs in chilled Cell Recovery Solution (Corning, cat. no. 354253) for 1h at 4 °C. The dissolved Matrigel was removed by rinsing 3 times in cold PBS. Total RNA was isolated using Direct-zol RNA MicroPrep Kit (Zymo Research) according to the manufacturer's protocol. RNA samples were collected in 2mL RNAse-free tubes and chilled on ice throughout the procedure. cDNA synthesis was performed using 900 ng of total RNA and the iScript Reverse Transcription Kit (BIO-RAD) according to the manufacturer's protocol. qPCR reactions were performed using IQ Multiplex Powermix (BIO-RAD) on a BIO-RAD 384-well machine (CXF384) with PrimePCR probe assays (BIO-RAD). Unique assay IDs for *UBE3A* primers and probe: qHsaCIP0031486. Primer pairs and probes for *UBE3A-ATS* (RT-17 designed by Runte and colleagues) (Runte et al., 2001), *HPRT* and *TBP* were custom designed and are listed below. Individual primer pairs and probes were tested before multiplexing reactions. Analysis of *UBE3A* and *UBE3A-ATS* expression along with two reference genes *TBP* and *HPRT* was performed in triplicate using Excel by calculating the ΔΔCt value. Data are presented as expression level ($2^{-\Delta\Delta Ct}$) relative to *TBP* or *HPRT*. For each qPCR sample, 3 independent experiments (n=3) with 3-5 organoids in each replicate from different culture dishes were collected.

Primers used in RT-qPCR experiments are listed below.

Target	Gene ID	Forward primer	Reverse primer	Probe	Amplicon size
UBE3A-ATS	104472715	GGCACTGAAAAT GTGGCATCCAG	GGTGTGTCAGCT GTGCTGGTGTC	AGCCAAAGAGTACTC TTCCTCAGTCATCCT	120
TBP	6908	GGGCACCACTCC ACTGTATC	CGAAGTGCAATG GTCTTTAGG	ATGACTCCCATGACC CCCATCACTCCT	100
HPRT	3251	TGACACTGGCAA AACAATGCA	GGTCCTTTTCAC CAGCAAGCT	TGCTTTCCTTGGTCAG GCAGTATAATCCA	94

SUPPLEMENTAL REFERENCES

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