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

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SUMMARY

Neurofibromatosis type 1 (NF1) is a common neurodevelopmental disorder caused by a spectrum of distinct germline NF1 gene mutations, traditionally viewed as equivalent loss-of-function alleles. To specifically address the issue of mutational equivalency in a disease with considerable clinical heterogeneity, we engineered seven isogenic human induced pluripotent stem cell lines, each with a different NF1 patient NF1 mutation, to identify potential differential effects of NF1 mutations on human central nervous system cells and tissues. Although all mutations increased proliferation and RAS activity in 2D neural progenitor cells (NPCs) and astrocytes, we observed striking differences between NF1 mutations on 2D NPC dopamine levels, and 3D NPC proliferation, apoptosis, and neuronal differentiation in developing cerebral organoids. Together, these findings demonstrate differential effects of NF1 gene mutations at the cellular and tissue levels, suggesting that the germline NF1 gene mutation is one factor that underlies clinical variability.

INTRODUCTION

Neurofibromatosis 1 (NF1; OMIM 162200) is a neurogenetic condition characterized by remarkable phenotypic variability, where affected children develop a wide variety of central nervous system (CNS) pathologies, ranging from brain tumors and motor delays to learning difficulties, attention deficits, and autism (Fisher et al., 2018; Hyman et al., 2006; Jett and Friedman, 2010; Korf, 2013; Morris and Gutmann, 2018). One of many potential factors underlying this clinical variability could be the specific NF1 germline mutation, a notion suggested by population-based studies (Anastasaki et al., 2017; Bolcekova et al., 2013; Kehrer-Sawatzki et al., 2017; Koczkowska et al., 2018; Pinna et al., 2015; Rojnueangnit et al., 2015; Sharif et al., 2011; Trevisson et al., 2019; Upadhaya et al., 2007). For example, patients harboring the c.2970–2971_delAAT, c.5425C > T, and c.3112A > G NF1 germline mutations lack dermal and plexiform neurofibromas, the signature peripheral nervous system tumors in NF1 (Pinna et al., 2015; Trevisson et al., 2019; Upadhaya et al., 2007).

Although these studies raise the possibility that not all NF1 gene mutations are functionally equivalent, they do not establish differential effects of NF1 patient germline mutations at the cellular or tissue levels, a critical step in interpreting the significance of reported genotype-phenotype associations. To specifically evaluate differential NF1 mutation effects on human CNS cells and tissues, while controlling for important confounding factors (e.g., sex, genomic differences), we generated an isogenic series of human induced pluripotent stem cells (hiPSCs) harboring seven representative NF1-patient NF1 mutations.

RESULTS AND DISCUSSION

Generation of Isogenic NF1-Mutant hiPSCs

The seven NF1 pathogenic mutations, derived from patients in our clinic population at Washington University/St. Louis Children’s Hospital, represent the spectrum of mutations typically seen in individuals with NF1. In this regard, the selected mutations were interspersed throughout the NF1 protein (neurofibromin) coding sequence, were both proximal and distal to the well-characterized RAS GTPase-activating protein (RAS-GAP) domain (GRD), and included four nonsense (c.1149C > A, c.2041C > T, c.6513T > A, c.6619C > T), one splice site (c.1185+1G > A), one missense (c.5425C > T), and one frameshift (c.3431-32_dupGT) mutation (Figures 1A, S1, and S2). All of the engineered isogenic hiPSCs harbored only a single NF1 mutation (“NF1-mutant”), retained expression of the remaining wild-type NF1 allele as confirmed by DNA and RNA sequencing (Figure S1), and expressed similar levels of NF1 mRNA (Figure 1B). For all hiPSC lines with two clones, identical results were obtained using numerous independently generated biological replicates, as well as with three NF1 patient-derived hiPSC lines generated from somatic cells (fibroblasts; Figure S3, Table S1).
A. NF1 gene
- c.2041C>T  p.Arg681X
- c.3431-32_dupGT  p.Thr1145Val_FS
- c.5425C>T  p.Arg1809Cys
- c.6513T>A  p.Tyr2171X
- c.6619C>T  p.Gln2207X
- c.1185+1G>A  skip exon 10

B. NFI (R.E.)
- Clones 1 and 2

C. Relative RAS-GTP
- Clones 1 and 2

D. Relative RAS-GTP
- Vehicle
- 10μg/mL FGF

E. Relative RAS-GTP
- Clones 1 and 2

F. Relative OD405 BrdU Labeling
- Clones 1 and 2

G. Cell Number (x10^5)
- Clones 1 and 2

H. Embryoid bodies
- CTL 1149C>A 1185+1G>A 3431-32_dupGT 5425C>T 6619C>T

I. Relative RAS-GTP
- Clones 1 and 2

J. SOX2+ NPCs per zone
- Clones 1 and 2

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Isogenic NF1-Mutant hiPSC-Derived NPCs and Astroglia Have Increased RAS Activity and Proliferation

To determine the consequence of the different NF1 gene mutations on neurofibromin signal transduction and function in human CNS cells, NF1-mutant and control hiPSCs were first differentiated into neural progenitor cells (NPCs) capable of generating both neurons (TUJ1+ cells) and glia (S100β cells) (Figure S2B). Because neurofibromin primarily functions as an RAS-GAP to control cell proliferation, we initially assessed RAS activity. Consistent with this negative RAS regulatory property, all NF1-mutant NPCs exhibited a comparable 1.8- to 2.2-fold increase in RAS-GTP relative to the isogenic control (Figure 1C). Importantly, the addition of growth factors (fibroblast growth factor [FGF] or brain-derived neurotrophic factor [BDNF]) did not further increase RAS activity in the NF1-mutant lines, but resulted in greater RAS-GTP levels in the control lines, equivalent to the levels observed in the unstimulated NF1-mutant lines (Figures 1D and 1E). These findings demonstrate that a heterozygous NF1 mutation phenocopies the effect of exogenous growth factor stimulation on RAS activation. In addition, all NF1-mutant NPCs exhibited increased cell division, as demonstrated by increased bromodeoxyuridine (BrdU) incorporation (2.6- to 3.2-fold increase; Figure 1F) and total cell number (1.9- to 2-fold increase; Figure 1G).

To evaluate the effects of distinct NF1 gene mutations on the production of NPCs in a 3D model of human brain development, we generated cerebral organoids from the control and NF1-mutant hiPSC lines. Despite repeated efforts, we were unable to derive organoids from two of the seven NF1-mutant hiPSC lines (c.2041C > T and c.6513T > A), but successfully generated organoids from the control and five of the seven NF1-mutant hiPSC lines (c.1149C > A, c.1185+1G > A, c.3431-32dupGT, c.5425C > T, c.6619C > T; Figure 1H). The organoids formed radially organized ventricle-like structures populated by SOX2+ NPCs by 16 days in vitro (DIV). Similar to the 2D cultures, all NF1-mutant organoids exhibited a 2.8- to 3.2-fold increase in RAS activity (Figure 1I), as well as a 1.6- to 2.2-fold increase in total NPCs per ventricular zone at 16 DIV (Figure 1J).

Next, we sought to determine whether these heterogeneous NF1 mutational effects were observed in another proliferating CNS cell type by differentiating the NPCs into astrocytes (Figure 2A). Similar to the NPCs, NF1-mutant astrocytes exhibited 2- to 2.3-fold increased RAS activity (Figure 2B), 2.3- to 2.7-fold increased cell division (Figure 2C), and 2.1- to 2.5-fold greater total cell number (Figure 2D) relative to the control line. Consistent with the 2D astrocytes, 56 DIV NF1-mutant organoids had more EAAT1- and GFAP-expressing cells (astrocytes) (Figure 2E) compared with control organoids. Importantly, isogenic NF1-mutant NPCs and organoids were similar to those of their respective patient-derived NPCs (c.1185+1G > A; c.5425C > T; c.6513T > A) and organoids (c.1185+1G > A; c.5425C > T) (Figures S3 and S4; Table S1) in RAS activity and NPC proliferation, as well as to whole-brain lysates from genetically engineered mice harboring the analogous germline NF1 gene mutations (c.1149C > A, c.2041C > T, c.3431-32dupGT, and c.5425C > T; Figure 3A). Taken together, these data illustrate that all heterozygous NF1 mutations increase RAS activity and RAS-regulated cell proliferation in both human and murine CNS cells.

Figure 1. Isogenic NF1-Mutant hiPSC-Derived NPCs Exhibit Increased RAS Activity and Cell Proliferation

(A) Schematic diagram illustrating the position of the engineered NF1 patient mutations within the NF1 gene. The location of the RAS-GAP domain is highlighted in black.

(B) Relative NF1 mRNA expression in isogenic NF1-mutant NPCs is similar to the controls.

(C–E) (C) Quantitation demonstrating increased RAS activity (RAS-GTP) in isogenic NF1-mutant NPCs relative to controls (CTL) before and after the addition of (D) 10 μg/mL FGF or (E) BDNF. A minimum of three independent replicates was performed for each treatment condition.

(F) BrdU incorporation is increased by 2.6- to 3.2-fold in NF1-mutant NPCs relative to control NPCs.

(G) 1.9- to 2-fold increases in total cell numbers were observed in NF1-mutant NPCs compared with controls.

(H) Representative bright-field images of embryoid bodies and cerebral organoids at 16 and 56 DIV.

(I and J) (I) Quantitation demonstrating increased RAS activity (2.8- to 3.2-fold) and (J) increased numbers of SOX2+ NPCs per ventricular zone (1.6- to 2.2-fold) in 16 DIV NF1-mutant cerebral organoids relative to control organoids. Each dot represents an independently generated data point derived from separate experiments and the two different clones for each line are denoted as black versus gray dots. All data are represented as means ± SEM. (B, C, F, G, I, and J) One-way ANOVA with Tukey post-test. (D and E) Two-way ANOVA with Bonferroni post-test. n.s., not significant. Scale bar, 1 mm.
Figure 2. hiPSC-Derived NF1-Mutant Astroglia Exhibit Increased RAS Activity and Cell Proliferation

(A) NF1-mutant and control NPCs were differentiated into GFAP⁺, S100⁺, EAAT1⁺, and EAAT2⁺ astrocytes in 2D cultures. Scale bar, 100 μm.

(B) RAS-GTP was increased by 2- to 2.3-fold in NF1-mutant astrocytes relative to controls (CTL).

(C) Proliferation of NF1-mutant astrocytes was 2.3- to 2.7-fold higher relative to controls.

(D) Direct cell counting demonstrated a 2.1- to 2.5-fold increase in NF1-mutant astrocytes compared with controls.

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GABA levels in NPC-derived GABAergic neurons (Figures 3B and S3E). In all NF1-mutant neurons (2D cultures), GABA levels were increased (6.5- to 7.8-fold) relative to isogenic control neurons, revealing a shared abnormality in all NF1-mutant GABAergic neurons.

In contrast, NF1-mutant NPCs in 2D cultures displayed striking differences in dopamine (DA) (Figure 3C) levels. DA levels were reduced by >70% in the c.1149C > A, c.2041C > T, and c.6619C > T NF1 mutants, but by <40% in the c.1185+1G > A, c.3431-32_dupGT, c.5425C > T, and c.6513T > A NF1 mutants relative to the control line. These differential effects mirror findings using patient-derived NPCs (Figures S3E and S3F; Table S1) (Anastasaki et al., 2015), as well as mice engineered with NF1 patient-specific Nf1 germline mutations (Figure 3D) (Toonen et al., 2016). Taken together, these findings demonstrate the existence of differential effects of NF1 germline mutations on neuronal differentiation in vitro.

Differential Effects of NF1 Mutations on Cerebral Organoid NPC Proliferation, Apoptosis and Differentiation

To further explore the differential effects of NF1 mutations in the developing human brain, we used the more contextually relevant cerebral organoid platform. Examination of NPC proliferation, apoptosis and neuronal differentiation in 16 DIV cerebral organoids revealed two distinct groups of NF1 mutants (Figure 4): group 1 (c.1185+1G > A; c.5425C > T; c.6619C > T) NF1 mutants exhibited increased NPC proliferation (1.3- to 1.4-fold) and apoptosis (2- to 3-fold), but had similar numbers of early (NeuroD1 +, TuJ1 +) and late (MAP2 +) immature neurons relative to controls. In this manner, group 1 NF1 mutations increased both proliferation and apoptosis during NPC differentiation, allowing neurogenesis to proceed normally.

In contrast, group 2 (c.1149C > A; c.3431-32_dupGT) NF1-mutant organoids had normal NPC proliferation, but reduced NPC apoptosis (70%–92% reduction) and very few immature neurons relative to the isogenic controls (73%–84% reduction). In this latter group, the reduction in NPC death was coupled with a delay in neurogenesis, suggesting that inappropriate survival of NPC subpopulations creates a barrier to initiating timely neuronal differentiation. Importantly, these observations persist in patient-derived cerebral organoids harboring the same mutations (Figure S4; Table S1).

(E) NF1-mutant cerebral organoids grown for 56 DIV had increased cells with GFAP+ fibers and increased numbers of EAAT1+ glial cells compared with isogenic controls. Scale bars, 50 μm.

Each dot represents an independently generated data point derived from separate experiments and the two different clones for each line are denoted as black versus gray dots. All data are represented as means ± SEM. One-way ANOVA with Tukey post-test.
Early immature neurons

Hoechst SOX2 Ki67

AB E D

SOX2 Cleaved caspase-3

Ventricular zone

Hoechst MAP2

Late immature neurons

NPC proliferation NPC apoptosis

Early immature neurons

Late immature neurons

NeuroD1 SOX2

Periventricular zone

Hoechst TUJ1

Hoechst MAP2

1149C>A

1185+1G>A

3431-32_dupGT

6619C>T

5425C>T

p<0.0001

p<0.0001

p<0.0001

n.s.

p=0.0103

p<0.0001

n.s.

p<0.0001

Clone 1

Clone 2

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Conclusions
The findings described in this report, in combination with compelling population-based genotype-phenotype associations, suggest that the germline NF1 gene mutation is one of the factors that underlies clinical heterogeneity in patients with NF1. Using an isogenic series of NF1-mutant hiPSC lines, we identified differential NF1 mutational effects on human CNS cells and tissues. Importantly, unlike previous studies, the use of an isogenic series of hiPSCs eliminates other contributing factors, such as sex and genomic variation (potential modifier genes), and permits a direct examination of the effects of different NF1 gene mutations. Moreover, this study raises several important points relevant to NF1 pathobiology.

First, we established that all heterozygous NF1 gene mutations similarly increase CNS NPC and astroglial cell proliferation and RAS activity, which is consistent with numerous reports demonstrating that neurofibromin controls cell proliferation largely by regulating RAS activity in mouse, swine, and Drosophila cells and tissues. Moreover, the regulation of RAS-mediated cell proliferation by neurofibromin is further supported by paired hiPSC-derived NPCs, heterozygous and homozygous for the same NF1 gene mutation, where a clear gene dose dependency was revealed (C. Anastasakis, Personal Communication). Therefore, the vast majority of human clinical trials for NF1-null tumors have appropriately used molecularly targeted therapies that inhibit RAS and RAS downstream effectors (e.g., MEK) (Dombi et al., 2016).

Second, we demonstrated differential effects of NF1 germline mutations on neuronal differentiation. These differential effects could reflect the fact that neurofibromin functions as a high-affinity dimer, where different mutations could change the overall architecture of the dimer interface (Sherekar et al., 2020). Because neurons from individuals with NF1 harbor only a single NF1 germline mutation, different NF1 mutations likely cause unique neuron-related pathologies. Therefore, the use of isogenic hiPSCs revealed differential effects of distinct NF1 gene mutations on NPC proliferation, apoptosis, and neuronal differentiation not previously reported in the developing Nfi-knockout (Nfi\textsuperscript{wt/neo}) mouse brain. Given the high degree of mutational specificity for autism symptomatology in children with NF1 (Morris and Gutmann, 2018), these findings suggest that investigations using Nfi mice with different patient germline Nfi mutations might uncover unique behavioral abnormalities not appreciated using conventional Nfi knockout mice (Costa et al., 2002; Omran et al., 2015) and identify causative underlying molecular mechanisms.

Third, the fact that the observed differences in neuronal differentiation in cerebral organoids and NPC DA levels do not correlate with RAS activation supports the existence of non-RAS-mediated neurofibromin functions. In this regard, neurofibromin also directly binds to several proteins important for neuronal differentiation, spinogenesis, and serotonin receptor activity, including collapsin response mediator protein-2 (Patrakitkomjorn et al., 2008), syndecan (Hsueh et al., 2001), and the 5-hydroxytryptamine-6 receptor (Deraredj Nadim et al., 2016), through domains distinct from the GRD. Moreover, the notion that non-RAS-mediated neurofibromin functions exist in neurons is reinforced by the presence of a neurofibromin isoform containing an additional amino terminal exon (11alt12), whose expression is restricted to postnatal brain neurons (Gutmann et al., 1999). Future investigations aimed at discovering novel neuron-specific neurofibromin binding partners will be critical to understanding how NF1 mutations differentially affect cognition and behavior in children with NF1.

Finally, although population and murine studies provided the first evidence for NF1 genotype-phenotype correlations, there had been no direct demonstration of the primary effect of the NF1 mutation at the cellular and tissue levels in humans. The use of this experimental human iPSC platform revealed NF1 mutational abnormalities in human NPCs and neurons. Collectively, these studies establish a foundational basis for future studies aimed at unraveling mechanistic etiologies responsible for NF1-specific CNS phenotypes, discovering new therapeutic targets, and assessing treatments relevant to precision medicine.

Figure 4. Differential Effects of NF1 Mutations on Cerebral Organoid Progenitor Cell Dynamics and Neurogenesis
(A and B) 50X\textsuperscript{2} NPCs in the ventricular zones of group 1 NF1-mutant cerebral organoids exhibit (A) 1.3- to 1.4-fold increased proliferation (Ki67\textsuperscript{+} cells; white arrowheads) and (B) 2- to 3-fold increased cell apoptosis (cleaved caspase-3; white arrowheads) compared with control and group 2 cerebral organoids at 16 DIV.
(C–E) Decreased numbers of (C and D) early immature neurons (NeuroD1; TUJ1 white arrowheads) and (E) late immature neurons (MAP2; white arrowheads) migrating into the periventricular zone of group 2 compared with group 1 and control cerebral organoids at 16 DIV.
(F) Quantifications of %Ki67\textsuperscript{+} NPCs, %cleaved caspase-3\textsuperscript{+} NPCs and NeuroD1\textsuperscript{+} immature neurons in NF1-mutant cerebral organoids compared with controls at 16 DIV.

Each dot represents an independently generated data point derived from separate experiments and the two different clones for each line are denoted as black versus gray dots. All data are represented as means ± SEM. One-way ANOVA with Dunnett post-test. n.s., not significant. Scale bars, 10 μm (C) and 50 μm (A, B, D, and E).
EXPERIMENTAL PROCEDURES

Human iPSCs
Seven distinct NF1-patient germline NF1 gene mutations (Transcript ID NM_000267; c.1149C > A, c.1185+1G > A, c.2041C > T, c.3431-32_dupGT, c.5425C > T, c.6513T > A, c.6619C > T) were individually engineered using CRISPR/Cas9 technology into a single commercially available male control human iPSC line (BJFF6) by the Washington University Genome Engineering and iPSC Core (GEIC) facility. Heterozygous mutations were confirmed by NGS sequencing (Bell et al., 2014), and two different clones were expanded for each of the six NF1-mutant and the control lines (Figures S1A–S1C). Only a single clone heterozygous for the c.6619C > T NF1 mutation could be generated without any additional genomic insertions or deletions. Retention of heterozygosity in the hiPSCs was confirmed by sequencing after five passages, as well as in all of the derivative cell lines by RAS activity assays. Similar results were obtained after each passage. In addition, iPSCs reprogrammed from the fibroblasts of three NF1 patients (c.1185+1G > A; c.5425C > T; c.6513T > A) and one control subject (Anastasaki et al., 2015) were used for subsequent analyses. For NPC differentiation, hiPSCs were passaged onto PLO/Laminin (Millipore Sigma)-coated plates using ReLeSR (STEMCELL Technologies), and seeded at 200,000 cells/cm² in NPC induction medium (50% DMEM F12 [Gibco], 50% Neurobasal medium [Gibco], supplemented with N2, B27 [Fisher], 2 mM GlutaMAX [Gibco], 10 ng/mL hLIF, 4 μM CHIR99021, 3 μM SB431541, and 0.1 μM Compound E [all from STEMCELL Technologies]). Cells were maintained in this medium supplemented with 2 μM dorsomorphin for 3 days and without dorsomorphin (STEMCELL Technologies) for an additional 5 days. NPCs were subsequently incubated in NPC maturation medium (50% DMEM/F12, 50% Neurobasal medium supplemented with N2, B27, 2 mM GlutaMAX, 10 ng/mL hLIF, 3 μM CHIR99021 and 2 μM SB431541), and were passaged weekly following Accutase (STEMCELL Technologies) dissociation according to the manufacturer’s instructions. NPCs were treated for 2.4 h with 10 μg/mL of FGF or BDNF (both STEMCELL Technologies) to assess growth factor-induced cell proliferation. GABAergic neurons were differentiated as described previously (Liu et al., 2013). For astrocytic differentiation, NPCs were plated on Primaria-coated plates in Astrocyte Growth Media (ScienCell) for a minimum of 2 weeks and a maximum of ten were plated on Primaria-coated plates in Astrocyte Growth Media (ScienCell) for a minimum of 2 weeks and a maximum of ten. Cerebral organoids were generated as described previously (Tcw et al., 2017). Cerebral organoids were maintained for up to 56 DIV. All experiments described previously (Toonen et al., 2016). Immunohistochemistry on cryosections of cerebral organoids was performed as described previously (Anastasaki et al., 2015) using the antibodies described (Table S2). RAS activity (Thermo Fisher Scientific), GABA, dopamine (both Rocky Mountain Diagnostics) detection (Anastasaki et al., 2015), BrdU proliferation assays (Roche), and direct cell counting were performed as described previously (Toonen et al., 2016). Immunohistochemistry on cryosections of cerebral organoids was performed as described previously (Sloan et al., 2018). A minimum of three independent samples representing different passages of two separate clones were used for each line.

Mice
All animals were maintained on an inbred C57BL/6 background using a 12-h light/dark cycle with ad libitum access to food and water. Heterozygous NF1 mice were generated to harbor point mutations corresponding to the human c.1149C > A, c.2041C > T, c.3431_32dupGT, and c.5425C > T mutations. The c.1149C > A, c.2041C > T, and c.3431_32dupGT mice were generated using CRISPR/Cas9 engineering on a C57BL/6 genetic background and heterozygous mutation was confirmed by direct sequencing. All mice were used in accordance with an approved Animal Studies Protocol at the Washington University School of Medicine.

Statistics
All statistical tests were performed using GraphPad Prism 5 software. We performed t tests, one- or two-way analysis of variance (ANOVA) with Dunnett or Bonferroni post-test correction using GraphPad Prism 5 software. Statistical significance was set at p < 0.05.

ACCESSION NUMBERS
The accession number for the deep sequencing data reported in this paper is GEO: GSE144601.
SUPPLEMENTAL INFORMATION

Supplemental Information can be found online at https://doi.org/10.1016/j.stemcr.2020.03.007.

AUTHOR CONTRIBUTIONS

C.A., M.L.W., and D.H.G. designed and analyzed the experiments. C.A., M.L.W., K.H., J.B.P., N.D.K., J.C., O.C., and J.D.D. conducted and/or interpreted the experiments. The article was assembled by C.A., M.L.W., and D.H.G. D.H.G. was responsible for the final production of the manuscript.

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REFERENCES


Supplemental Information

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**Supplementary Figures**

**Figure S1.** Isogenic *NF1*-mutant hiPSC sequencing and allele expression analysis of isogenic and patient-derived *NF1*-mutant hiPSCs.

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

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

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

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

**Table S2.** Primary antibodies used.

**Supplementary Methods**
Figure S1. Isogenic NF1-mutant hiPSC sequencing and allele expression analysis of isogenic and patient-derived NF1-mutant hiPSCs.
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 S100β+ 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.

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.

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<th>Antibody</th>
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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: GTGACTGGAGTTCCAGACGTTGCTCTCCGATCTCTACTTGGTCATGGTGG
1149C>A REV: AACTCTTTCCCTACACGAGCTCTCCGATCTCTCAATGGGATAGCTCTTCGATCT
2041C>T FW: GTGACTGGAGTTCCAGACGTTGCTCTCCGATCTCTATTACTACGACTTCCTGGGAC
2041C>T REV: AACTCTTTCCCTACACGAGCTCTCCGATCTCTGGCAAGAGGTATTAGCAGCTAC
3431-32_dupGT FW: GTGACTGGAGTTCCAGACGTTGCTCTCCGATCTCGACTGCAGTGAAGTTGAC
3431-32_dupGT REV: AACTCTTTCCCTACACGAGCTCTCCGATCTCGACTGGGTAGGAT
3431-32_dupGT REV: AACTCTTTCCCTACACGAGCTCTCCGATCTCGACTGTCCAGTCTATC
5425C>T FW: GTGACTGGAGTTCCAGACGTTGCTCTCCGATCTCTGGTACAAGTTCAGCACAG
5425C>T REV: AACTCTTTCCCTACACGAGCTCTCCGATCTCTGGTACAAGTTAAGGCACACAG
6513T>A FW: GTGACTGGAGTTCCAGACGTTGCTCTCCGATCTCTAGTTCTGGTCAGGGAT
6513T>A REV: AACTCTTTCCCTACACGAGCTCTCCGATCTCTGGTACAAGTTAAGGCACACAG
6619C>T FW: GTGACTGGAGTTCCAGACGTTGCTCTCCGATCTCTAGTTCTGGTCAGGGAT
6619C>T REV: AACTCTTTCCCTACACGAGCTCTCCGATCTCTAGTTCTGGTCAGGGAT

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: AATGATACGGCGACCACCCGAGATCTACACACACTCTTTCCCTACACGACGCTTC
Primer 1.0 SIC2 FW: AATGATACGGCGACCACCCGAGATCTACACACACTCTTTCCCTACACGACGCTTC

Common REV: GTGACTGGAGTTCCAGACGTTGCTCTCCGATCT
Index 1 REV: CAAGCAGAAGACGGCATAACAGAGATAGATGGTTAAGGGTACTGAGGT