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Supplemental Information

Human iPSC-Derived Neurons and Cerebral Organoids Establish Differential Effects of Germline *NF1* Gene Mutations

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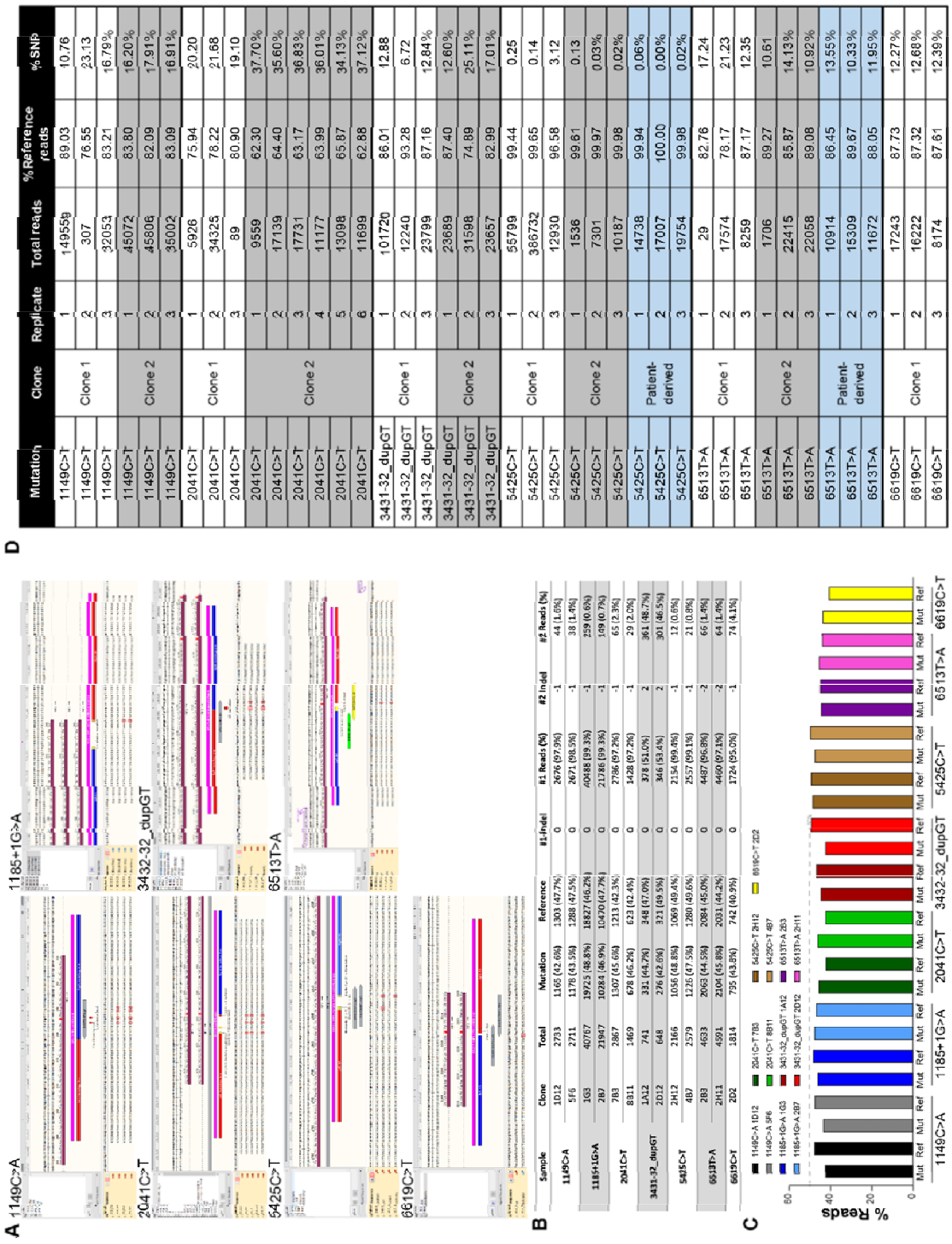

Mutation	Clone	Replicate	Total reads	% Reference reads	% SNP
1149C>T	Clone 1	1	149559	89.03	10.76
1149C>T		2	307	76.55	23.13
1149C>T		3	32053	83.21	16.79%
1149C>T	Clone 2	1	45072	83.80	16.20%
1149C>T		2	45806	82.09	17.91%
1149C>T		3	35002	83.09	16.91%
2041C>T	Clone 1	1	5926	75.94	20.20
2041C>T		2	34325	78.22	21.68
2041C>T		3	89	80.90	19.10
2041C>T	Clone 2	1	9559	62.30	37.70%
2041C>T		2	17139	64.40	35.60%
2041C>T		3	17731	63.17	36.83%
2041C>T		4	11177	63.99	36.01%
2041C>T		5	13098	65.87	34.13%
2041C>T		6	11699	62.88	37.12%
3431-32_dupGT	Clone 1	1	101720	86.01	12.88
3431-32_dupGT		2	12240	93.28	6.72
3431-32_dupGT		3	23799	87.16	12.84%
3431-32_dupGT	Clone 2	1	23689	87.40	12.60%
3431-32_dupGT		2	31598	74.89	25.11%
3431-32_dupGT		3	23657	82.99	17.01%
5425C>T	Clone 1	1	55799	99.44	0.25
5425C>T		2	386732	99.65	0.14
5425C>T		3	12930	96.58	3.12
5425C>T	Clone 2	1	1536	99.61	0.13
5425C>T		2	7301	99.97	0.03%
5425C>T		3	10187	99.98	0.02%
5425C>T	Patient-derived	1	14738	99.94	0.06%
5425C>T		2	17007	100.00	0.00%
5425C>T		3	19754	99.98	0.02%
6513T>A	Clone 1	1	29	82.76	17.24
6513T>A		2	17574	78.17	21.23
6513T>A		3	8259	87.17	12.35
6513T>A	Clone 2	1	1706	89.27	10.61
6513T>A		2	22415	85.87	14.13%
6513T>A		3	22058	89.08	10.92%
6513T>A	Patient-derived	1	10914	86.45	13.55%
6513T>A		2	15309	89.67	10.33%
6513T>A		3	11672	88.05	11.95%
6619C>T	Clone 1	1	17243	87.73	12.27%
6619C>T		2	16222	87.32	12.68%
6619C>T		3	8174	87.61	12.39%

Figure S1. Sequencing of *NF1*-mutant hiPSCs. (**A**) Snapgene view of NGS sequencing of all *NF1*-mutant hiPSC clones. (Block only: control; Block/mod: introduced *NF1* gene mutation). (**B-C**) Table and histogram summarizing the percentage of sequence reads detected for the mutant and reference (wild-type) alleles at the mutation site at the genomic level. (**D**) Analysis of reference and mutant (SNP) allele expression in each *NF1*-mutant hiPSC line at the RNA level demonstrates that the wild-type reference allele is over-represented in all *NF1*-mutant hiPSCs relative to the mutation-bearing (SNP) allele. Data are represented as the percentages of reference and mutant reads relative to the total reads.

Figure S2. Analysis of isogenic hiPSCs, NPCs, and cerebral organoids.

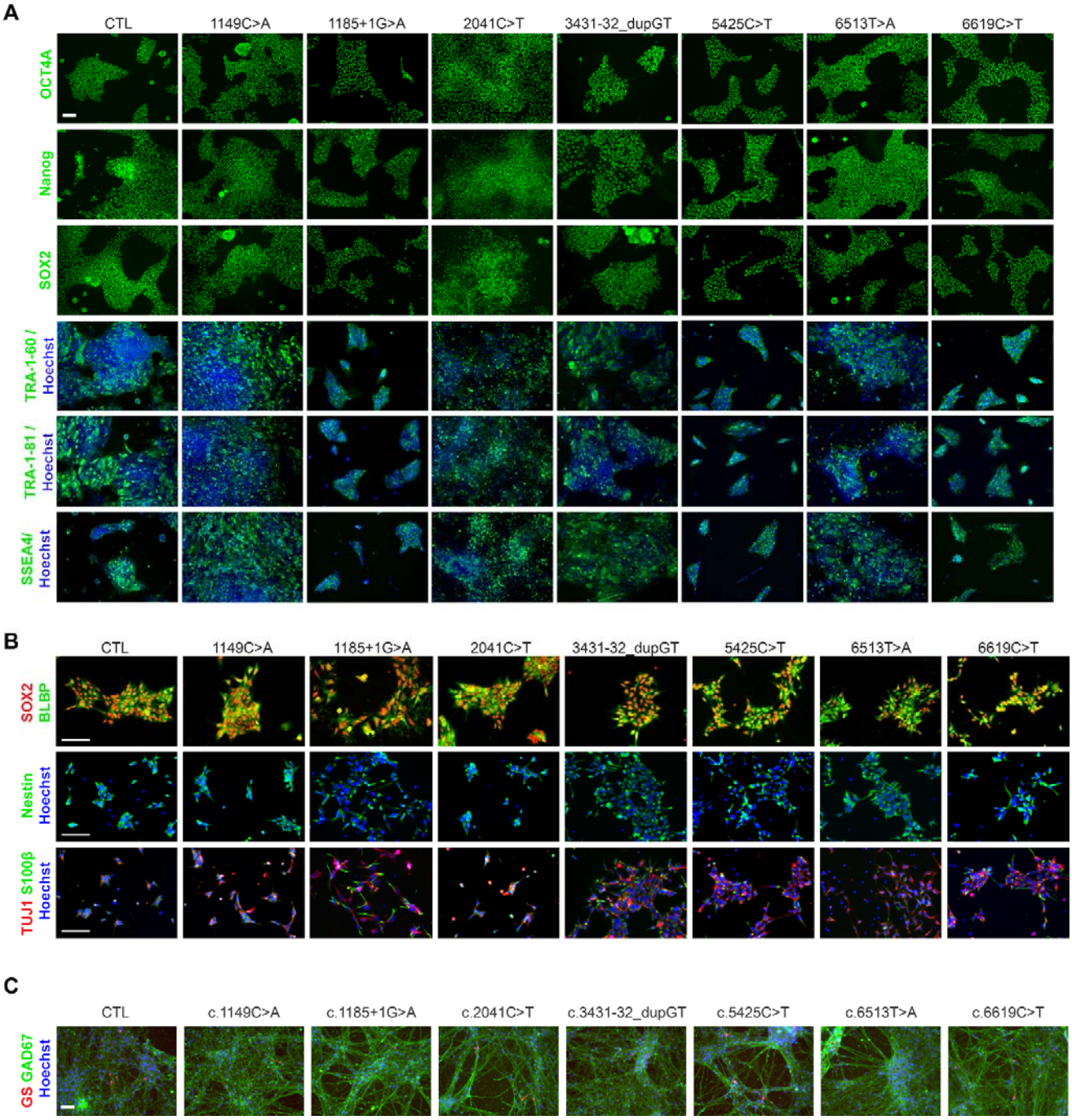

Figure S2. (**A**) Immunofluorescence analysis of all hiPSCs with the NANOG, SOX2, OCT4A, SSEA-4, TRA-1-60, and TRA-1-81 pluripotency markers. Scale bar, 100μm. (**B**) NPCs were

immunopositive for the SOX2, BLBP and Nestin neural stem cell markers (top two panels), and were multipotent, as illustrated by $TUJ1^+$ and $S100\beta^+$ double labeling (bottom panel). Scale bars, 100μm. (**C**) Control and *NF1*-mutant NPC-differentiated GABAergic neuronal cultures were immunopositive for GAD67 (green) and immunonegative for the excitatory neuron marker glutamate synthetase (GS; red). Scale bar, 100μm.

Figure S3. Comparisons between isogenic and patient-derived *NF1*-mutant hiPSC-NPCs.

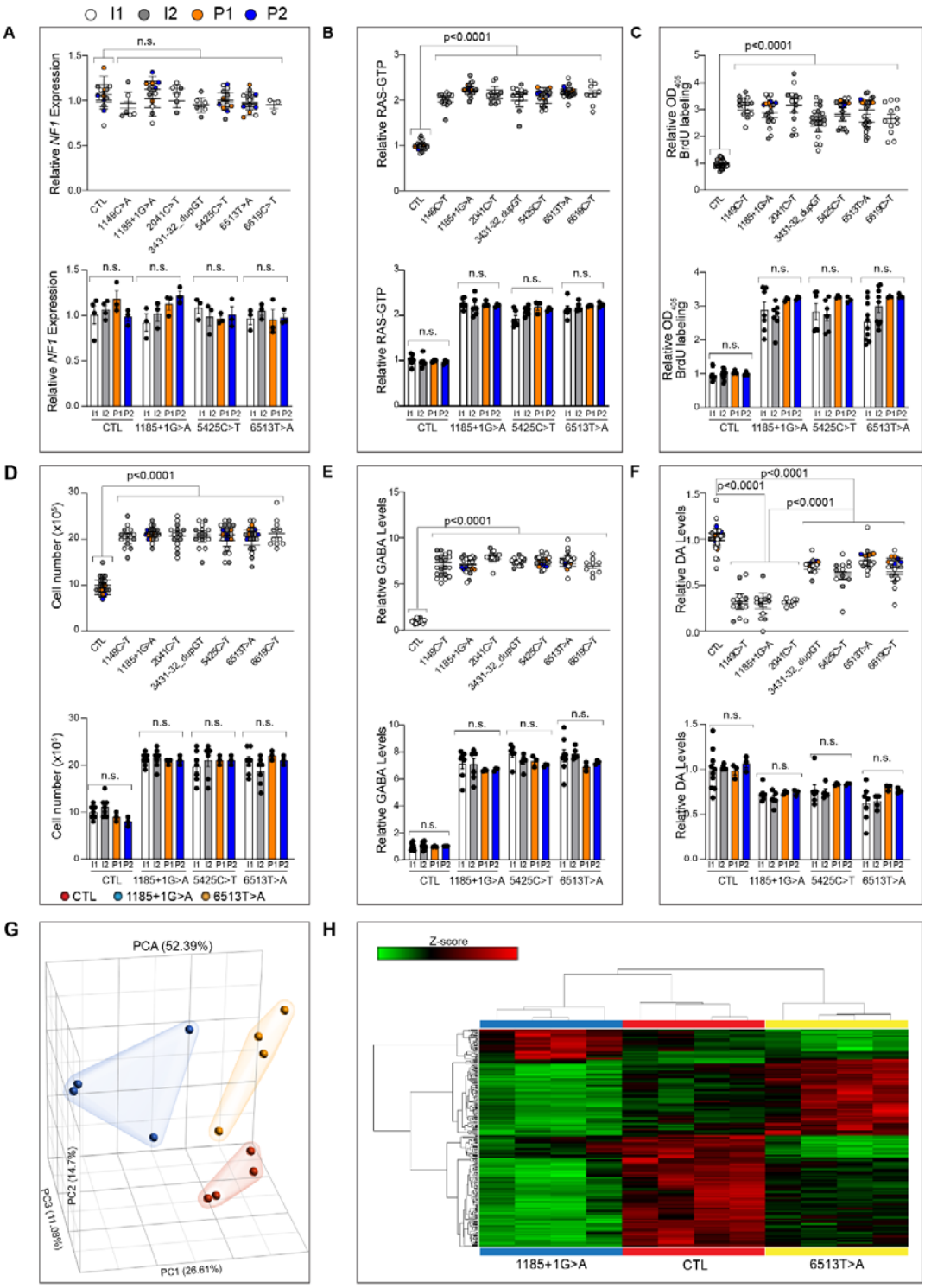

Figure S3. (**A**) Relative *NF1* mRNA expression, (**B**) RAS activity, (**C**) BrdU incorporation, and (**D**) cell numbers are similar between the isogenic and their respective patient-derived NPC lines that harbor the same *NF1* gene mutation. The top panels illustrate all the clones (Isogenic clones: I1, I2; white, grey circles, respectively; Patient-derived clones: P1, P2; orange and blue circles, respectively) employed for each assay, and represent comparisons between all *NF1*-mutant NPCs and controls. The bottom panels illustrate the individual comparisons between the isogenic (I1, I2) and their respective patient-derived (P1, P2) NPCs (CTL; 1185+1G>A; 5425C>T; 6513T>A). Relative (**E**) GABA levels and (**F**) DA levels are similar between the isogenic and their respective patient-derived NPC lines harboring the same *NF1* gene mutation. The top panels illustrate all the clones (Isogenic clones: I1, I2; white, grey circles, respectively; Patient-derived clones: P1, P2; orange and blue circles, respectively) employed for each assay, and represent the comparisons between all *NF1*-mutant NPCs and controls. The bottom panels illustrate the individual comparisons between the isogenic (I1, I2) and their respective patient-derived (P1, P2) NPCs (CTL; 1185+1G>A; 5425C>T; 6513T>A). All data are represented as means ± SEM; One-way ANOVA with Tukey post-test. ns, not significant. (**G**) PCA plot and (**H**) histogram analysis illustrate differential clustering of gene expression between patient-derived and isogenic NPCs harboring two separate *NF1* mutations (1185+1G>A; 6513T>A) or no *NF1* mutation (control; CTL), following next-generation sequencing.

Figure S4. Comparisons between isogenic and patient-derived *NF1*-mutant hiPSC-organoids.

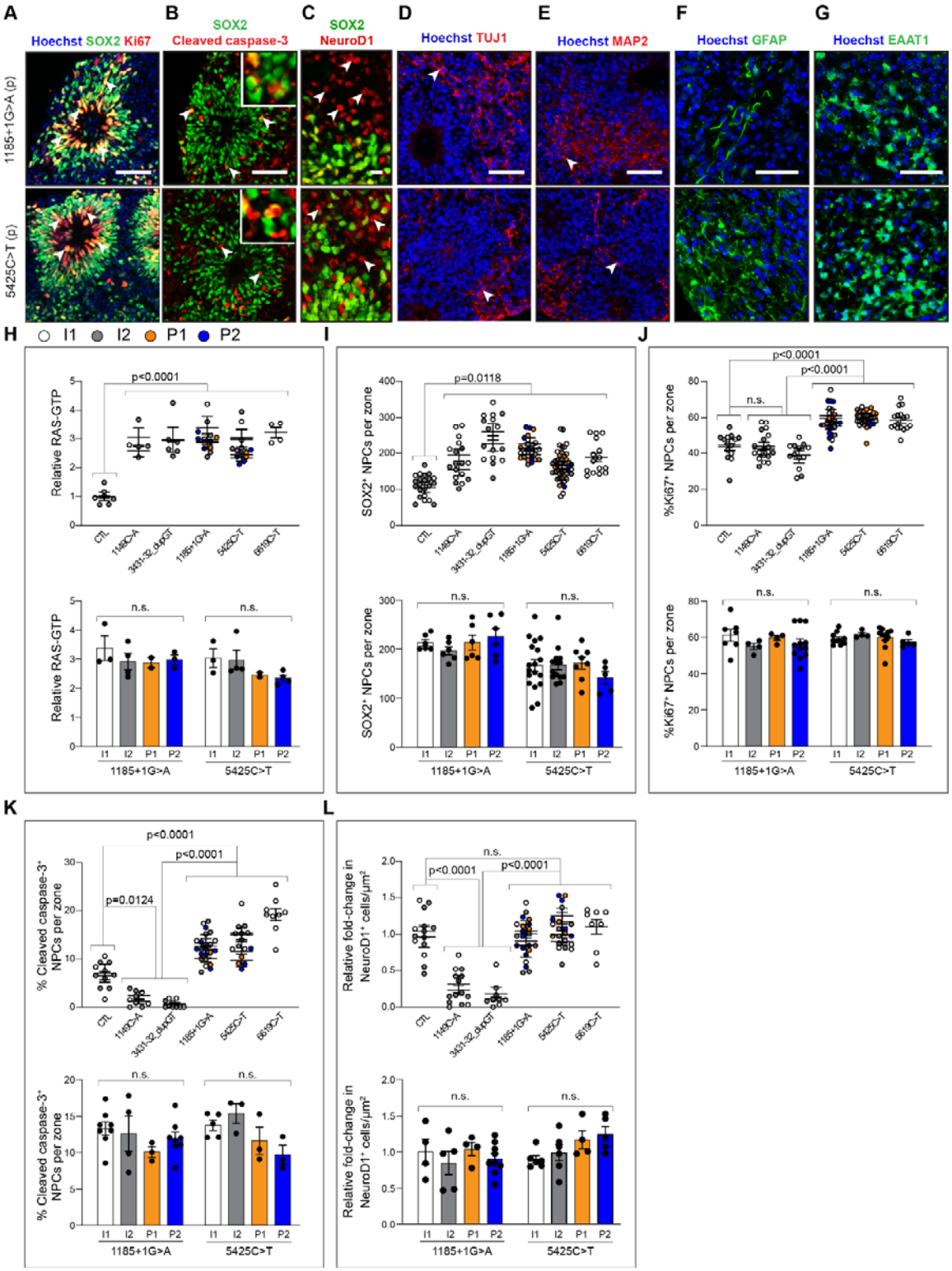

Figure S4. Representative images of (**A**) NPC proliferation (Ki67$^+$ cells; white arrowheads), (**B**) NPC apoptosis (cleaved caspase-3; white arrowheads), (**C, D**) early immature neurons (NeuroD1; TUJ1; white arrowheads), (**E**) late immature neurons (MAP2; white arrowheads) at 16DIV, and the production of (**F**) EAAT1$^+$ glial cells and (**G**) GFAP$^+$ fibers at 56DIV in cerebral organoids generated from 1185+1G>A and 5425C>T patient-derived hiPSC lines. Scale bars: (**C**) 10μm, (**A, B, D, E, F, G**) 50μm. There were no differences in (**H**) RAS activity or (**I**) SOX2$^+$ NPCs per ventricular zone (VZ) between the isogenic *NF1*-mutant cerebral organoids and their corresponding patient-derived at 16DIV. There were no differences in (**J**) %Ki67$^+$ NPCs per VZ, (K) %cleaved caspase-3$^+$ NPCs per VZ, or (**L**) NeuroD1$^+$ immature neurons between the isogenic *NF1*-mutant and their corresponding patient-derived cerebral organoids at 16DIV. The top panels illustrate all the clones (Isogenic clones: I1, I2; white, grey circles, respectively; Patient-derived clones: P1, P2; orange and blue circles, respectively) employed for each assay, and represent the comparisons between all *NF1*-mutant organoids and controls. The bottom panels illustrate the individual comparisons between the isogenic (I1, I2) and their respective patient-derived (P1, P2) NPCs (1185+1G>A; 5425C>T). All data are represented as means ± SEM; One-way ANOVA with Tukey post-test. ns, not significant.

Table S1. Inter-clone analysis of control and *NF1*-mutant hiPSC-derived cells and organoids.

	NPCs *NF1* Relative Expression		Isogenic NPCs *			Isogenic Astrocytes *		
	Isogenic *	Isogenic vs Patient-derived **	RAS assays	BrdU Assays	Direct cell count	RAS assays	BrdU Assays	Direct cell count
CTL	0.598	0.479 / 0.634	0.559	0.929	0.258	0.317	0.566	0.775
1149C>T	0.153	n/a	0.383	0.155	0.657	0.604	0.395	0.690
1185+1G>A	0.953	0.046 / 0.956	0.564	0.552	0.758	0.532	0.504	0.563
2041C>T	0.381	n/a	0.078	0.939	>0.999	0.847	0.543	0.142
3431-32_dupGT	0.570	n/a	0.363	0.146	0.686	0.709	0.426	0.553
5425C>T	0.151	3.380 / 0.104	0.065	0.785	0.491	0.742	0.458	0.060
6513T>A	0.846	0.941 / 0.441	0.590	0.093	0.204	0.148	0.096	>0.999

	Isogenic vs Patient-derived NPCs **			NPCs GABA		NPCs DA	
	RAS assays	BrdU Assays	Direct cell count	Isogenic *	Isogenic vs Patient-derived **	Isogenic *	Isogenic vs Patient-derived **
CTL	0.309 / 0.742	0.593 / 0.573	1.207 / 0.343	0.598	0.479 / 0.634	0.121	0.925 / 0.431
1149C>T	n/a	n/a	n/a	0.153	n/a	0.716	n/a
1185+1G>A	0.372 / 0.704	1.399 / 0.318	2.600 / 0.154	0.953	0.046 / 0.956	0.371	0.017 / 0.984
2041C>T	n/a	n/a	n/a	0.381	n/a	0.963	n/a
3431-32_dupGT	n/a	n/a	n/a	0.570	n/a	0.879	n/a
5425C>T	0.598 / 0.580	1.058 / 0.404	1.000 / 0.422	0.151	3.380 / 0.104	0.766	0.188 / 0.833
6513T>A	1.289 / 0.322	3.975 / 0.058	0.500 / 0.622	0.846	0.941 / 0.441	0.469	0.986 / 0.410

	Isogenic Organoids *					Isogenic vs Patient-derived **				
	Ki67$^+$	RAS assays	NSCs per PZ	NeuroD1$^+$	Cleaved Caspase 3$^+$	Ki67$^+$	RAS assays	NSCs per PZ	NeuroD1$^+$	Cleaved Caspase 3$^+$
CTL	0.803	0.943	0.367	0.651	0.603	n/a	n/a	n/a	n/a	n/a
1149C>T	0.711	0.354	0.125	0.517	0.433	n/a	n/a	n/a	n/a	n/a
1185+1G>A	0.217	0.383	0.138	0.527	0.784	0.830/ 0.491	0.561/ 0.656	0.632/ 0.599	0.488/ 0.695	0.838/ 0.491
3431-32_dupGT	0.944	0.951	0.291	0.636	0.895	n/a	n/a	n/a	n/a	n/a
5425C>T	0.216	0.909	0.982	0.466	0.288	0.780/ 0.516	1.850/ 0.208	0.632/ 0.599	2.675/ 0.080	4.029/ 0.037

Table S1. Table detailing the inter-clone analyses of isogenic iPSC-derived NPCs and organoids, as well as isogenic versus patient-derived hiPSC-derived NPCs and organoids harboring the same germline *NF1* mutations. There are no statistically significant differences in the relative *NF1* mRNA expression in NPCs, RAS activity, BrdU incorporation, or cell number in NPCs or astrocytes, GABA and dopamine (DA) levels in NPCs, %Ki67$^+$ progenitor cells per ventricular zone (VZ), RAS activity, NPCs per ventricular zone (VZ), NeuroD1$^+$ cells or %cleaved caspase-3$^+$ progenitor cells per VZ in cerebral organoids. *t-test; P-values reported, **One-Way ANOVA; F / P-values reported, respectively. n/a: not applicable.

Table S2. Primary antibodies used.

Antibody	Vendor	Catalog No	Host	Application (dilution)
BLBP	Millipore	ABN14	Rabbit	ICC (1:200)
Cleaved caspase-3	Cell Signaling	9664	Rabbit	IF (1:250)
EAAT1	Abcam	ab416	Rabbit	ICC, IF (1:500)
EAAT2	Abcam	ab41621	Rabbit	ICC (1:500)
GFAP	Abcam	ab4648	Mouse	ICC, IF (1:500)
Ki67	Fisher Scientific	BDB556003	Mouse	IF (1:100)
MAP2	Abcam	ab11267	Mouse	IF (1:500)
Nanog (D73G4)	Cell Signaling	9656S	Rabbit	ICC (1:200)
Nestin	Abcam	ab92391	Rabbit	ICC (1:250)
NeuroD1	Abcam	ab60704	Mouse	IF (1:500)
Oct-4A (C30A3)	Cell Signaling	9656S	Rabbit	ICC (1:200)
S100β	Abcam	ab41548	Rabbit	ICC (1:200)
SOX2	Cell Signaling	4900S	Mouse	ICC (1:1000); IF (1:250)
Sox2 (D6D9)	Cell Signaling	9656S	Rabbit	ICC (1:200)
SOX2	Abcam	ab92494	Rabbit	IF (1:250)
SSEA4 (MC813)	Cell Signaling	9656S	Mouse	ICC (1:200)
TRA-1-60(S)	Cell Signaling	9656S	Mouse	ICC (1:200)
TRA-1-81	Cell Signaling	9656S	Mouse	ICC (1:200)
TUJ-1	Abcam	ab78078	Mouse	ICC (1:1000)

Supplemental Methods

Next Generation RNA Sequencing and Analysis. RNA was extracted from three independently-generated samples of isogenic CTL or *NF1*-mutant (1185+1G>A; 6513T>A) NPCs, and one sample each from the non-isogenic CTL and patient-derived *NF1*-mutant NPCs harboring the same *NF1* mutations. Samples were prepared according to library kit manufacturer's protocol, indexed, pooled, and sequenced on an Illumina HiSeq. Basecalls and demultiplexing were performed with Illumina's bcl2fastq software and a custom python demultiplexing program with a maximum of one mismatch in the indexing read. RNA-seq reads were then aligned to the Ensembl release 76 primary assembly with STAR version 2.5.1a. Gene counts were derived from the number of uniquely aligned unambiguous reads by Subread:featureCount version 1.4.6-p5. Isoform expressions of known Ensembl transcripts were estimated with Salmon version 0.8.2. Sequencing performance was assessed for the total number of aligned reads, total number of uniquely aligned reads, and features detected. The ribosomal fraction, known junction saturation, and read distribution over known gene models were quantified with RSeQC version 2.6.2. The raw gene count matrix was then imported into Partek Flow software, version 8.0. Normalization size factors were calculated for all gene counts by CPM to adjust for differences in sequencing depth. Ribosomal genes and genes not expressed in the smallest group size minus samples greater than one count-per-million were excluded from further analysis. Gene-specific analysis was then performed using the lognormal with shrinkage model (limma-trend method) to analyze for differential expression between the three groups of samples. Principle component analysis (PCA) was conducted in Partek Flow using normalized gene counts. The "grouping" is simply a post hoc highlighting of the genotypes for assistance in visualizing that the different samples clustered together by genotype during the principle component. For further visualization, a heatmap was generated using the differential genes for each group filtered at p-values ≤ 0.05 and log fold-changes more extreme or equal to ±2.

Features and samples were clustered using Pearson Correlation as a distance metric. Deep sequencing data has been submitted to GEO with accession number GSE144601.

Allele-Specific Analysis Primers Used. The primers used for the first PCR reaction including the Illumina adaptor sequences were the following:

1149C>A FW: GTGACTGGAGTTCAGACGTGTGCTCTTCCGATCTCTACTTGTTCAGTCCATGGTGG

1149C>A REV: ACACTCTTTCCCTACACGACGCTCTTCCGATCTCCAATGCGGAATTGGTGATGA

2041C>T FW: GTGACTGGAGTTCAGACGTGTGCTCTTCCGATCTAATTACTACGTACTCCTGGAGC

2041C>T REV: ACACTCTTTCCCTACACGACGCTCTTCCGATCTGCAAGAGGTTATGCACTGAC

3431-32_dupGT FW: GTGACTGGAGTTCAGACGTGTGCTCTTCCGATCTGACTGCAGTGAAGTTGA AGATG

3431-32_dupGT REV: ACACTCTTTCCCTACACGACGCTCTTCCGATCTGTAGCTCTTGTCTGGAGAT CC

5425C>T FW: GTGACTGGAGTTCAGACGTGTGCTCTTCCGATCTGAAGCCATTGTCCAGTCTATC

5425C>T REV: ACACTCTTTCCCTACACGACGCTCTTCCGATCTGGTACAAGTTAAGGCACACAG

6513T>A FW: GTGACTGGAGTTCAGACGTGTGCTCTTCCGATCTGACTCAGTCTGACAGAGTTCTC

6513T>A REV: ACACTCTTTCCCTACACGACGCTCTTCCGATCTCTAGTTCTGTCCACTGGTCC

6619C>T FW: GTGACTGGAGTTCAGACGTGTGCTCTTCCGATCTTGCCTTCCGTTCCAGTTACC

6619C>T REV: ACACTCTTTCCCTACACGACGCTCTTCCGATCTGAGCTCTTGGTTGCAGGGAT

The primers used for the second amplification PCR reaction which include the unique indexes employed to identify different sequencing products were the following:

Primer 1.0 FW: AATGATACGGCGACCACCGAGATCTACACACACTCTTTCCCTACACGACGCTCTTC CGATCT

Primer 1.0 SIC2 FW: AATGATACGGCGACCACCGAGATCTACACAATGAAACACTCTTTCCCTACA CGACGCTCTTCCGATCT

Common REV: GTGACTGGAGTTCAGACGTGTGCTCTTCCGATCT

Index 1 REV: CAAGCAGAAGACGGCATACGAGATAAATAGATGGTGACTGGAGTTC

Index 2 REV: CAAGCAGAAGACGGCATACGAGATCAGAGGAGAGTGACTGGAGTTC

Index 3 REV: CAAGCAGAAGACGGCATACGAGATCGTATAGATGTGACTGGAGTTC

Index 4 REV: CAAGCAGAAGACGGCATACGAGATAAGGGCTCAGTGACTGGAGTTC