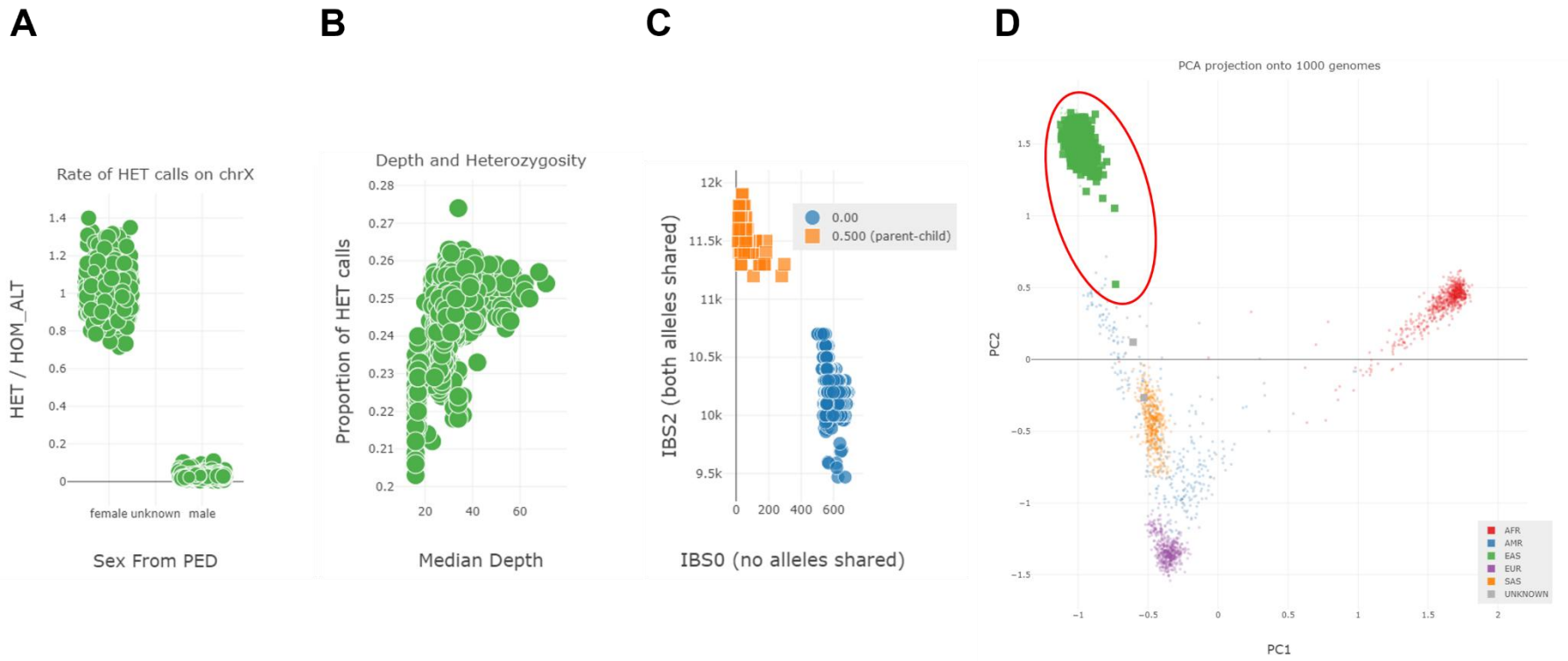
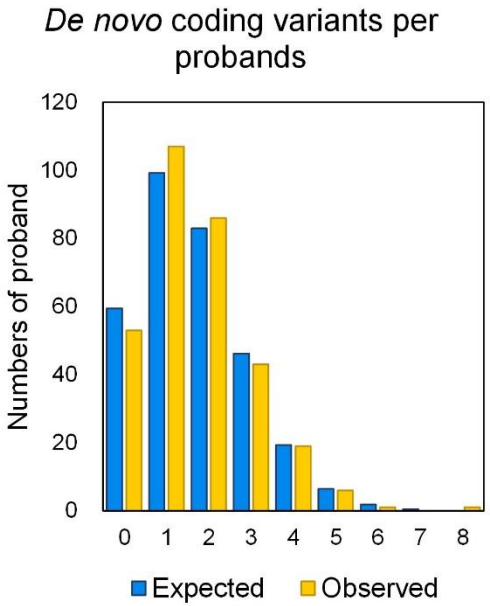


Fig. S1: Quality control and detection of sample anomalies using Peddy.

(A) The rate of heterozygous calls on the X chromosome for the identification of sex. **(B)** Distribution of median depth for the heterozygous calls. **(C)** Relatedness checks using the IBS (identity-by-state) plot. **(D)** Bold green dots indicate ancestry analysis results on the principal component analysis (PCA) plot. The results show that there were no violations of sex, reported relatedness, and sample quality in the tested 1,050 exomes.



Variant class	Observed	Expected	Causative	Observed - Causative	(Observed / Expected) ratio	*P* value	(Observed - Causative / Expected) ratio	*P* value
Synonymous	112	106.32	1	111	1.05	0.303	1.04	0.338
Missense	324	238.8	69	255	1.36	9.57×10^{-8}	1.07	0.155
Loss-of-funtion (LOF)	73	33.12	35	38	2.2	1.54×10^{-9}	1.15	0.22
Missense + LOF	397	271.92	104	293	1.46	7.43×10^{-13}	1.08	0.107
Coding variant	524	378.36	106	418	1.38	8.58×10^{-13}	1.1	0.023

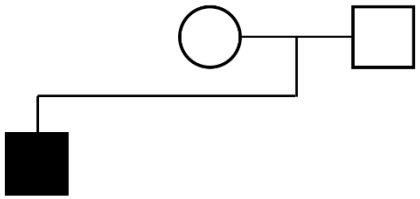
Fig. S2: *De novo* variant burden analysis in 316 trios.

We identified a total of 524 *de novo* protein-coding variants, comprising 468 single-nucleotide variants (SNVs), 55 insertions/deletions (indels), and one multi-nucleotide variant. The observed distribution of *de novo* variant counts per proband within coding regions closely aligns with the expected *Poisson* distribution. Importantly, causative variants showed a marked enrichment, particularly among loss-of-function variants, with a 2.20-fold increase ($P = 1.54 \times 10^{-9}$), predominantly driven by causative variants. These results highlight the significant role of *de novo* events in contributing to the burden of causative variants in this cohort.

MCPH020: Proband-only WES > Sanger PCR

HC Z score: -4.10

Global DD, Autistic behavior
Seizure, Visual defect

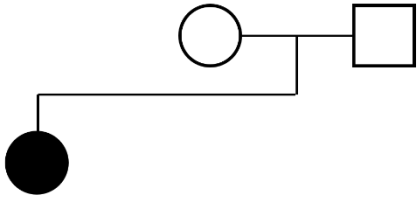


	MCPH020.1	MCPH020.2	MCPH020.3
***CTNNB1* c.1399C>T p.R467X**	C/**T**	C/C	C/C
***COL4A2* c.3089_3090del p.G1030Afs*41**	WT/**del**	WT/WT	WT/WT

MCPH034: Proband-only WES > Sanger PCR

HC Z score: -5.80

Simplified gyral pattern
Global DD/ID
Early-onset epileptic encephalopathy
Autistic behavior, Bruxism
Involuntary movement
Corneal opacity

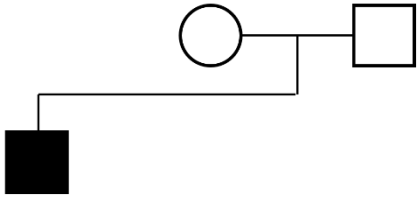


	MCPH034.1	MCPH034.2	MCPH034.3
***CYFIP2* c.260G>T p.R87L**	G/**T**	G/G	G/G
***TUBB3* c.1207+2T>G splicing defect**	T/**G**	T/T	T/T

MCPH094: Trio WES

HC Z score: -4.51

Agenesis of corpus callosum
Global DD, Seizure
Hearing abnormality

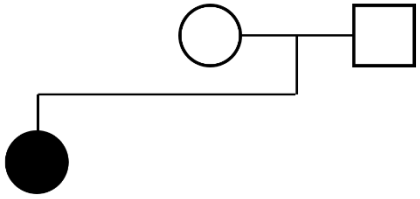


	MCPH094.1	MCPH094.2	MCPH094.3
***BPTF* deletion**	WT/**del**	WT/WT	WT/WT
***PAK3* c.1627C>G p.R543G**	**G**/-	C/C	C/-

MCPH202: Proband-only WES > Sanger PCR

HC Z score: -2.78

Diffuse brain atrophy
Severe retardation
Developmental arrest
Facial dysmorphism: large ear
Exotropia

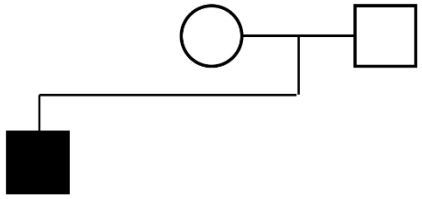


	MCPH202.1	MCPH202.2	MCPH202.3
***DHX30* c.2428C>T p.R810W**	C/**T**	C/C	C/C
***NALCN* c.3452T>G p.M1151R**	T/**G**	T/T	T/T

MCPH204: Proband-only WES > Sanger PCR

HC Z score: -2.88

Facial dysmorphism: coarse, large ear
Cerebellar atrophy
Global DD
Seizure
Infantile esotropia

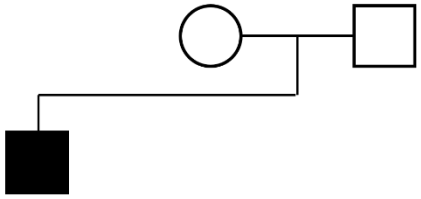


	MCPH204.1	MCPH204.2	MCPH204.3
***NALCN* c.4519_4520del p.Q1508Afs*20**	WT/**del**	WT/WT	WT/WT
***PMM2* c.307C>T p.R103X**	C/**T**	C/C	C/**T**
***PMM2* c.440G>C p.R147P**	G/**C**	G/**C**	G/G

MCPH223: Trio WES

HC Z score: -3.67

Facial dysmorphism: lip eversion, low set ears, short philtrum, thick eye brow, protruded tongue
Thinning of corpus callosum
Global DD
Hearing loss
Atrial septal defect



	MCPH223.1	MCPH223.2	MCPH223.3
***KMT2A* c.4819+1G>T splicing defect**	G/**T**	G/G	G/G
***ATRX* c.622C>T p.R208C**	**T**/-	C/**T**	C/-

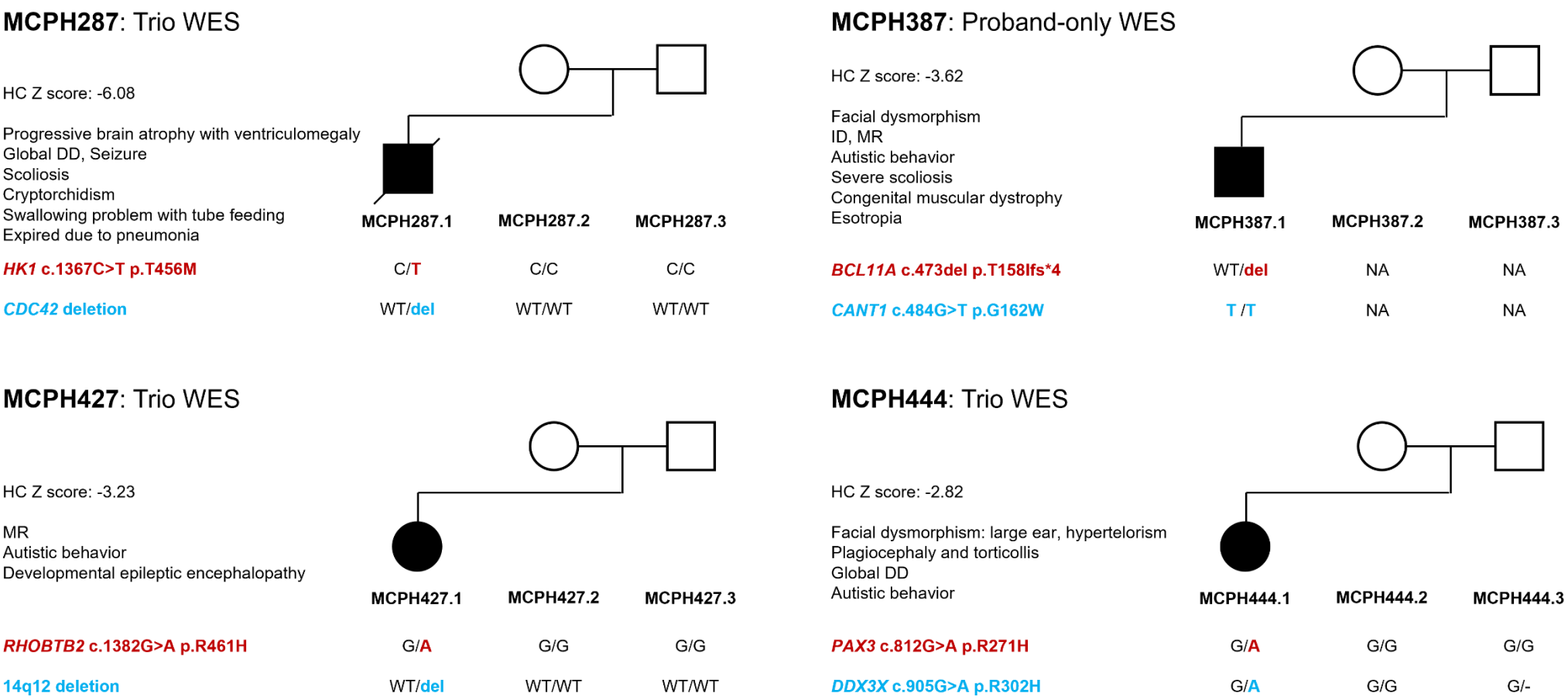


Fig. S3: Pedigree and genotype information for individuals with multi-locus pathogenic variations.

We identified 10 probands (2.4%) with multi-locus pathogenic variations (MPVs) in our cohort. These cases mostly exhibited syndromic features with phenotypic expansions or blended phenotypes of dual genetic disorders. The combinations included three cases of AD + AD, two cases each of AD + AR, AD + XL, AD + CNV, and one case of XL + CNV events.

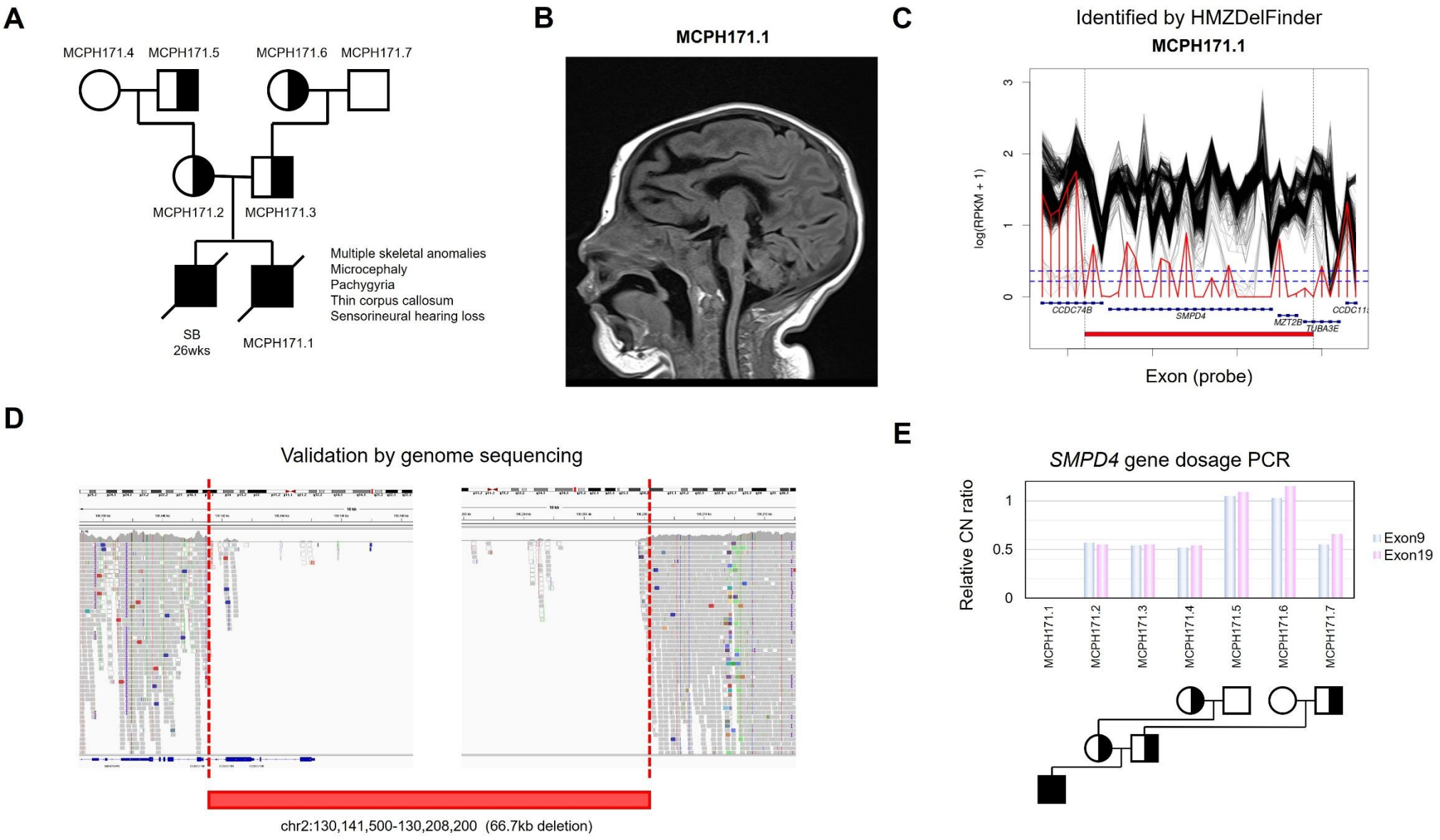

A
MCPH171.4
MCPH171.5
MCPH171.6
MCPH171.7
MCPH171.2
MCPH171.3
SB
26wks
MCPH171.1
Multiple skeletal anomalies
Microcephaly
Pachygyria
Thin corpus callosum
Sensorineural hearing loss
B
MCPH171.1
C
Identified by HMZDelFinder
MCPH171.1
log(RPKM + 1)
CCDC74B
SMPD4
MZT2B
TUBA3E
Exon (probe)
D
Validation by genome sequencing
chr2:130,141,500-130,208,200 (66.7kb deletion)
E
SMPD4 gene dosage PCR
Relative CN ratio
Exon9
Exon19
MCPH171.1
MCPH171.2
MCPH171.3
MCPH171.4
MCPH171.5
MCPH171.6
MCPH171.7

Fig. S4: Identification of *SMPD4* homozygous deletion in a Korean family with recurrent pregnancy loss and perinatal death.

(A) Pedigree information and clinical characteristics of the affected patient (MCPH171). The parents experienced a stillbirth at 26 weeks with the first baby, who had pachygyria on brain sonography. The second baby (MCPH171.1) showed no abnormal prenatal history but presented multiple skeletal anomalies, microcephaly, pachygyria, thin corpus callosum, and sensorineural hearing loss soon after birth. **(B)** Brain MRI findings of the patient show pachygyria, microcephaly, and a thin corpus callosum. **(C)** Copy-number variant analysis using whole-exome sequencing (WES) data, facilitated by HMZDelFinder, identifies a homozygous deletion encompassing the entire *SMPD4* gene. **(D)** Integrative Genomic Viewer (IGV) findings from whole-genome sequencing (WGS, 30X) validated this deletion and identified its size (66.7 kb) and approximate breakpoints (chr2:130,141,500–130,208,200). Due to the highly polymorphic sequences around this region, exact breakpoints could not be determined. **(E)** Gene dosage PCR targeting exon 9 and exon 19 of the *SMPD4* gene confirms the copy number status of the family members spanning three generations. This deletion was transmitted from the maternal grandmother (MCPH171.4) to the mother (MCPH171.2) and the patient's paternal grandfather (MCPH171.7) to the father (MCPH171.3). Identifying the genetic cause and following genetic counseling could help the parents perform preimplantation genetic testing (PGT) and plan for a third baby, as they had given up on pregnancy due to their recurrent history.

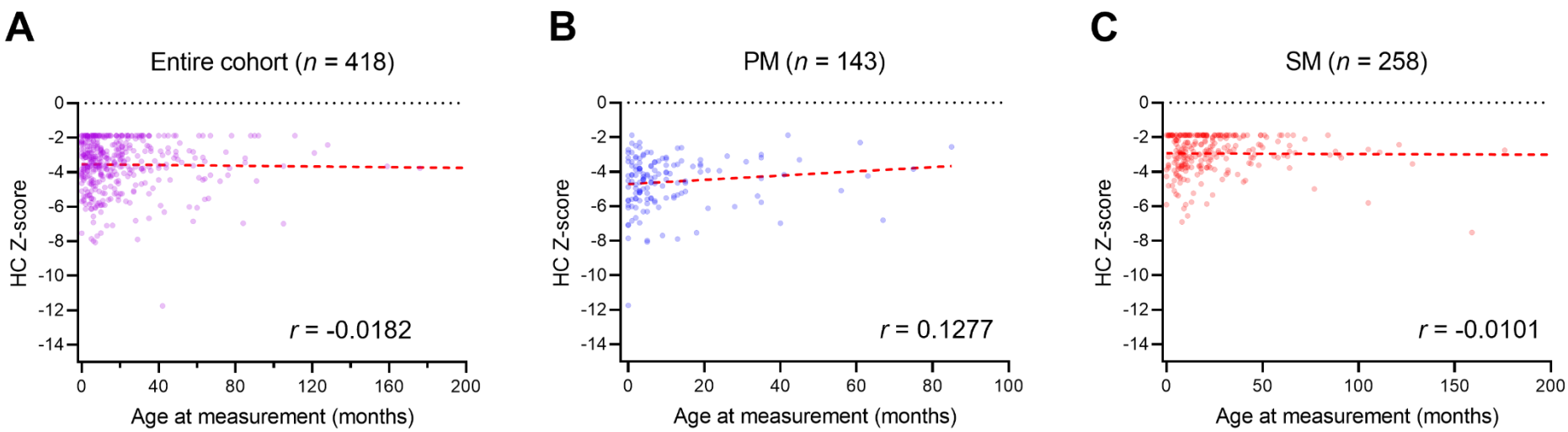


Fig. S5: Correlation analysis between head circumference and age at measurement.

(A) Scatter plot of head circumference (HC) Z-scores vs. age at measurement across the entire cohort reveals no significant correlation, with a negligible *Pearson* correlation coefficient (r = -0.0182). **(B, C)** Age at measurement shows no significant correlation with microcephaly severity in both the primary microcephaly (PM) and secondary microcephaly (SM) groups, with *Pearson* correlation coefficients (r) of 0.1277 and -0.0101, respectively.

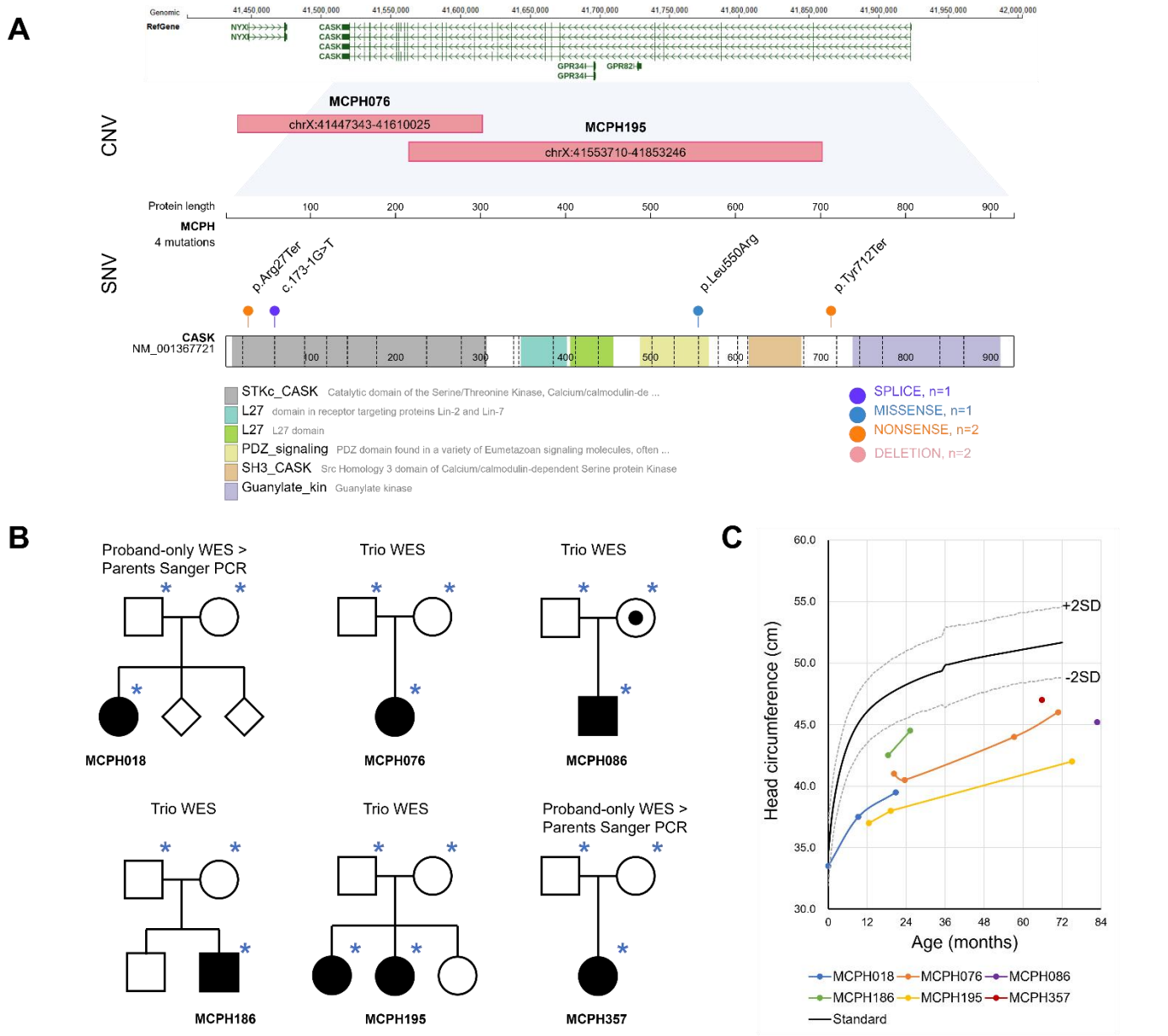

A
Genomic
RefGene
41,450,000
41,500,000
41,550,000
41,600,000
41,650,000
41,700,000
41,750,000
41,800,000
41,850,000
41,900,000
41,950,000
42,000,000
NYX
CASK
GPR34
GPR82
CNV
MCPH076
chrX:41447343-41610025
MCPH195
chrX:41553710-41853246
Protein length
100
200
300
400
500
600
700
800
900
MCPH
4 mutations
SNV
p.Arg27Ter
c.173-1G>T
p.Leu550Arg
p.Tyr712Ter
CASK
NM_001367721
STKc_CASK Catalytic domain of the Serine/Threonine Kinase, Calcium/calmodulin-de ...
L27 domain in receptor targeting proteins Lin-2 and Lin-7
L27 L27 domain
PDZ_signaling PDZ domain found in a variety of Eumetazoan signaling molecules, often ...
SH3_CASK Src Homology 3 domain of Calcium/calmodulin-dependent Serine protein Kinase
Guanylate_kin Guanylate kinase
SPLICE, n=1
MISSENSE, n=1
NONSENSE, n=2
DELETION, n=2
B
Proband-only WES > Parents Sanger PCR
Trio WES
Trio WES
MCPH018
MCPH076
MCPH086
Trio WES
Trio WES
Proband-only WES > Parents Sanger PCR
MCPH186
MCPH195
MCPH357
C
Head circumference (cm)
60.0
55.0
50.0
45.0
40.0
35.0
30.0
+2SD
-2SD
0
12
24
36
48
60
72
84
Age (months)
MCPH018
MCPH076
MCPH086
MCPH186
MCPH195
MCPH357
Standard
D
MCPH018
MCPH076
MCPH086
MCPH186
MCPH195
MCPH357

Fig. S6: *CASK*-related disorders are associated with severe microcephaly.

(A) Schematic of variants identified in *CASK*, the third most frequently altered gene in our cohort (*n* = 6, 2.4%). The variants include two large deletions and four single-nucleotide variants (SNVs). **(B)** Pedigrees of the six individuals with *CASK* variants. Blue asterisks denote samples tested for genotype. All variants were *de novo* except for the p.Leu556Arg variant in MCPH086, which was maternally inherited. The female (MCPH195) with a *de novo* Xp11.4 deletion (300 kb) had a similarly affected older sister with the same deletion, suggesting potential parental germline mosaicism. **(C)** The measured head circumferences in centimeters of the six individuals are plotted on the growth curve, indicating profound microcephaly. **(D)** Brain magnetic resonance imaging (MRI) findings of the six individuals. Five show pontine and cerebellar hypoplasia consistent with microcephaly and pontine-cerebellar hypoplasia (MICPH; OMIM #300749).

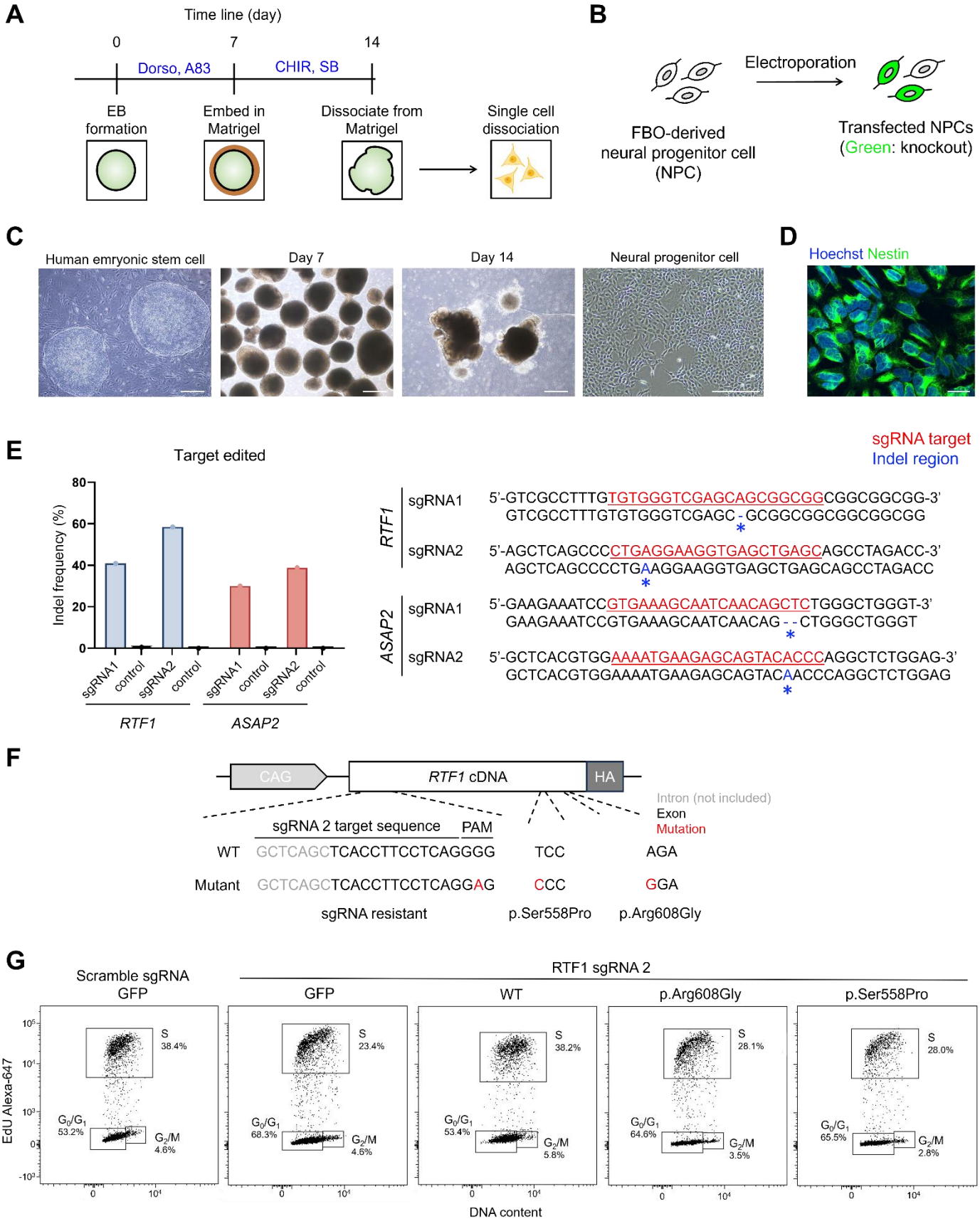

A
Time line (day)
0
7
14
Dorso, A83
CHIR, SB
EB formation
Embed in Matrigel
Dissociate from Matrigel
Single cell dissociation
B
Electroporation
FBO-derived neural progenitor cell (NPC)
Transfected NPCs (Green: knockout)
C
Human emryonic stem cell
Day 7
Day 14
Neural progenitor cell
D
Hoechst Nestin
E
Target edited
sgRNA target
Indel region
Indel frequency (%)
sgRNA1
control
sgRNA2
control
RTF1
ASAP2
RTF1
sgRNA1 5'-GTCGCCTTTGTGTGGGTCGAGCAGCGGCGGCGGCGGCGGCGG-3'
GTCGCCTTTGTGTGGGTCGAGC-GCGGCGGCGGCGGCGG
sgRNA2 5'-AGCTCAGCCCCTGAGGAAGGTGAGCTGAGCAGCCTAGACC-3'
AGCTCAGCCCCTGAAGGAAGGTGAGCTGAGCAGCCTAGACC
ASAP2
sgRNA1 5'-GAAGAAATCCGTGAAAGCAATCAACAGCTCTGGGCTGGGT-3'
GAAGAAATCCGTGAAAGCAATCAACAG--CTGGGCTGGGT
sgRNA2 5'-GCTCACGTGGAAAATGAAGAGCAGTACACCCAGGCTCTGGAG-3'
GCTCACGTGGAAAATGAAGAGCAGTACAACCCAGGCTCTGGAG
F
CAG
RTF1 cDNA
HA
Intron (not included)
Exon
Mutation
sgRNA 2 target sequence
PAM
WT GCTCAGCTCACCTTCCTCAGGGG TCC AGA
Mutant GCTCAGCTCACCTTCCTCAGGAG CCC GGA
sgRNA resistant
p.Ser558Pro
p.Arg608Gly
G
Scramble sgRNA
GFP
RTF1 sgRNA 2
GFP
WT
p.Arg608Gly
p.Ser558Pro
EdU Alexa-647
DNA content
S 38.4%
G0/G1 53.2%
G2/M 4.6%
S 23.4%
G0/G1 68.3%
G2/M 4.6%
S 38.2%
G0/G1 53.4%
G2/M 5.8%
S 28.1%
G0/G1 64.6%
G2/M 3.5%
S 28.0%
G0/G1 65.5%
G2/M 2.8%

Fig. S7: Characterization of human forebrain organoid-derived NPCs and assessment of knockout efficiency post-electroporation.

(A) Schematic illustration of the generation of neural progenitor cells (NPCs) from human forebrain organoids (FBO). **(B)** Schematic illustration of the FBO-derived NPC electroporation procedure. GFP labeling indicates cells co-electroporated with Cas9 and sgRNA-expressing plasmids. **(C)** Bright-field images showing human embryonic stem cells in feeder-dependent culture, embryoid bodies (EB), brain organoids at Day 14, and NPCs derived from forebrain organoids (scale bar, 100 μm). **(D)** Confocal image of NPCs stained with Nestin, a cytoskeletal marker specific for NPCs, confirming strong Nestin expression (scale bar, 10 μm). **(E)** Analysis of indel frequency to assess the genome editing efficacy of Cas9 in DNA from sorted GFP^+ cells post-electroporation. The left panel quantifies the frequencies (%), while the right panel illustrates the most prevalent indel regions within the sgRNA target sites of *RTF1* and *ASAP2*. Original sequences with target sites highlighted in red are shown above, and genome-edited sequences and indel mutations highlighted in blue are shown below. **(F)** Schematic of the piggyBac transposon-based vector containing sgRNA-resistant *RTF1* cDNA. The degenerate mutation within the PAM sequence is indicated in red. **(G)** Representative flow cytometry plots showing cell cycle phase distribution in NPCs. Overexpression of wild-type (WT) *RTF1* restored S-phase entry, whereas patient-derived variants (p.Arg608Gly and p.Ser558Pro) exhibited diminished rescue efficiency.