Figure S1

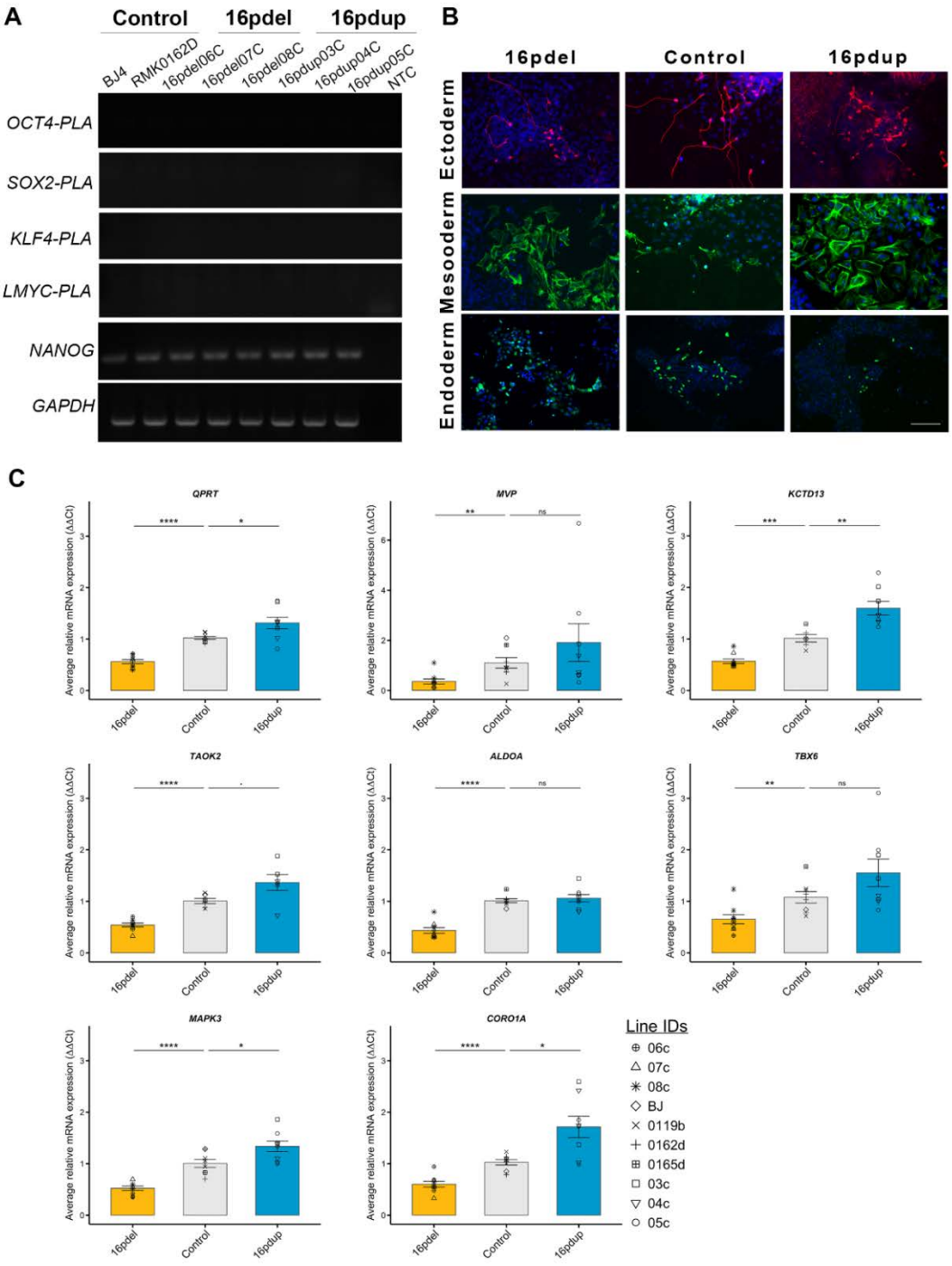

Figure S1. Related to Figure 1. Random or spontaneous differentiation of iPSCs to the three germ layers.
(A) RT-PCR analyses for iPSC lines for plasmid-derived (*OCT4-PLA*, *SOX2-PLA*, *KLF4-PLA* and *c-MYC-PLA)* and endogenous (*OCT4* and *NANOG)* reprogramming factors using specific PCR primers. *GAPDH* was used as a loading control. (B) Images depict immunostaining for specific markers for the three layers – ectoderm (b-III-tubulin), mesoderm (smooth muscle actin) and endoderm (SOX17). Nuclei counterstained with Hoechst 33342. (C) Individual qRT-PCR analyses for selected 16p11.2 genes in iPSCs.[§]
Values are mean ± SE. *p<0.05; **p<0.01; ***p<0.001; ****p<0.0005; .p<0.1; ns=not significant; scale bar, 100μm.
[§] Symbols in bar plots represent all lines used in the study. See Table S4 for details about lines (3 16pdel; 3 16pdup; 2-4 controls), clones (1-2/line) and repeated experiments (1-3).

Figure S2

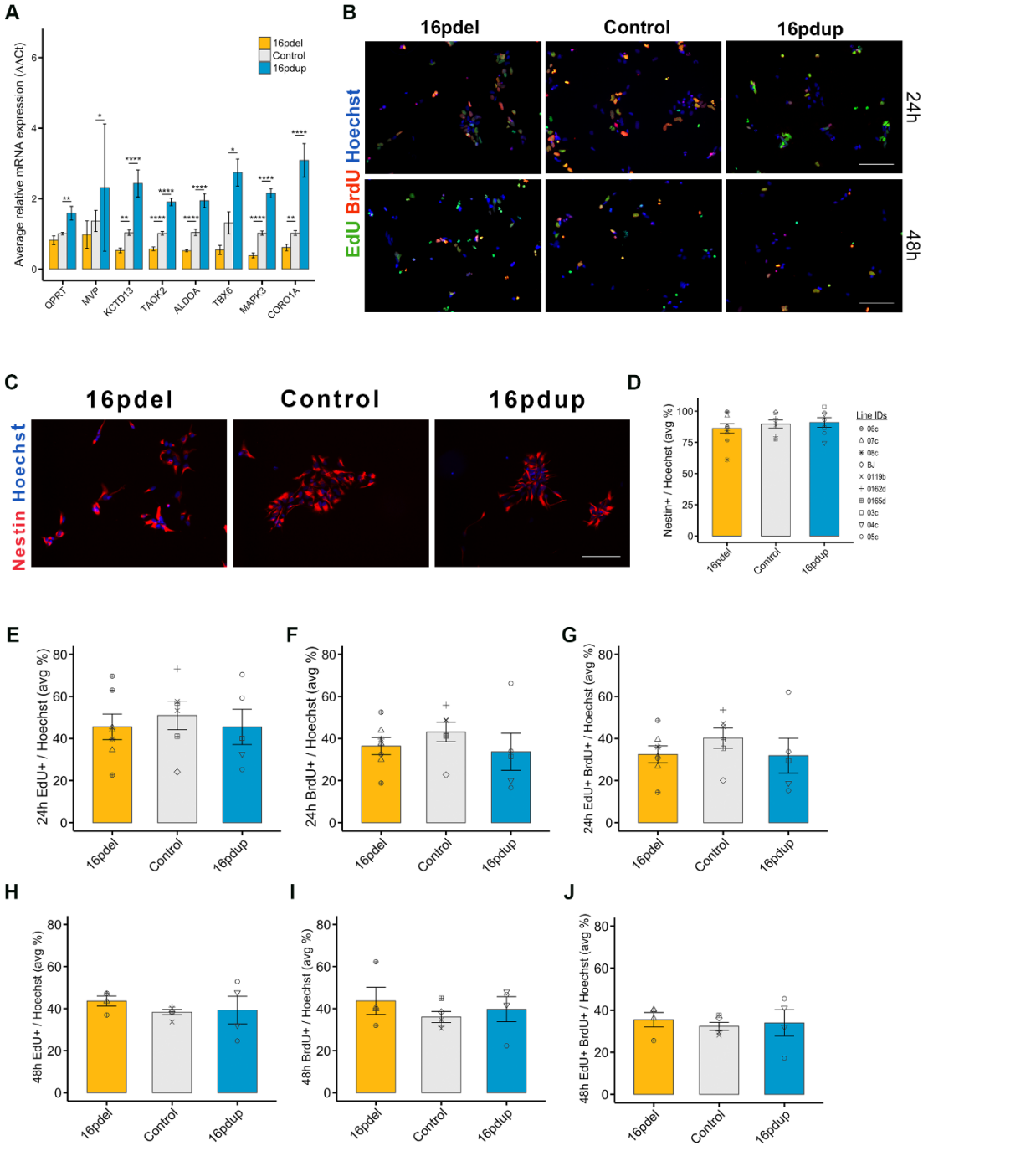

Figure S2. Related to Figure 1. Characterization of NPCs.
(A) Relative mRNA expression of selected 16p11.2 genes by qRT-PCR. (B) Immunostaining depicting EdU/BrdU double labeling in NPCs at 24h and 48h. (C) Immunostaining of NPCs with nestin. Nuclei counterstained with Hoechst 33342. (D) Quantification of nestin+ NPCs from 16pdel, control and 16pdup lines.[§] (E-J) Quantification of EdU/BrdU double labeling assay at 24h and 48h in 16pdel, control and 16pdup iPSC-derived NPCs. [§]
Values are mean ± SE. *p<0.05; **p<0.01; ***p<0.001; ****p<0.0005; ns=not significant; scale bar, 100µm.
[§] Symbols in bar plots represent all lines used in the study. See Table S4 for details about lines (3 16pdel; 3 16pdup; 3-4 controls), clones (2-3/line) and repeated experiments (3-4).

Figure S3

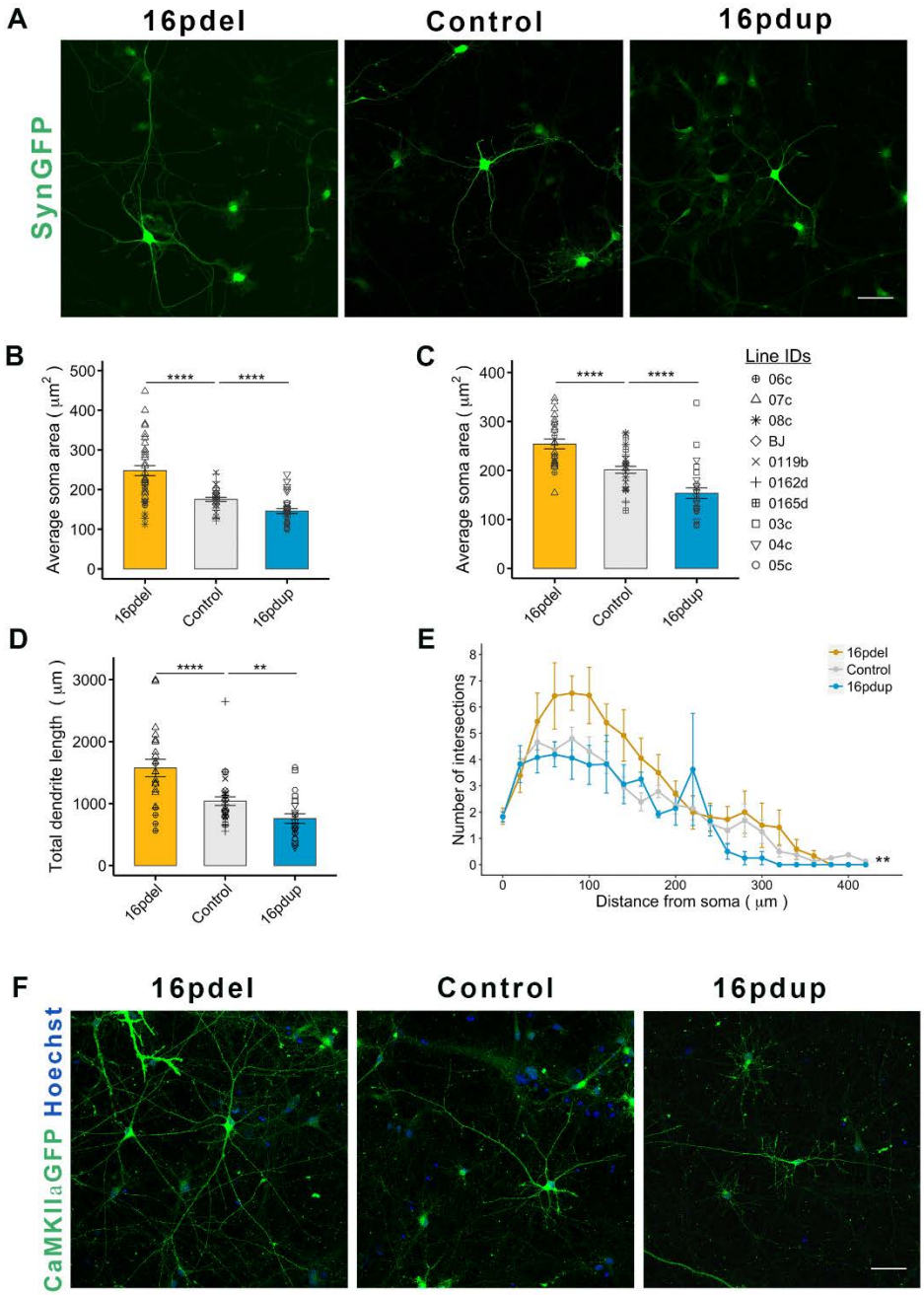

Figure S3. Related to Figure 2. Morphometric characteristics of iPSC-derived neurons at 3 and 6 wpd.
(A) Representative images of SynGFP transduced neurons with their dendritic arbors, 3wpd. (B) Average soma area of SynGFP+ neurons from 16pdel, control and 16pdup lines at 3wpd.§ (C) Average soma area of CaMKIIaGFP+ neurons from 16pdel, control and 16pdup lines at 6wpd.§ (D) Total dendrite length of CamKIIaGFP+ neurons from 16pdel, control and 16pdup lines at 6wpd.§ E) Sholl analysis of CaMKIIaGFP+ 16pdel, control and 16pdup neurons at 6wpd. (F) Representative images of CaMKIIaGFP transduced neurons with their dendritic arbors, 6wpd.
Values are mean ± SE. **p<0.01; ****p<0.0005; ns=not significant. Scale bar, 50µm. §Symbols in bar plots represent all lines used in the study. See Table S4 for details about lines (2 16pdel; 2-3 16pdup; 3-4 controls), clones (1-3/line) and repeated experiments (3-4).

Figure S4

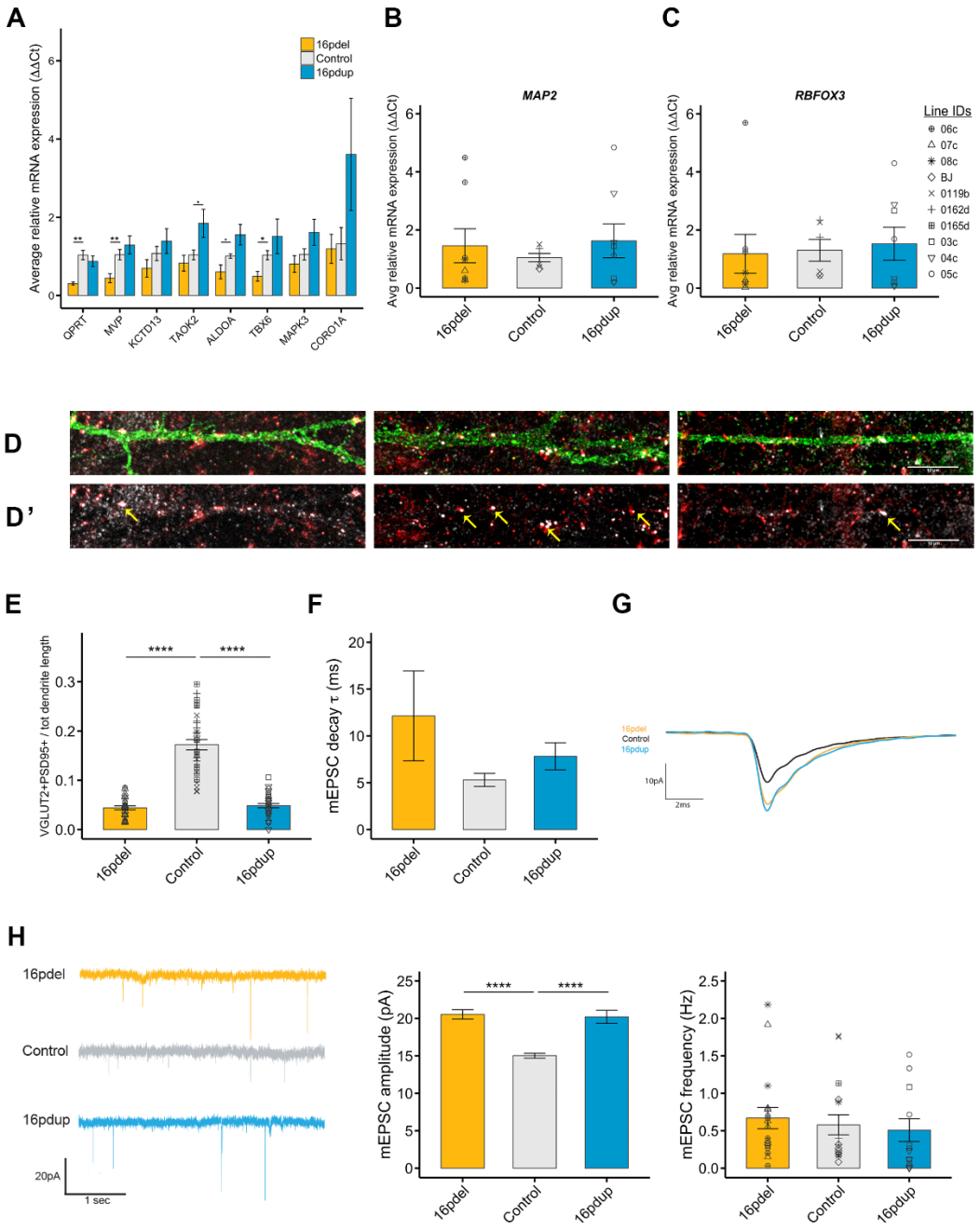

Figure S4. Related to Figures 2-4. Synaptic development of iPSC-derived neurons.
(A) Relative mRNA expression of selected 16p11.2 genes by qRT-PCR at 6wpd. (B-C) Relative mRNA expression of neuronal genes *MAP2* and *RBFOX3* by qRT-PCR at 6wpd. (D) High magnification images of CaMKIIaGFP+ dendrites of 16pdel, control and 16pdup neurons, 6wpd (green), presynaptic marker VGLUT2 (red) and postsynaptic marker PSD95 (white). B' depicts $VGLUT2^{+}PSD95^{+}$ co-localized punctae (yellow arrows). (E) Density of excitatory synapses quantified as number of $VGLUT2^{+}PSD95^{+}$punctae/ dendrite divided by total dendrite length.[§] (F) Decay time constant (τ) of mEPSCs in 16pdel, control and 16pdup neurons (averaged by events). (G) Overlay of example averaged raw traces of mEPSCs from 16pdel, control and 16pdup neurons. (H) Summary of mEPSC amplitude (averaged by events) and frequency in 16pdel, control and 16pdup neurons (averaged by cells). [§]
Values are mean ± SE. ****p<0.0005. Scale bar, 10µm. [§]Symbols in bar plots represent all lines used in the study. See Table S4 for details about lines (3 16pdel; 3 16pdup; 3-4 controls), clones (1-2/line) and repeated experiments (3).

Supplemental Experimental Procedures

Directed neural differentiation of iPSCs and characterization of progenitors

The iPSCs were differentiated into forebrain-specific neural stem cells as previously described (Zhang et al., 2001). Briefly, iPSCs were treated with 1 U/ml dispase (Stem Cell Technologies), scraped and transferred to uncoated T-25 flasks in mTeSR media containing 10uM ROCK inhibitor (Stem Cell Technologies) for formation of embryoid bodies (EBs). At 24 hours after suspension in mTeSR media, EBs were transferred to neural media containing DMEM/F-12, 1% N-2 supplement, 1% Nonessential amino acids, 2μg/ml Heparin, 1% Penicillin/Streptomycin (PenStrep; Life Technologies) supplemented with small molecule inhibitors of the TGF-β and SMAD pathways, 5μM SB431542 and 0.25μM LDN-193189 (Stemgent). On day 3, EBs were plated for attachment on Matrigel-coated 6-well plates for rosette formation in neural media (without inhibitors). On day 11, rosettes were manually picked and transferred to T75 flasks in neural media and grown as neurospheres for a further two weeks. Neural progenitor cells (NPCs) from 16pdel, control and 16pdup lines were immunostained for specific forebrain NPC markers such as PAX6 and nestin. The NPCs were subjected to qRT-PCR for selected 16p11.2 genes.

Generation of cortical neurons

On day 25, neurospheres were dissociated with Accutase (Stem Cell Technolgoies) and plated for differentiation to neurons in neuronal differentiation media containing Neurobasal-A, 1% Glutamax, 1% PenStrep, 2% B-27 and 1% N-2 supplements (Life Technologies) and supplements 20ng/ml BDNF, 20ng/ml GDNF (Peprotech), 200μM Ascorbic acid, 1μM cyclic-AMP and 1μg/ml Laminin (Sigma-Aldrich). The next day, cells were treated with the γ-secretase inhibitor, 0.2μM Compound E (EMD Millipore) to inhibit cell division. For long-term neuronal maturation (upto 14 weeks), neurons were plated in Neurobasal-A media with supplements and two weeks later, neuronal differentiation media was switched to BrainPhys™ Neuronal Medium (Stem Cell Technologies), 1% PenStrep, 2% B-27 and 1% N-2 supplement with the same supplements as before.

RNA extraction

Total RNA was isolated from iPSCs, NPCs or neurons using RNeasy Mini kit (Qiagen) following manufacturer's instructions. Genomic DNA was eliminated using RNase-free DNase 1 (Qiagen) according to manufacturer's protocol. Resulting RNA was quantified using IMPLEN nanophotometer and integrity of RNA was confirmed by agarose gel electrophoresis.

qRT–PCR analysis

About 2-3μg RNA was reverse transcribed (RT) using Superscript-III First-strand Synthesis Supermix (Life Technologies) and was subjected to quantitative RT-PCR analysis using Taqman gene expression assay primer-probes (Life Technologies) and the 7900HT Fast Real-Time PCR System (Applied Biosystems). The mRNA expression levels of selected genes spanning the 16p11.2 CNV were assessed. The 16p11.2 genes (Taqman probe ID) are - *QPRT* (Hs00204757_m1), *MVP* (Hs00911188_m1), *KCTD13* (Hs00923251_m1), *TAOK2* (Hs00191170_m1), *ALDOA* (Hs00605108_g1), *TBX6* (Hs00365539_m1), *MAPK3* (Hs00946872_m1) and *CORO1A* (Hs00200039_m1). The genes *GUSB* (Hs00939627_m1), *B2M* (Hs00187842_m1) and *PPIA* Hs04194521_s1) were used as internal controls. Fold change in expression was calculated using the ΔΔCt method.

Semi-quantitative RT-PCR for episomal transgene expression

RNA was extracted from iPSCs and NPCs as previously described. Complementary DNA was generated using super script III reverse transcriptase according to manufacturer's instructions. PCR was performed using Apex™ RED Taq DNA Polymerase Master Mix, 2.0X, 1.5mM MgCl2 (Genesee Scientific) and primers listed below. Annealing temperature differed for the primer pairs of individual genes (Bharathan et al., 2017; Okita et al., 2011). Water was used as a negative template control for amplification.PCR conditions - 95°C for 2 minutes; 35 repeats of 95°C for 30 seconds, T_A for 30 seconds and 72°C for 30 seconds; and finally, 72°C for 5 minutes. . Primer pairs - *OCT4-PLA:* For - 5'- TTA AGG ATC CCA GTG TGG TG -3', Rev - 5'- TAG CCA GGT CCG AGG ATC AA -3'; *SOX2-PLA:* For - 5'- TTC ACA TGT CCC AGC ACT ACC AGA -3', Rev - 5'- TTT GTT TGA CAG GAG CGA CAAT -3'; *KLF4-PLA*: For - 5'- CCA CCT CGC CTT ACA CAT GAA GA -3', Rev - 5'- TTT GTT TGA CAG GAG CGA CAAT -3'; *MYC-PLA*: For - 5'- GGCTGA GAA GAG GAT GGCTAC -3', Rev - 5'- TTT GTT TGA CAG GAG CGA CAAT -3'; *NANOG*: For - 5'- GCT TGC CTT GCT TTG AAG CA -3', Rev - 5'- TTC TTG ACT GGG ACC TTG TC -3'; *GAPDH*: For - 5'- ACC ACA GTC CAT GCC ATC AC -3', Rev - 5'- TCC ACC ACC CTG TTG CTG TA -3'.

Immunocytochemistry

Cells were fixed in 4% paraformaldehyde (PFA)+4% sucrose for 20 min, washed with PBS three times and incubated in blocking buffer (10% normal goat serum in 0.2% Triton X-100 in PBS) for 1h at room temperature. Cells were incubated overnight in primary antibodies diluted in blocking buffer. The next day, cells were washed with PBS three times, incubated with secondary antibodies (Alexa Fluor 488, 555 and 647, Life Technologies, and 1:2000) for 1 h at room temperature and washed with PBS three times. Nuclei were stained using Hoechst 33342. Coverslips were mounted in Fluoromount-G for imaging. Primary antibodies used are listed in Table S3.

Imaging

iPSCs and NPCs were imaged using EVOS FL cell imaging system with four LED light cubes (DAPI, GFP, RFP, Cy®5) and 10x and 20x objectives. Fixed neuronal samples were imaged on an upright Leica SP5 laser-scanning confocal microscope with a 40X or 63X (NA1.4) oil objective. Images of dendritic arbors were acquired as confocal stacks of 1024x1024 pixels using the 40X objective with 0.3μm z-section. Images of dendrites for synaptic scoring were acquired using the 63X objective with 1-4X digital zoom.

Cell proliferation

Neural PCs were seeded at a density of $4x10^4$ in neural differentiation medium containing Neurobasal-A, 1% N-2 supplement and 2% B-27 without Vitamin A (Life Technologies). The next day, cells were pulsed with 10μM 5-ethynyl-2'-deoxyuridine (EdU) (Thermofisher) for four hours followed by a 24-h chase. Cells were fixed with 4%PFA+4%sucrose and the EdU label was detected using the Click-iT® EdU Alexa Fluor® 488 Imaging Kit. For EdU/BrdU dual labeling, NPCs were seeded as at a density of $4x10^4$ in neural differentiation medium. Two to three hours post seeding once the cells had attached, 10μM EdU was added to the cultures. At 24 and 48h after EdU addition, 20μM 5-Bromo-2′-deoxyuridine (BrdU) was added to the cultures. After 1h incubation in BrdU, cells were fixed. For simultaneous revelation of EdU and BrdU, first EdU was detected by Click chemistry and then BrdU was detected using copper ions using a published protocol (Liboska et al., 2012). Briefly, the cells were incubated in a freshly-prepared solution of 10 mM sodium ascorbate, and 4 mM copper(II) sulfate for 10 minutes and then in 20 mM EDTA for 30 minutes. After a brief wash in PBS, cells were incubated overnight in primary antibody against BrdU (see Table S3) in the presence of exonuclease III. Primary antibody was diluted in exonuclease III buffer containing 0.1u/ml enzyme (Thermofisher). All proliferation experiments were analyzed in a blinded fashion.

Morphometric analysis of iPSC-derived neurons

Six-week old iPSC-derived neurons were transduced with lentivirus encoding green fluorescent protein (GFP) under the human synapsin promoter (SynGFP) or calcium/calmodulin dependent protein kinase II alpha promoter and upstream CMV promoter expressing Archer1-EGFP fusion (CaMKIIaGFP) generated by the Viviana Gradinaru lab (Addgene Plasmid #6042). Cells were fixed three days later for immunocytochemistry and imaging. All morphometric analyses were performed using ImageJ software. For soma area calculation, the perimeter of the GFP+ cell body was outlined and the area within was measured. For total dendrite length, each dendrite or its branch was traced separately and the dendrite length was calculated by addition of all the individual lengths. For dendritic arbors, dendrites were subjected to Sholl analysis using the Simple Neurite Tracer plugin (Ferreira et al., 2014). At least one experiment for each morphometric analysis was analyzed blind.

Synaptic puncta quantification

Three channel z-stack images of dendrites from neurons transfected with SynGFP or CaMKIIaGFP were acquired to quantify synaptic density of neurons or specifically excitatory neurons, respectively. Immunostaining with GFP (A488 channel) antibody marked the dendrites of neurons, the Cy3 channel was antibody labeling for presynaptic protein (synapsin1 or VGLUT2) and the Cy5 channel was labeling for postsynaptic protein (homer1 or PSD95) respectively. Scoring of synapses was performed manually. A maximum projection image of each stack was generated, and the GFP channel was inverted to allow easy visualization of the presynaptic and postsynaptic punctae that were on the dendritic membrane. The punctae where the presynaptic and postsynaptic proteins co-localized, were counted as synapses. Synaptic density was calculated as the number of synaptic punctae on the dendrite divided by the total length of the dendrite in each image. For each genotype, excitatory synaptic density from at least three carrier lines (1-2 clones per line) was analyzed. Excitatory synaptic density for each genotype was calculated by averaging the density of all the neurons pooled from the different lines of each genotype. Details of genotypes, clones and number of cells analyzed are listed in Table S4. At least one experiment for each synaptic density was quantified blind.

Electrophysiology

To isolate electrophysiological analysis to neurons, 14-week-old cultures were transduced with SynGFP lentivirus. Cultures were transferred to the recording chamber installed over an upright light microscope (Nikon Eclipse). During electrophysiological recordings, cultures were immersed in a recording solution (140mM NaCl, 5mM KCl, 10mM HEPES, 10mM D-glucose, 2mM $MgCl_2$, 2mM $CaCl_2$) at room temperature. For recording of mEPSCs from 8-9 week-old CaMKIIaGFP+ neurons, 1μM tetrodotoxin and 20μM bicuculline were added to the bath solution. Visually identified neurons (GFP+) were subjected to whole-cell patch-clamp electrophysiology in both current- and voltage-clamp configurations. For current-clamp and voltage-clamp recordings for intrinsic excitability, electrodes were filled with a K-gluconate-based internal solution (123mM K-gluconate, 10mM KCl, 1mM $MgCl_2$, 10mM HEPES-KOH pH 7.2, 1mM EGTA, 3mM Mg-ATP, 0.3mM Na_4GTP and 4mM D-glucose). For synaptic current recordings in voltage-clamp configuration, electrodes were filled with a CsCl-based internal solution (120mM CsCl, 5mM NaCl, 1mM $MgCl_2$, 10mM HEPES-KOH pH 7.2, 10mM EGTA, 0.1mM $CaCl_2$, 1mM K_2ATP, and 0.2mM Na_4GTP). Potassium current density was calculated by dividing potassium currents by whole cell capacitance. For mEPSC recordings in voltage-clamp configuration, electrodes were filled with a CsCl-based internal solution (135mM CsCl, 10mM HEPES-CsOH pH 7.43, 1mM EGTA). A patch clamp amplifier (Multiclamp 700B, Axon Instruments/ Molecular Devices, Sunnyvale, CA USA) and digitizer (Digidata 1440A, Axon Instruments/ Molecular Devices) were used to acquire electrophysiological signals. Signals were filtered and sampled at 10 and 25 kHz, respectively, for current-clamp recordings. For voltage-clamp recordings, signals were filtered and sampled at 4 and 10 kHz, respectively. For mEPSC recordings, signals were sampled 20 kHz. For acquisition and analysis, signals were filtered at 10kHz and 1kHz, respectively. Proper whole-cell capacitance and series resistance compensation was used. For mEPSC quantification, analysis was done only of recordings in which the access resistance remained lower than 30MΩ. For amplitude and decay tau of mEPSC, individual synaptic events were averaged within each condition. All recordings and analyses were performed blind.

Supplemental References

Bharathan, S.P., Manian, K.V., Aalam, S.M.M., Palani, D., Deshpande, P.A., Pratheesh, M.D., Srivastava, A., and Velayudhan, S.R. (2017). Systematic evaluation of markers used for the identification of human induced pluripotent stem cells. Biol. Open *6*, 100–108.

Ferreira, T.A., Blackman, A. V, Oyrer, J., Jayabal, S., Chung, A.J., Watt, A.J., Sjöström, P.J., and van Meyel, D.J. (2014). Neuronal morphometry directly from bitmap images. Nat. Methods *11*, 982–984.

Liboska, R., Ligasová, A., Strunin, D., Rosenberg, I., and Koberna, K. (2012). Most Anti-BrdU Antibodies React with 2′-Deoxy-5-Ethynyluridine — The Method for the Effective Suppression of This Cross-Reactivity. PLoS One *7*, e51679.

Okita, K., Matsumura, Y., Sato, Y., Okada, A., Morizane, A., Okamoto, S., Hong, H., Nakagawa, M., Tanabe, K., Tezuka, K., et al. (2011). A more efficient method to generate integration-free human iPS cells. Nat. Methods *8*, 409–412.

Supplemental Tables

Table S1. Description of 16p11.2 CNV carrier- and controls. Related to Figure 1.

Individual ID	16p11.2 CNV status	Age in years	Gender	ASD Diagnosis	Head size diagnosis
16pdup03C	Duplication de-novo	6	Male	ASD	Not diagnosed
16pdup04C	Duplication de-novo	36	Male	Non-spectrum	N/A
16pdup05C	Duplication inherited	2	Female	Non-spectrum	Microcephaly
16pdel06C	Deletion de-novo	14	Female	autism	Macrocephaly
16pdel07C	Deletion unknown	8	Male	autism	Macrocephaly
16pdel08C	Deletion inherited	13	Female	autism	Not diagnosed
RMK0162D	copy normal	12	Female	-	-
RMK0165D	copy normal	15	Female	-	-
RMK0119B	copy normal	47	Male	-	-
BJ	copy normal (Matsumoto et al., 2013)	2	Male	-	-

Table S2. Characterization of carrier and control-derived iPSCs. Related to Experimental Procedures.

iPSC Line	Clone#	16p11.2 CNV	Pathogenic changes	Other VUS >200kb
16pdup03C	2, 14	Duplication	None	2q24.3 copy loss 633 kb: VUS
16pdup05C	1, 3, 5	Duplication	None	None
16pdel06C	5, 14	Deletion	None	None
16pdel07C	4, 2	Deletion	None	Xp22.33 copy gain 600 kb; VUS
16pdel08C	2, 6	Deletion	None	3p26.3 copy gain 830kb: VUS
RMK0165D	3, 6	None	None	None
RMK0119B	2, 10	None	XYY karyotype	10q21 Duplication 300 kb; VUS

Clone#=clone number; VUS=variant of unknown significance.

Table S3. Primary antibodies used for immunocytochemistry. Related to Experimental Procedures.

Antigen	**Host**	**Source**	**Catalog#**	**Dilution**
Nanog	mouse	Cell Signaling Technology	4893	1:400
OCT4	rabbit	Cell Signaling Technology	2750	1:400
SOX2	rabbit	Millipore	AB5603	1:500
SSEA-4	mouse	Millipore	MAB4304	1:400
PAX6	mouse	Millipore	MAB5552	1:200
PAX6	rabbit	Abcam	ab5790	1:500
Nestin	mouse	Stem Cell Technologies	60091	1:1000
5-bromo-2'-deoxyuridine (BrdU)	Mouse (MoBU-1)	Life Technologies	B35133	1:50
β-III-tubulin	mouse	R & D Systems	MAB1195	1:1000
β-III-tubulin	mouse	Stem Cell Technologies	01409	1:200
MAP2	chicken	Abcam	ab5790	1:500
smooth muscle actin	rabbit	Abcam	ab5694	1:100
SOX17	goat	R & D Systems	963121	1:250
GFP	chicken	Aves	GFP-1020	1:1000
Synapsin 1	rabbit	Cell Signaling Technology	5297S	1:500
Homer1	mouse	Synaptic Systems	160011	1:200
VGLUT2	rabbit	Synaptic Systems	135403	1:200
PSD95	mouse	Thermofisher Scientific	MA1-045	1:500