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Supplemental Information

Recapitulation of Human Neural Microenvironment Signatures in iPSC-Derived NPC 3D Differentiation

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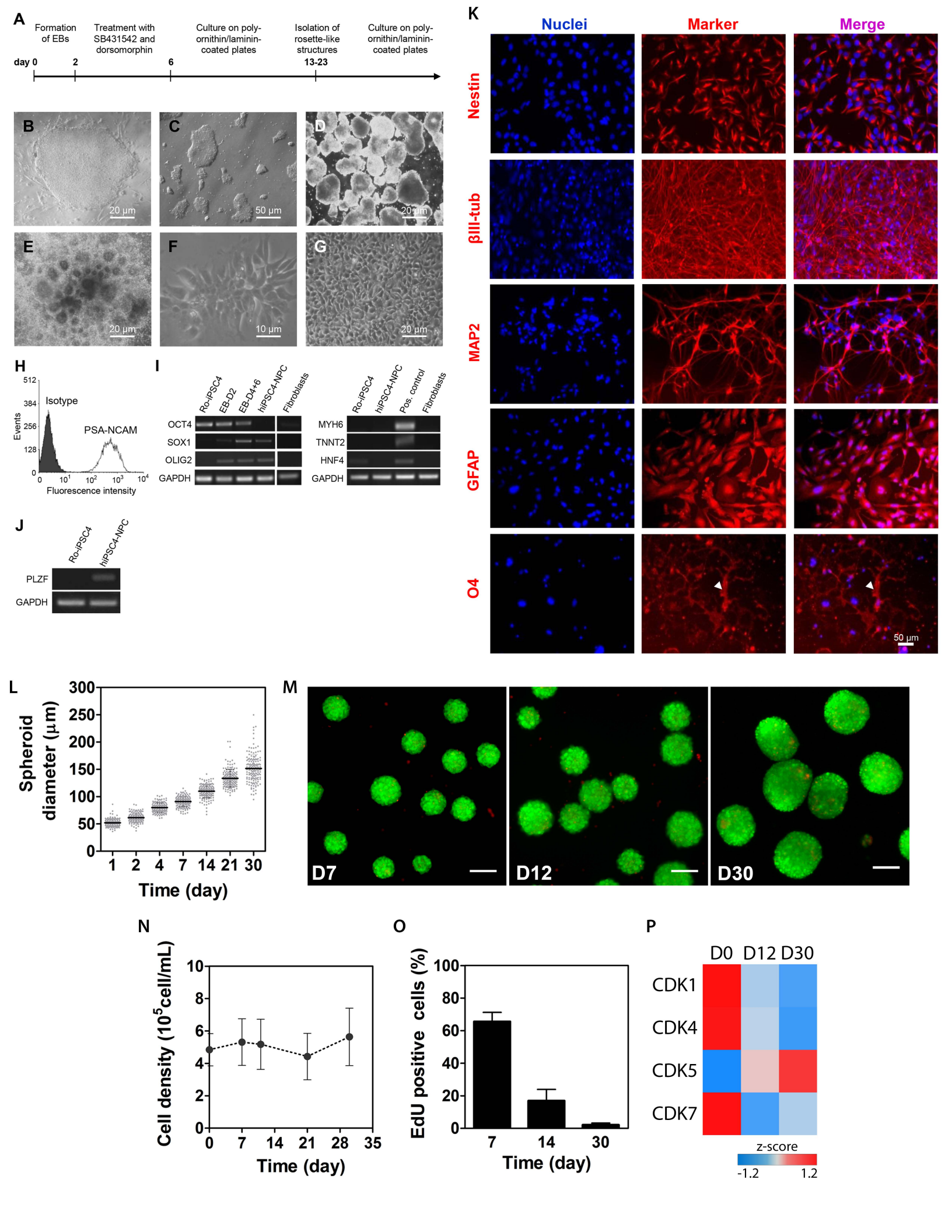
A
Formation of EBs
Treatment with SB431542 and dorsomorphin
Culture on poly-ornithin/laminin-coated plates
Isolation of rosette-like structures
Culture on poly-ornithin/laminin-coated plates
day 0
2
6
13-23
B
C
D
E
F
G
20 μm
50 μm
10 μm
H
Isotype
PSA-NCAM
Events
Fluorescence intensity
I
Ro-iPSC4
EB-D2
EB-D4+6
hiPSC4-NPC
Fibroblasts
Pos. control
OCT4
SOX1
OLIG2
MYH6
TNNT2
HNF4
GAPDH
J
PLZF
K
Nuclei
Marker
Merge
Nestin
βIII-tub
MAP2
GFAP
O4
50 μm
L
Spheroid diameter (μm)
Time (day)
M
D7
D12
D30
N
Cell density (10^5cell/mL)
Time (day)
O
EdU positive cells (%)
Time (day)
P
D0
D12
D30
CDK1
CDK4
CDK5
CDK7
z-score
-1.2
1.2

Figure S1 – Characterization of hiPSC-NPC generation, aggregation and differentiation, related to Figure 1 and Figure 2. (A) Schematic diagram for neural differentiation of hiPSCs. **(B)** R1-hiPSC4 colony grown on MEF feeders (passage 50) and **(C)** after being adapted to grow on Matrigel (passage 55). **(D)** EBs formed by R1-hiPSC4 after collagenase IV treatment cultured in mTesR1 medium for 24 hours. **(E)** Rosette-like structures formed after plating of EBs on PLOL-coated plates for 7-10 days. **(F)** Neuroectodermal cells obtained after plating the dissociated rosette-like structures. **(G)** Confluent monolayer of hiPSC-derived NPCs at passage 10. **(H)** Flow cytometry analysis showing expression of PSA-NCAM on more than 97% of NPCs (passage 10). **(I)** Gene expression of indicated transcripts in undifferentiated R1-hiPSC4, day 2 EBs (EB-d2), day 4-6 EBs (EB-d4-6) and established hiPSC-NPC line as demonstrated by semi-quantitative RT-PCR and agarose gel electrophoresis of PCR products, which were visualized by ethidium bromide staining. Human dermal fibroblasts were used as negative control. hiPSC-NPCs do not express mesodermal markers MYH6 and TNNT2 (expressed in hiPSC-derived cardiomyocytes positive control) and the endodermal marker HNF4-α (expressed in hepatocytes differentiated from hESC line H9 as positive control). **(J)** Gene expression analysis demonstrating that hiPSC-NPCs express PLZF, a marker of early-derived rosette-type neural stem cells, at passage 19. **(K)** Immunocytochemical analysis of R1-hiPSC4-derived NPC demonstrating their neural progenitor identity with the expression of nestin. hiPSC-NPCs cultured for 30 days in neuronal lineage-specific differentiation medium gave rise to neurons expressing pan-neuronal markers βIII-tubulin and MAP2. hiPSC-NPCs differentiate into astrocytes when cultured for 10 days in medium promoting astrocyte differentiation as demonstrated by GFAP staining. Rare O4-positive oligodendrocyte (arrowhead) derived from hiPSC-NPCs after culture in oligodendrocyte-specific medium for 21 days. **(L)** Neurospheroid diameter distribution as a function of time. **(M)** Neurospheroid viability assay, where live cells are stained with fluorescein diacetate (FDA; green) and non-viable cells are labeled with propidium iodide (PI; red). Scale bars, 100 μm. **(N)** Cell density along culture time, evaluated by DNA quantification. Data is presented as mean ± s.e.m. **(O)** Percentage of actively proliferating cells in culture along culture time, assessed by incorporation of EdU. **(P)** Heatmap of cyclin-dependent kinases (CDK) expression levels at D0, D12 and D30. Z-score values were color coded from blue to red, corresponding to downregulation or upregulation, respectively. Data shown represent at least three pooled independent biological experiments (four for panel P, two independent experiments of two cell lines).

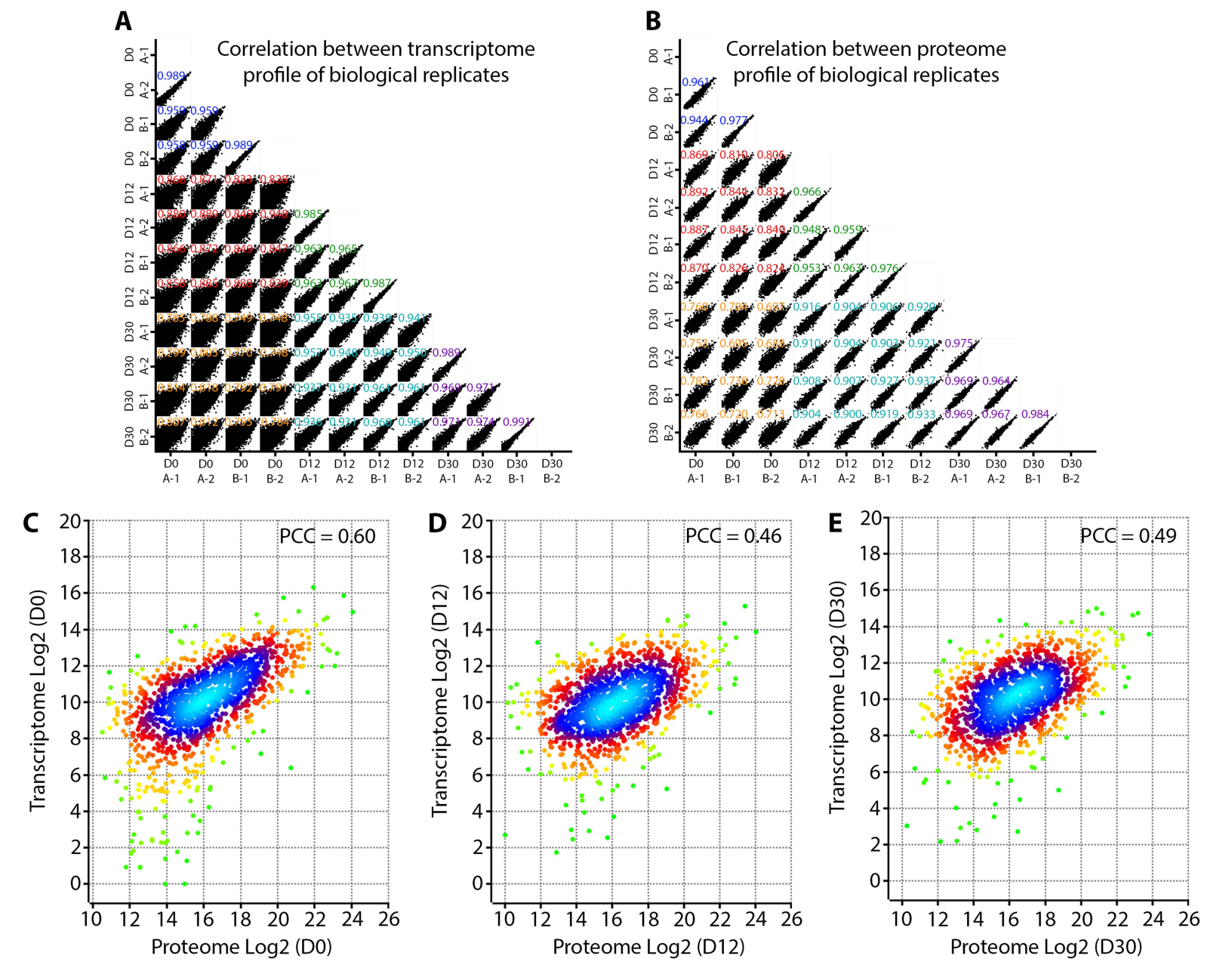
A
Correlation between transcriptome profile of biological replicates
B
Correlation between proteome profile of biological replicates
C
PCC = 0.60
Transcriptome Log2 (D0)
Proteome Log2 (D0)
D
PCC = 0.46
Transcriptome Log2 (D12)
Proteome Log2 (D12)
E
PCC = 0.49
Transcriptome Log2 (D30)
Proteome Log2 (D30)

Figure S2 – Correlation of quantitative data obtained from transcriptome and proteome analyses, related to Figure 1. (A-B) Comparison of the different biological replicates and cell lines (A and B refers to R1-hiPSC1-NPC and R1-hiPSC4-NPC, respectively) at the transcriptome **(A)** and proteome **(B)** level. Each dot represents the quantified levels of a specific transcript/protein. The Pearson correlation coefficients are indicated in each scatterplot. **(C-E)** The density scatterplots describe the correlation obtained for the transcriptome or proteome abundances at day 0 **(C)**, 12 **(D)** and 30 **(E)** of 3D differentiation. Each dot represents the quantified levels of a specific transcript/protein. The Pearson correlation coefficient (PCC) is indicated in the scatterplot. The color code represents the density of dots included in a region of the scatterplot, ranging from high density in blue to low density in green. Data shown represent four pooled independent biological experiments (two independent experiments of two cell lines).

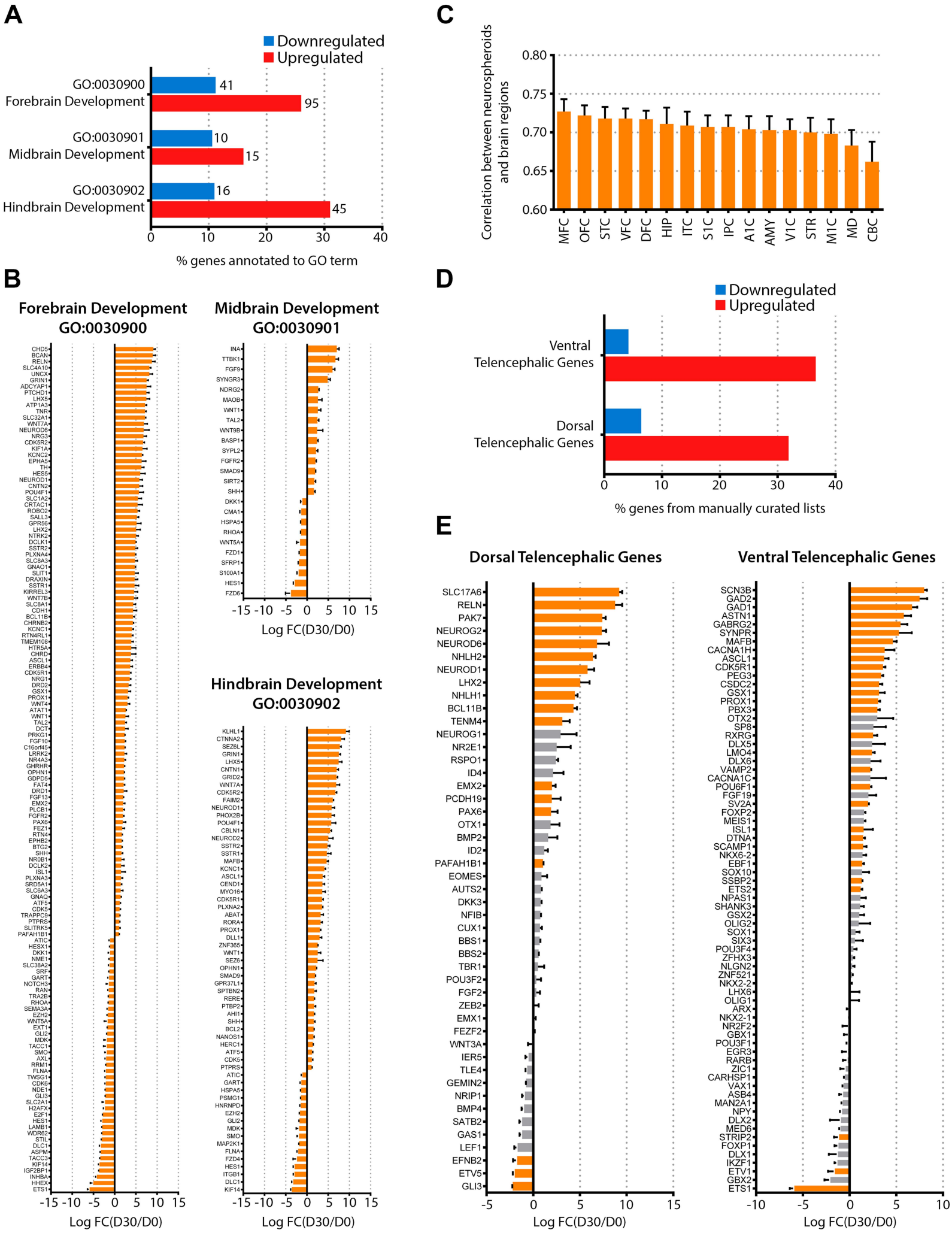
A
Downregulated
Upregulated
GO:0030900
Forebrain Development
41
95
GO:0030901
Midbrain Development
10
15
GO:0030902
Hindbrain Development
16
45
0
10
20
30
40
% genes annotated to GO term
B
Forebrain Development
GO:0030900
Midbrain Development
GO:0030901
Hindbrain Development
GO:0030902
-15 -10 -5 0 5 10 15
Log FC(D30/D0)
C
Correlation between neurospheroids and brain regions
0.80
0.75
0.70
0.65
0.60
MFC
OFC
STC
VFC
DFC
HIP
ITC
S1C
IPC
A1C
AMY
V1C
STR
M1C
MD
CBC
D
Downregulated
Upregulated
Ventral Telencephalic Genes
Dorsal Telencephalic Genes
0
10
20
30
40
% genes from manually curated lists
E
Dorsal Telencephalic Genes
SLC17A6
RELN
PAK7
NEUROG2
NEUROD6
NHLH2
NEUROD1
LHX2
NHLH1
BCL11B
TENM4
NEUROG1
NR2E1
RSPO1
ID4
EMX2
PCDH19
PAX6
OTX1
BMP2
ID2
PAFAH1B1
EOMES
AUTS2
DKK3
NFIB
CUX1
BBS1
BBS2
TBR1
POU3F2
FGF2
ZEB2
EMX1
FEZF2
WNT3A
IER5
TLE4
GEMIN2
NRIP1
BMP4
SATB2
GAS1
LEF1
EFNB2
ETV5
GLI3
-5 0 5 10 15
Log FC(D30/D0)
Ventral Telencephalic Genes
SCN3B
GAD2
GAD1
ASTN1
GABRG2
SYNPR
MAFB
CACNA1H
ASCL1
CDK5R1
PEG3
CSDC2
GSX1
PROX1
PBX3
OTX2
SP8
RXRG
DLX5
LMO4
DLX6
VAMP2
CACNA1C
POU6F1
FGF19
SV2A
FOXP2
MEIS1
ISL1
DTNA
SCAMP1
NKX6-2
EBF1
SOX10
SSBP2
ETS2
NPAS1
SHANK3
GSX2
OLIG2
SOX1
SIX3
POU3F4
ZFHX3
NLGN2
ZNF521
NKX2-2
LHX6
OLIG1
ARX
NKX2-1
NR2F2
GBX1
POU3F1
EGR3
RARB
ZIC1
CARHSP1
VAX1
ASB4
MAN2A1
NPY
DLX2
MED6
STRIP2
FOXP1
DLX1
IKZF1
ETV1
GBX2
ETS1
-10 -5 0 5 10
Log FC(D30/D0)

Figure S3 – Regional specification analysis of differentiated neurospheroids, related to Figure 2. (A) Expression profile summary of genes annotated to the gene ontology (GO) terms for forebrain, midbrain and hindbrain development (365, 94 and 145 genes, respectively). Data are presented as percentage of upregulated (red) or downregulated (blue) genes that are annotated to each GO term, between day 0 and day 30. The number of modulated genes is presented on top of each bar. **(B)** Transcript levels of the genes found to be modulated for each GO term. Data is presented as mean ± s.e.m. of the Log2 fold change between day 0 and 30. **(C)** Correlation analysis between the expression profile of neurospheroids at day 30 and human brain tissue samples from BrainSpan Developmental Transcriptome Dataset. Data is presented as mean ± s.e.m. of correlation coefficient. MFC – anterior (rostral) cingulate (medial prefrontal) cortex; OFC – orbital frontal cortex; STC – posterior (caudal) superior temporal cortex; VFC – ventrolateral prefrontal cortex; DFC – dorsolateral prefrontal cortex; HIP – hippocampus; ITC – inferolateral temporal cortex; S1C – primary somatosensory cortex; IPC – posteroventral (inferior) parietal cortex; A1C – primary auditory cortex; AMY – amygdaloid complex; V1C – primary visual cortex (striate cortex); STR – striatum; M1C – primary motor cortex; MD – mediodorsal nucleus of thalamus; CBC – cerebellar cortex. **(D)** Expression profile summary of dorsal and verntral telencephalic genes. Gene lists were manually curated based on previous publication (Mariani et al., 2012). Data are presented as percentage of upregulated (red) or downregulated (blue) genes, between day 0 and day 30. **(E)** Transcript levels of the dorsal and telencephalic genes. Significantly modulated genes ($p<0.05$ and fold change ±2) are colored orange and non-modulated genes are gray. Data is presented as mean ± s.e.m. of the Log2 fold change between day 0 and 30. Data shown was obtained from four pooled independent biological experiments (two independent experiments of two cell lines).

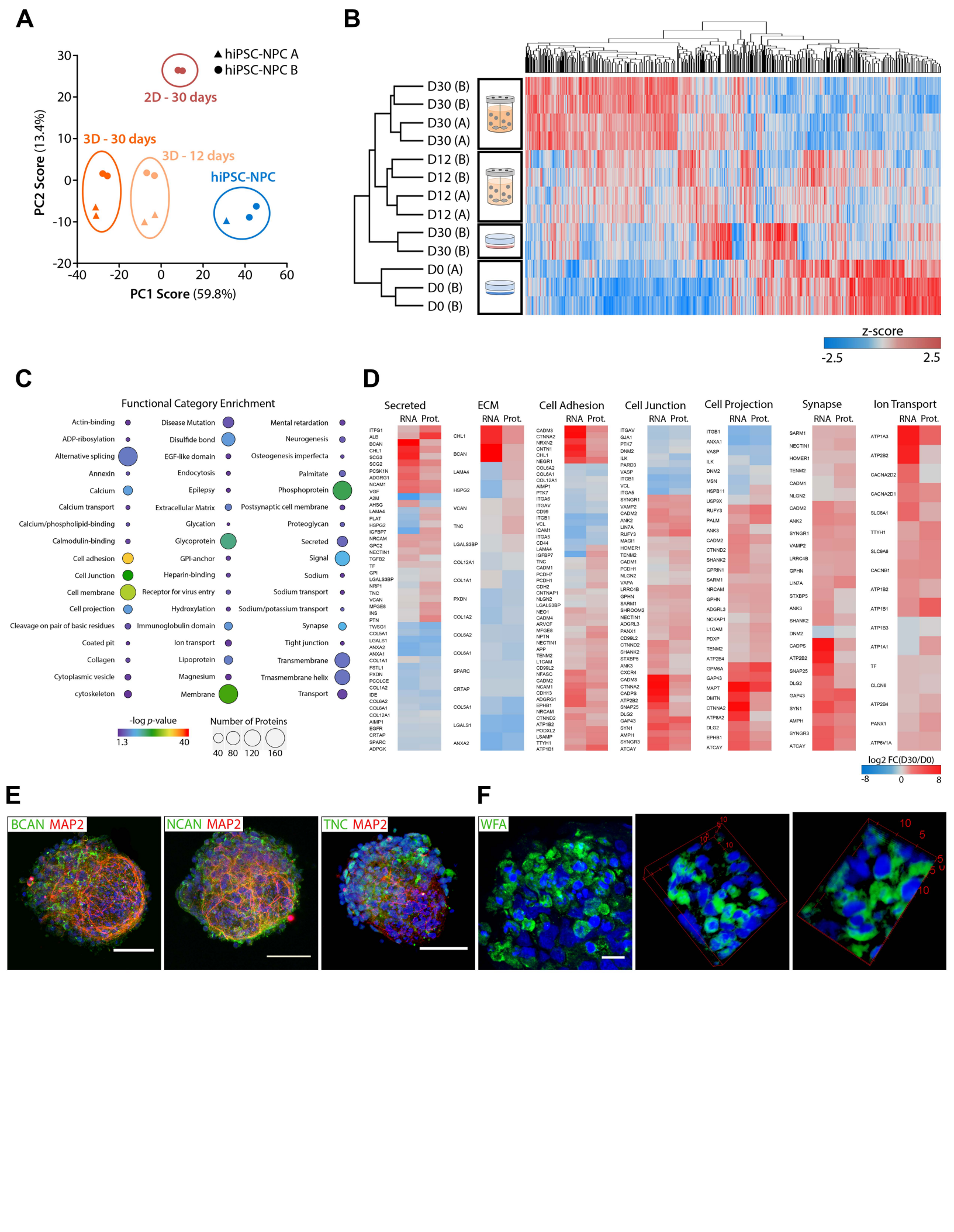
A
▲ hiPSC-NPC A
● hiPSC-NPC B
2D - 30 days
3D - 30 days
3D - 12 days
hiPSC-NPC
PC2 Score (13.4%)
PC1 Score (59.8%)
B
D30 (B)
D30 (B)
D30 (A)
D30 (A)
D12 (B)
D12 (B)
D12 (A)
D12 (A)
D30 (B)
D30 (B)
D0 (A)
D0 (B)
D0 (B)
z-score
-2.5
2.5
C
Functional Category Enrichment
Actin-binding
ADP-ribosylation
Alternative splicing
Annexin
Calcium
Calcium transport
Calcium/phospholipid-binding
Calmodulin-binding
Cell adhesion
Cell Junction
Cell membrane
Cell projection
Cleavage on pair of basic residues
Coated pit
Collagen
Cytoplasmic vesicle
cytoskeleton
Disease Mutation
Disulfide bond
EGF-like domain
Endocytosis
Epilepsy
Extracellular Matrix
Glycation
Glycoprotein
GPI-anchor
Heparin-binding
Receptor for virus entry
Hydroxylation
Immunoglobulin domain
Ion transport
Lipoprotein
Magnesium
Membrane
Mental retardation
Neurogenesis
Osteogenesis imperfecta
Palmitate
Phosphoprotein
Postsynaptic cell membrane
Proteoglycan
Secreted
Signal
Sodium
Sodium transport
Sodium/potassium transport
Synapse
Tight junction
Transmembrane
Trnasmembrane helix
Transport
-log p-value
1.3
40
Number of Proteins
40 80 120 160
D
Secreted
RNA Prot.
ITFG1
ALB
BCAN
CHL1
SCG3
SCG2
PCSK1N
ADGRG1
NCAM1
VGF
A2M
AHSG
LAMA4
PLAT
HSPG2
IGFBP7
NRCAM
GPC2
NECTIN1
TGFB2
TF
GPI
LGALS3BP
NRP1
TNC
VCAN
MFGE8
INS
PTN
TWSG1
COL5A1
LGALS1
ANXA2
ANXA1
COL1A1
FSTL1
PXDN
PCOLCE
COL1A2
IDE
COL6A2
COL6A1
COL12A1
AIMP1
EGFR
CRTAP
SPARC
ADPGK
ECM
RNA Prot.
CHL1
BCAN
LAMA4
HSPG2
VCAN
TNC
LGALS3BP
COL12A1
COL1A1
PXDN
COL1A2
COL6A2
COL6A1
SPARC
CRTAP
COL5A1
LGALS1
ANXA2
Cell Adhesion
RNA Prot.
CADM3
CTNNA2
NRXN2
CNTN1
CHL1
NEGR1
COL6A2
COL6A1
COL12A1
AIMP1
PTK7
ITGA6
ITGAV
CD99
ITGB1
VCL
ICAM1
ITGA5
CD44
LAMA4
IGFBP7
TNC
CADM1
PCDH7
PCDH1
CDH2
CNTNAP1
NLGN2
LGALS3BP
NEO1
CADM4
ARVCF
MFGE8
NPTN
NECTIN1
APP
TENM2
L1CAM
CD99L2
NFASC
CADM2
NCAM1
CDH13
ADGRG1
EPHB1
NRCAM
CTNND2
ATP1B2
PODXL2
LSAMP
TTYH1
ATP1B1
Cell Junction
RNA Prot.
ITGAV
GJA1
PTK7
DNM2
ILK
PARD3
VASP
ITGB1
VCL
ITGA5
SYNGR1
VAMP2
CADM2
ANK2
LIN7A
RUFY3
MAGI1
HOMER1
TENM2
CADM1
PCDH1
NLGN2
VAPA
LRRC4B
GPHN
SARM1
SHROOM2
NECTIN1
ADGRL3
PANX1
CD99L2
CTNND2
SHANK2
STXBP5
ANK3
CXCR4
CADM3
CTNNA2
CADPS
ATP2B2
SNAP25
DLG2
GAP43
SYN1
AMPH
SYNGR3
ATCAY
Cell Projection
RNA Prot.
ITGB1
ANXA1
VASP
ILK
DNM2
MSN
HSPB11
USP9X
RUFY3
PALM
ANK3
CADM2
CTNND2
SHANK2
GPRIN1
SARM1
NRCAM
GPHN
ADGRL3
NCKAP1
L1CAM
PDXP
TENM2
ATP2B4
GPM6A
GAP43
MAPT
DMTN
CTNNA2
ATP8A2
DLG2
EPHB1
ATCAY
Synapse
RNA Prot.
SARM1
NECTIN1
HOMER1
TENM2
CADM1
NLGN2
CADM2
ANK2
SYNGR1
VAMP2
LRRC4B
GPHN
LIN7A
STXBP5
ANK3
SHANK2
DNM2
CADPS
ATP2B2
SNAP25
DLG2
GAP43
SYN1
AMPH
SYNGR3
ATCAY
Ion Transport
RNA Prot.
ATP1A3
ATP2B2
CACNA2D2
CACNA2D1
SLC8A1
TTYH1
SLC9A6
CACNB1
ATP1B2
ATP1B1
ATP1B3
ATP1A1
TF
CLCN6
ATP2B4
PANX1
ATP6V1A
log2 FC(D30/D0)
-8
0
8
E
BCAN MAP2
NCAN MAP2
TNC MAP2
F
WFA

Figure S4 – Proteome remodeling during hiPSC-NPC differentiation, related to Figure 3. (A) Principal component analysis of SWATH-MS data demonstrating a clear clustering of the different experimental groups. **(B)** Heatmap of the proteins significantly modulated during 3D and 2D differentiation (total of 2079 proteins). Hierarchical clustering was performed for rows and columns, where rows represent the different biological replicates of each time point and cell line (A and B refers to R1-hiPSC1-NPC and R1-hiPSC4-NPC, respectively), while each column represents one protein. For the identification of significantly modulated proteins, multi-sample ANOVA test with a permutation-based FDR cutoff of 0.05 was applied on the logarithmized intensities. Z-score values were color coded from blue to red, corresponding to downregulation or upregulation, respectively. **(C)** Functional categories significantly overrepresented (Benjamini Hochberg < 0.05) in proteins annotated as extracellular space or plasma membrane components and significantly modulated during 3D differentiation. The *p*-values and number of proteins are graphically represented by different colors and sphere sizes, respectively. **(D)** Heatmaps of the expression profiles at transcript and protein level of selected categories. Logarithmized fold-changes between D0 and D30 were color coded from blue to red, corresponding to downregulation or upregulation, respectively. Data shown represent four pooled independent biological experiments (two independent experiments of two cell lines). **(E)** Confocal imaging of whole-mount immunostaining, demonstrating the accumulation of important neural ECM components, as brevican (BCAN), neurocan (NCAN) and tenascin-C (TNC). MAP2-positive neuronal cells were also labelled. Scale bars, 50 μm. **(F)** Confocal imaging of wisteria floribunda agglutinin (WFA) labeling in cryosections and 3D rendering insets, demonstrating the presence of neural ECM proteoglycans. Scale bars, 10 μm.

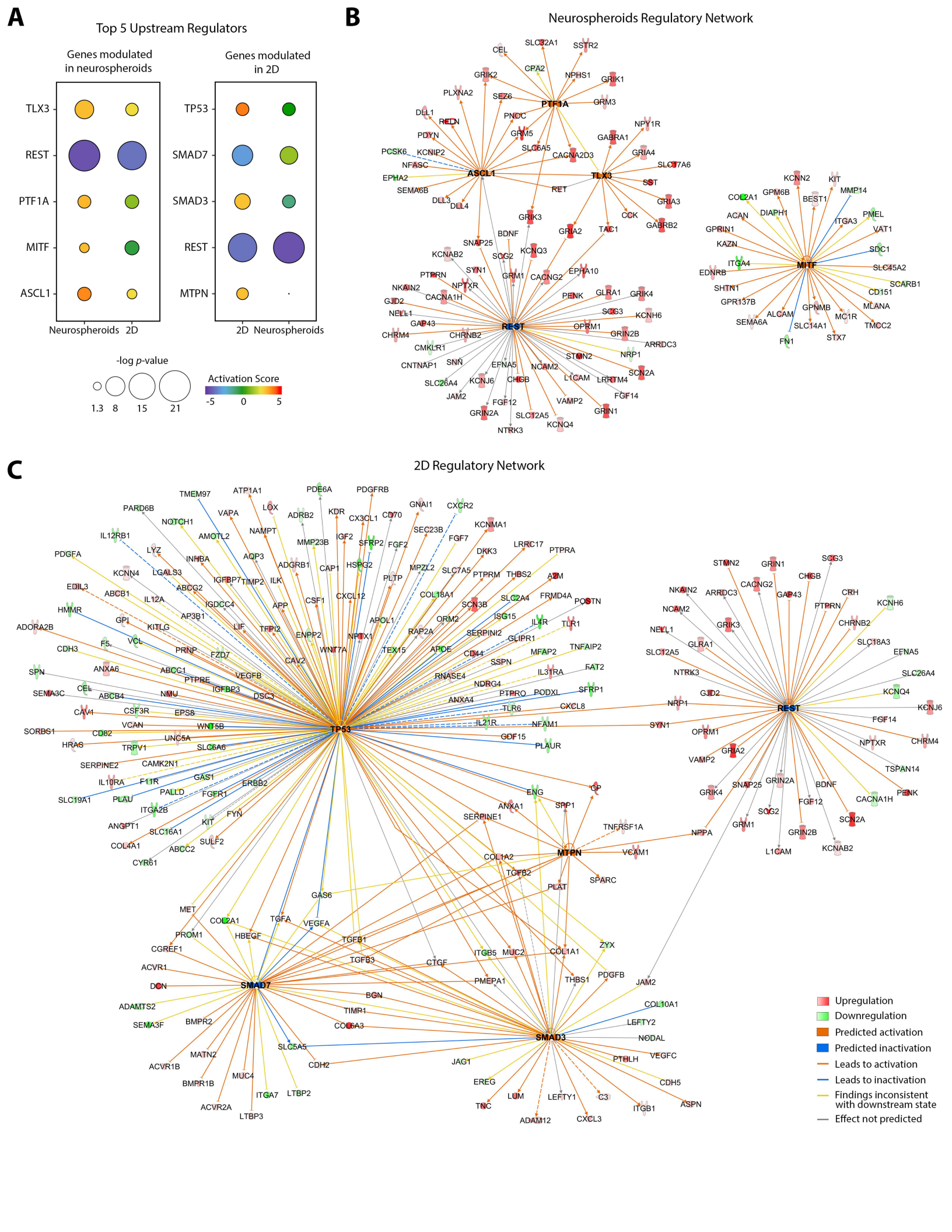
A
Top 5 Upstream Regulators
Genes modulated in neurospheroids
Genes modulated in 2D
TLX3
REST
PTF1A
MITF
ASCL1
TP53
SMAD7
SMAD3
REST
MTPN
Neurospheroids 2D
2D Neurospheroids
-log p-value
1.3 8 15 21
Activation Score
-5 0 5
B
Neurospheroids Regulatory Network
PTF1A
ASCL1
TLX3
REST
MITF
C
2D Regulatory Network
TP53
SMAD7
SMAD3
MTPN
REST
Upregulation
Downregulation
Predicted activation
Predicted inactivation
Leads to activation
Leads to inactivation
Findings inconsistent with downstream state
Effect not predicted

Figure S5 – Upstream regulators analysis for neurospheroids and 2D differentiation, related to Figure 5. (A) Top 5 upstream regulators predicted in Ingenuity Pathway Analysis (IPA) to be responsible for the modulation of all exclusively modulated genes in 3D differentiation (neurospheroids) and 2D differentiation (Srikanth et al., 2015). The activation scores and *p*-values are graphically represented by different colors and sphere sizes, respectively. **(B-C)** Regulatory networks derived for 3D **(B)** and 2D **(C)** data based on IPA predictions for the top 5 upstream regulators.

Supplemental Tables

Table S1 – Two-dimensional annotation enrichment analysis data, indicating the GO-BP terms significantly modulated during 3D differentiation at the transcriptome level and in comparison with the proteome, related to Figure 2

Table S2 – One-dimensional annotation enrichment analysis data, indicating the GO-BP terms significantly modulated in 3D versus 2D differentiation at proteome level, related to Figure 3.

Table S3 – Summary of transcriptome data from the different culture systems, related to Figure 4.

Supplemental Experimental Procedures

Human induced pluripotent stem cells (hiPSC) culture

Human iPSCs (Royan iPSC clone 1 (R1-hiPSC1) and clone 4 (R1-hiPSC4), also known as RIi001-A and RIi007-A, respectively) were derived from dermal fibroblasts as described previously (Totonchi et al., 2010). hiPSC were expanded on Growth Factor Reduced Matrigel Matrix (BD Biosciences) in mTeSR1 medium (StemCell Technologies) under feeder-free culture conditions. Complete medium exchange was performed every day. Cells were maintained under humidified atmosphere with 5 % CO_2, at 37 °C.

Generation and expansion of hiPSC-derived neural progenitor cells (hiPSC-NPC)

hiPSC were detached by treatment with 2 mg/ml of type IV collagenase (Life Technologies) for 15 min at 37°C followed by formation of embryoid bodies (EB) in mTeSR1 medium for 2 days (Figure S1). EBs were treated with 10 µM of SB431542 (TGF-β/Activin/Nodal pathway inhibitor; Selleckchem) and 5 µM dorsomophin (bone morphogenetic protein signaling inhibitor; Sigma-Aldrich) in mTeSR1 for 4 days. On day 6, EBs were plated on previously prepared poly-L-ornithine-laminin (PLOL)-coated surfaces. PLOL coating was prepared by performing a 3 hour incubation at 37°C with 0.16 mg/mL solution of poly-L-ornithine in PBS (with Ca^{2+} and Mg^{2+}), followed by a washing step and a 3 hour incubation at 37°C with 1 µg/mL solution of laminin in PBS (with Ca^{2+} and Mg^{2+}). Cells were then maintained for 7-10 days in DMEM/F12 media with Glutamax (Life Technologies) supplemented with 1% N2 supplement (Life Technologies), 0.1% B27 supplement (Life Technologies), 1.6 µg/mL glucose (Sigma-Aldrich), 20 µg/mL insulin (Sigma-Aldrich), 20 ng/mL rhu-bFGF (Peprotech) (Koch et al., 2009). To establish NPC lines, differentiated EBs were treated with 0.1 mg/ml dispase (Life Technologies) for 15 min at 37°C to isolate the rosette structures from the surrounding flat cells. Isolated cell clusters were then washed once with PBS without Ca^{2+} and Mg^{2+} and treated with 1 mg/ml collagenase IV for 5 min at 37°C. After removing the enzyme, loose clusters were washed once with PBS without Ca^{2+} and Mg^{2+} and dissociated with 0.05% trypsin-EDTA (Life Technologies) for 3 min. Cells were then plated on PLOL-coated surfaces in the NPC expansion medium, composed of DMEM/F12 media with Glutamax (Life Technologies) supplemented with 1% N2 supplement (Life Technologies), 0.1% B27 supplement (Life Technologies), 1.6 µg/mL glucose (Sigma-Aldrich), 20 µg/mL insulin (Sigma-Aldrich), 20 ng/mL rhu-bFGF (Peprotech) and 20 ng/ml rhu-EGF (Sigma-Aldrich). Medium was changed every other day until confluent cell culture was obtained. For further expansion and maintenance hiPSC-NPC were split typically every 4-5 days at 90-100% confluence. Cells were dislodged through incubation with 0.05% Trypsin-EDTA for 1-2 minutes, ressuspended in DMEM supplemented with 10% FBS (Life Technologies), sedimented by centrifugation and the resultant pellet ressuspended in NPC expansion medium. Cell concentration and viability were determined by the trypan blue exclusion method in a Fuchs-Rusenthal hemocytometer. The cell suspension was used to inoculate PLOL-coated T-flasks, at a cell density of 3 x 10^4 cell/cm^2. A 50 % media exchange was performed at day 2 of culture. Cells were maintained under humidified atmosphere, in a multi-gas cell incubator (Sanyo), with 5 % CO_2 and 3 % O_2, at 37 °C.

hiPSC-NPC lineage-specific 2D differentiation

To differentiate hiPSC-NPC to neuronal cells, 1x10^4 cells/cm^2 were plated on PLOL-coated plates in the NPC expansion medium. Next day, medium was changed to DMEM/F12 supplemented with neurobasal medium (1:1; Life Technologies), 1% N-2 Supplement, 1% B-27 Supplement, 200 µM ascorbic acid (Wako, Neuss, Germany) and cells cultured for 2 weeks. Half of the medium was replaced every other day. After 2 weeks of culture, the ratio of neurobasal to DMEM/F12 medium was changed to 3:1, N-2 Supplement was reduced to 0.5% and 10 ng/ml BDNF of brain derived neurotrophic factor (BDNF; R&D, Wiesbaden-Nordenstadt, Germany) was added and cells cultured for one additional week. Half of the medium was changed every other day. To differentiate hiPSC-NPC to astrocytes, 5x10^4 cells/cm^2 were plated on PLOL-coated plate. After 24 hours, medium was changed to DMEM/F12 containing 10% FBS, 1% minimum essential medium NEAA (MEM-NEAA) and 1% L-glutamine (all from Life Technologies) for 7-10 days. 75% of medium was changed every other day. For differentiation of hiPSC-NPC to oligodendrocytes, 5x10^4 cells/cm^2 were plated on PLOL-coated plate in the NPC expansion medium. Next day, half of the medium was changed to DMEM/F12 medium supplemented with 2% B-27 Supplement, 2 mM L-glutamine, 10 µg/ml insulin, 10 µg/ml putrescine, 63 ng/ml progesterone, 50 ng/ml sodium selenite, 40 ng/ml triiodothyronine (T3), 50 µg/ml holo-transferrin and 20

ng/ml EGF (all from Sigma-Aldrich). After one week, the cells were dissociated with 0.05% trypsin–EDTA, $1.5x10^4$ cells/cm^2 were plated on PLOL- coated plates and further cultured for 2 weeks. Medium was changed every other day.

hiPSC-NPC 3D and 2D differentiation

hiPSC-NPC were expanded and dislodged with trypsin into a cell suspension, as described above. This cell suspension was passed through a 70 µm nylon strainer (Millipore) prior to bioreactor inoculation, in order to eliminate cell clumps. The obtained single cell suspension was diluted for a cell density of $4x10^5$ cell/mL in aggregation medium (AM), with the same composition as EM, except for reduced EGF/FGF concentration (5 ng/mL) and the addition of 5 µM Y-27632. Cells were then inoculated into software-controlled stirred-tank DASGIP® Bioblock bioreactor system (Eppendorf), as described previously (Simão et al., 2016). Culture conditions were set to maintain cells under 3 % dissolved oxygen (15 % of air with 21 % of oxygen), pH 7.4, 37 ºC and a stirring rate of 70 rpm. In order to control the aggregate size and avoid aggregate fusion, the stirring rate was gradually increased up to 90 rpm with 10 rpm steps, based on visual inspection of the culture. After 48 hours of culture, perfusion operation mode was activated, with a dilution rate of 0.33 day^{-1} (i.e., 33 % working volume exchange *per* day) under gravimetric control. To prevent the loss of aggregates through the outlet perfusion line, a metallic filter of 20 µm pore size was adopted as cell retention device (Simão et al., 2016). After a 7 day aggregation period with AM, differentiation was induced by replacing the perfusion medium with differentiation medium (DM), maintain the culture for further 23 days (total of 30 days). DM was prepared by supplementing DMEM/F12 with Glutamax with 2 % B27 supplement, 1.6 µg/mL glucose, 10 µg/mL insulin, 10 µg/mL putrescin, 63 ng/mL progesterone, 50 µg/mL apotransferrin, 50 ng/mL sodium selenium (all from Sigma-Aldrich) and 200 mM ascorbic acid (Wako). For 2D differentiation, cells were plated in PLOL-coated surfaces at a cell density of 3×10^4 cell/cm^2 in EM. On the day after culture medium was changed to DM and every other day half of the medium was exchanged to fresh DM. At day 7, cells were dislodged by incubating with 0.05% Trypsin-EDTA for 1-2 minutes and replated on PLOL-coated surfaces at a cell density of 5×10^4 cell/cm^2 in DM. Cells were then further cultured for 23 days (total of 30 days), where half of the medium was exchanged to fresh DM every other day.

Immunofluorescence microscopy

Cells were fixed in 4% paraformaldehyde + 4% sucrose in phosphate-buffered saline (PBS) for 20 min at room temperature and washed three times with PBS. For cryosectioning samples were dehydrated with 30% (w/v) sucrose overnight, frozen at -80 ºC in Tissue-Tek O.C.T. (Sakura) and sectioned at a thickness of 10 mm using a cryomicrotome (Cryostat I, Leica). For immunofluorescence, cells were permeabilized with 0.5 M ammonium chloride and 0.25% Triton X-100 in PBS (Figure S1K) or with 0.1% TritonX-100 in PBS (Figures 2C, 3F and S1P). Cells were blocked for 1 hour with 5% goat or rabbit serum in PBS (Figure S1K) or for 30 min with 0.2% fish skin gelatin in PBS (Figures 2C, 3F). Primary antibodies were then incubated overnight at 4°C diluted in 0.8% bovine serum albumin (BSA) in PBS (Figure S1K) or for 2 hours diluted in 0.1% TritonX-100 + 0.125% fish skin gelatin in PBS (Figures 2C, 3F). Cells were washed three times with PBS and incubated for 1 hour with secondary antibodies diluted in 0.8% BSA in PBS (Figure S1K) or 0.125% fish skin gelatin in PBS (Figures 2C, 3F). Primary and secondary antibodies were used as follows: anti-nestin (AB5922, Millipore), anti-Sox2 (AB5603, Millipore), anti-βIII-tubulin (1:200, MAB1637, Millipore; or 1:1000, sc-80005, Santa Cruz), anti-synaptophysin (1:200, MAB5258, Millipore), anti-MAP2 (1:10 000, ab5392, Abcam; or 1:500, sc-20172, Santa Cruz), anti-GFAP (1:200, AB5804, Millipore; or 1:500, G3893, Sigma-Aldrich), anti-O4 (1:500, O7139, Sigma-Aldrich), AlexaFluor 488 goat anti-mouse IgG, AlexaFluor 594 goat anti-chicken IgY or AlexaFluor 594 goat anti-rabbit IgG, AlexaFluor 488 goat anti-rabbit IgG (1:500; all from Life Technologies). Cell nuclei were counterstained with Hoechst, DAPI or TO-PRO-3 (Life Technologies). Coverslips were mounted in ProLong Gold antifade reagent with DAPI (Life Technologies). Preparations were visualized on an Axiovert 200M fluorescence microscope (Carl-Zeiss) or point-scan confocal microscope (SP5, Leica). The obtained images were processed using FIJI software (Schindelin et al., 2012) and only linear manipulations were performed.

Flow cytometry

Single-cell suspensions of hiPSC-NPC were prepared by 0.05% trypsin-EDTA treatment as described above. The dissociated cells were centrifuged, washed and filtered through a 40 µm cell strainer (BD Pharmingen). Cell density was determined and cells were resuspended in the appropriate

volume of staining buffer (PBS supplemented with 0.1% FBS) to obtain a cell density of 0.5 $x10^6$ cells/50 µl. Cells were then dispensed into 1.5 ml microcentrifuge tubes (50 µl/tube) and stained either with isotype control or antigen-specific PSA-NCAM antibody (1:100, MAB5324, Millipore), followed by 30 minutes incubation with AlexaFluor 555 secondary antibody (dilution 1:1000). The cells were washed two times with staining buffer and data collected on the flow cytometer (Attune® Acoustic Focusing Cytometer, Thermo Fisher Scientific). Cells were gated on forward and side scatter dot plots. 10,000 events per sample were acquired and the data were analyzed with Attune software.

Viability assay

For cell viability assessment, neurospheres were incubated with 20 mg/mL fluorescein diacetate, which stains viable cells, and 10mg/mL propidium iodide, a membrane-impermeable DNA dye that stains nonviable cells in PBS for 5 min, washed with PBS, and observed using fluorescence microscopy (DMI6000, Leica).

DNA quantification-based cell concentration determination

For cell density determination, 0.5 mL samples were collected, sedimented by centrifugation at 1000 x g, 5 min and resuspended in ultrapure distilled water. Complete cell lysis was achieved through a freeze-thaw cycle in liquid nitrogen coupled to 15 min sonication into an ultrasound bath at 35 kHz. DNA quantification was performed using the Quant-iT™ PicoGreen® dsDNA Assay Kit (Life Technologies), according to the manufacturer's instructions. Fluorescence intensity was measured in spectrofluorometer at 480 nm excitation and 520 nm emission wavelengths. The obtained values were correlated with calibration curves performed by serial dilutions of standard DNA solutions and cell lysates from samples with known cell concentrations.

Aggregate Size Determination

Neural spheroid size determination was performed by imaging analysis of phase contrast images, using the open source FIJI software (Schindelin et al., 2012). Briefly, the aggregate boundaries were defined by manual threshold adjustment and the Feret's diameter was automatically determined for each one of the neurospheres.

Cell proliferation Assessment

The percentage of proliferative cells within neurospheres was determined through the EdU (5-ethynyl-2´-deoxyuridine) incorporation assay, using the Click-iT® EdU Alexa Fluor® 488 Assay Kit (Life Technologies). Briefly, neurospheres were allowed to adhere to PLOL-coated glass coverslips and incubated with 10 µM EdU in culture media for 20 h. After incubation, cells were fixed with 4 % paraformaldehyde (PFA) and 4 % sucrose in PBS for 20 min, washed twice with PBS with 1 % bovine serum albumin (BSA) and permeabilized with saponin-based working reagent for 20 to 30 min, depending on neural spheroid size. Cells were incubated with AlexaFluor488 anti-EdU antibody, according to manufacturer's instructions, for 45 min to 1 h 15 min, depending on neural spheroid size. Coverslips were mounted in ProLong Gold antifade reagent with DAPI (Life Technologies). Preparations were visualized in spinning disk microscope (Nikon Eclipse Ti-E, confocal scanner: Yokogawa CSUx1) and resultant images processed in FIJI software. For quantitative analysis, Edu-positive cells and total DAPI-labeled nuclei were determined using cell counter plug-in in FIJI (Schindelin et al., 2012).

RT-PCR

Total RNA was isolated from hiPSC, EB and hiPSC-NPC by TRIzol reagent (Life Technologies) following the manufacturer's recommendations. RNA concentration was measured with a Nanodrop 1000 (Thermo Scientific, Frankfurt, Germany) and the quality was assessed by agarose gel electrophoresis. cDNA samples were synthesized from 1 µg of total RNA using the SuperScript II First-Strand Synthesis Kit and random hexamers (Life Technologies) for priming. RT-PCR was carried out using DreamTaq Green PCR Master Mix (Thermo Scientific, Frankfurt, Germany) and PCR products were analyzed by agarose gel electrophoresis with a DNA ladder mix (SM0331, Life Technologies) to determine the PCR product sizes. The following primers were used: OCT4 (fwd 5'-AGGGCAAGCGATCAAGCA-3' and rev 5'-AGGGCAAGCGATCAAGCA-3'); SOX1 (fwd 5'-CAATGCGGGGAGGAGAAGTC-3' and rev 5'-CTCTGGACCAAACTGTGGCG-3'); OLIG2 (fwd 5'-

CAGAAGCGCTGATGGTCATA-3' and rev 5'-TCGGCAGTTTTGGGTTATTC-3'); MYH6 (fwd 5'-TGTCCCGGGAAGGGGGCAAA-3' and rev 5'-CCGGCTCGTGCAGGAAGGTC-3'); TNNT2 (fwd 5'-GGTGCCTCCCAAGATCCCCG-3' and rev 5'-GATGCGCTGCTGCTCGGCCC-3'); HNF4 (fwd 5'-CTGCTCGGAGCCACCAAGAGATC-3' and rev 5'-ATCATCTGCCAGGTGATGCTCTGCA-3'); PLZF (fwd 5'-CTATGGGCGAGAGGAGAGTG-3' and rev 5'-TCAATACAGCGTCAGCCTTG-3'); GAPDH (fwd 5'-GGACTCATGACCACAGTCCAT-3' and rev 5'-ACCTTGCCCACAGCCTTG-3').

Sample preparation for mass spectrometry

Samples from 3D cultures were directly harvested from the bioreactor. 2D differentiated cells were harvested by gentle mechanical dislodgment using a cell scrapper. Cells were then sedimented by centrifugation at 300 x*g*, 5 min. Supernatant was discarded and resulting cell pellet was washed with PBS, followed by centrifugation at 300 x*g*, 5 min. Cells were lysed in Triton X-100 lysis buffer (50 mM Tris, 5 mM EDTA, 150 mM NaCl, 1% Triton X-100 (all from Sigma-Aldrich) and 1x complete protease inhibitors cocktail (Roche)), for 45 minutes at 4ºC. Protein quantification was performed using Micro BCA™ Protein Assay Kit (Thermo Fisher Scientific) following manufacturer's instructions. Proteins were precipitated using methanol, as previously described (Wessel and Flügge, 1984). Briefly, proteins were precipitated in four-fold excess of methanol, centrifuged at 9 000 x*g* for 10 seconds and followed by the addition of 2 parts of chloroform with subsequent centrifugation. For phase separation, 3 parts of deionized water were added to the samples, homogenized by vigorous vortex and centrifuged at 9 000 x*g* for 1 min. The upper phase was discarded and 3 parts of methanol were added. Samples were mixed, and centrifuged at 9 000 x*g* for 2 min to pellet precipitated protein. Supernatant was removed and precipitates were dried by heating at 60 °C with lids slightly ajar. For surfactant assisted in-solution protein digestion, precipitated proteins were solubilized in 0.1% of RapiGest SF Surfactant (Waters). Prior to digestion, samples were reduced with 5 mM of dithiothreitol (DTT) for 30 mins at 60 °C, alkylated with 15 mM iodoacetamide (IAA) for 30 min in dark and boiled at 100 °C for 5 mins. After cooling to room temperature, protein digestion was performed by overnight incubation with trypsin (Promega; 1.2 µg per 100 µg protein) at 37 °C. Trypsin inactivation was achieved by acidification with trifluoroacetic acid (TFA) at 0.5 % and incubation at 37 °C for 45 min. Samples were centrifuged at 16 000 x*g* for 10 min, supernatants were collected into new tubes and dried using a Savant™ Universal SpeedVac™ concentrator (Thermo Fisher Scientific).

Generation of the spectral reference library

Seven samples from different experimental group were used for information-dependent acquisition (IDA) analysis by NanoLC-MS using TripleTOF 6600 (ABSciex, Framingham, MA, USA). A reversed phase nanoLC with a trap and elution configuration, using a Nano cHiPLC Trap column (200 µm × 0.5 mm ChromXP C18-CL 3 µm 120 Å) and nano column (75 µm × 15 cm ChromXP C18-CL 3 µm 120 Å) was performed. Water with 0.1% (v/v) formic acid (solvent A) and acetonitrile with 0.1% (v/v) formic acid (solvent B) were used. Sample was loaded in the trap column at a flow rate of 2 µL.min-1 for 10 min using 100% (v/v) solvent A. Peptide separation was performed in the nano column at a flow rate of 300 $\mu L.min^{-1}$ applying a 90 min linear gradient of 5% to 30% (v/v) of solvent B. Each sample was subjected to two IDA runs. The mass spectrometer was set for IDA scanning full spectra (400–2000 m/z) for 250 ms. The top 50 ions were selected for subsequent MS/MS scans (150–1800 m/z for 40 ms each) using a total cycle time of 2.3 s. The selection criteria for parent ions included a charge state between +2 and +5, and counts above a minimum threshold of 125 counts per second. Ions were excluded from further MS/MS analysis for 12 s. Fragmentation was performed using rolling collision energy with a collision energy spread of 5.

The spectral library was created by combining all IDA raw files using ProteinPilot™ software (v5.0 ABSciex) with the Paragon algorithm and with the following search parameters: search against Homo sapiens from Uniprot/SwissProt database (release 2015_05); trypsin digestion; iodoacetamide cysteine alkylation; through identification efforts. After a false discovery rate (FDR) analysis, only FDR<1% were considered (3272 proteins). The output of these searches, in the form of a group file, was used as the reference spectral library. An extended spectral library was built by merging the spectral library containing the IDA runs with an in-house spectral library of human samples, using SwathXtend R package (Wu and Pascovici, 2015). This spectral ion library contains 5458 proteins.

Quantitative proteomic analysis

For quantitative analysis, 2.5 µg of each sample were subjected to three SWATH runs (Sequential Windowed data independent Acquisitions of the Total High-resolution Mass Spectra). Similar chromatographic conditions to the previously described IDA runs were used. The mass spectrometer was operated in a cyclic product ion data independent acquisition (DIA). A variable windows calculator and SWATH acquisition method editor (AB SCIEX) were used to setup the SWATH acquisition. A set of 32 overlapping windows (containing 1 m/z for the window overlap) was constructed, covering the precursor mass range of 400 – 1200 m/z. A 50 ms survey scan was acquired at the beginning of each cycle, and SWATH MS/MS spectra were collected for 96 ms resulting in a cycle time of 3.172 s. Rolling collision energy with a collision energy spread of 15 was used. The spectral alignment and targeted data extraction of DIA samples were performed using PeakView v.2.1 (AB SCIEX; Framingham, US) with the reference spectral library. For data extraction the following parameters were used: six peptides/protein, six transitions/peptide, peptide confidence level of >99%, FDR threshold of 1%, excluded shared peptides, and extracted ion chromatogram (XIC) window of 10 min and width set at 20 ppm. A total of 3728 proteins were quantified under these conditions. One sample that did not meet quality standards was removed from further analysis (day 0 from cell line R1-hiPSC1-NPC). The mass spectrometry proteomics data have been deposited to the ProteomeXchange Consortium via the PRIDE (Vizcaíno et al., 2016) partner repository with the dataset identifier PXD007130.

Next Generation Sequencing

Total RNA was extracted using the RNeasy Mini Kit (Qiagen), according to the manufacturer's instructions and quantified using a NanoDrop 2000c (Thermo Scientific). The RNA was sequenced using a Illumina HiSeq 4000 instrument at the Transcriptome and Genome Analysis Laboratory of the Universitaetsmedizin Goettingen. The raw sequences were mapped to the Ensembl Human Genome Assembly GRCh38.p10 using StrangNGS software (http://www.strand-ngs.com/). After mapping and counting, the data were normalized using quantile using StrandNGS. The accession number for the RNA sequencing data reported in this paper is NCBI GEO: GSE102139.

Proteome and transcriptome data analysis

Bioinformatic data analysis was performed using Perseus software environment (Tyanova et al., 2016). Statistical analysis of transcriptome and proteome was performed on logarithmized intensities for transcripts/proteins identified in at least one experimental condition. Significantly modulated transcripts/proteins were identified by performing ANOVA or t-test with a permutation-based FDR cutoff of 0.05. Annotation matrix algorithm (Cox and Mann, 2012) was used to identify the gene ontology biological processes (GO-BP) significantly overrepresented in the datasets (Benjamini-Hochberg FDR lower than 0.02 or 0.05, as indicated in figure captions) and correlated these with an expression level score. The identification of GO-BP terms enriched in the top 1000 principal component analysis loadings was performed using DAVID 6.8 (Huang et al., 2009). The pathway analysis, transcription factor activity prediction and transcriptional network analysis were performed using Ingenuity Pathway Analysis (IPA) as described (Murdoch et al., 2016; Raimundo et al., 2012). Datasets of hiPSC-NPC 2D differentiation were downloaded from GSE71289. Datasets containing samples from hPSC, embryoid bodies, cortical organoids and fetal cortex (male, 19 weeks gestation) were downloaded from GSE82022. Batch normalization was performed using ComBat function (Johnson et al., 2007) on Surrogate Variable Analysis R package within R/Bioconductor (Gentleman et al., 2004). BrainSpan Developmental Transcriptome Dataset (publicly accessible via the Allen Brain Atlas data portal - www.brainspan.org) was used for correlation analysis between the transcriptome profile of neurospheroids and human brain tissue samples. Batch normalization was performed using ComBat function in R and correlation coefficients were determined using CORREL function of Microsoft Excel. Each neurospheroid sample at day 30 was compared with each tissue sample and correlation coefficients for the four neurospheroid samples were averaged. Data was then averaged for brain region, excluding regions with less than 20 samples.

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