Mutations in the HECT domain of NEDD4L lead to AKT–mTOR pathway deregulation and cause periventricular nodular heterotopia

Loïc Broix1–5,22, Hélène Jagline1–4,22, Ekaterina I Ivanova1–4, Stéphane Schmucker1–4, Nathalie Drouot1–4, Jill Clayton-Smith6, Alistair T Pagnamenta7, Kay A Metcalfe6, Bertrand Isidor8, Ulrike Walther Louvrier9, Annapurna Poduri10, Jenny C Taylor7, Peggy Tilly1–4, Karine Poirier5, Yoann Saillour5, Nicolas Lebrun5, Tristan Stemmelen1–4, Gabrielle Rudolf1–4, Giuseppe Muraca3, Benjamin Saintpierre3, Adrienne Elmorjani5, Deciphering Developmental Disorders study11, Martin Moïse12, Nathalie Bednarek Weirauch13, Renzo Guerrini14, Anne Boland15, Robert Olaso15, Cecile Masson16, Ratna Tripathy17, David Keays17, Cherif Beldjord18, Laurent Nguyen12, Juliette Godin1–4, Usha Kini19, Patrick Nischké16, Jean-François Deleuze15, Nadia Bahi-Buisson20, Izabela Sumara1–4, Maria-Victoria Hinckelmann1–4 & Jamel Chelly1–4,21

Neurodevelopmental disorders with periventricular nodular heterotopia (PNH) are etiologically heterogeneous, and their genetic causes remain in many cases unknown. Here we show that missense mutations in NEDD4L mapping to the HECT domain of the encoded E3 ubiquitin ligase lead to PNH associated with toe syndactyly, cleft palate and neurodevelopmental delay. Cellular and expression data showed sensitivity of PNH-associated mutants to proteasome degradation. Moreover, an in utero electroporation approach showed that PNH-related mutants and excess wild-type NEDD4L affect neurogenesis, neuronal positioning and terminal translocation. Further investigations, including rapamycin-based experiments, found differential deregulation of pathways involved. Excess wild-type NEDD4L leads to disruption of Dab1 and mTORC1 pathways, while PNH-related mutations are associated with deregulation of mTORC1 and AKT activities. Altogether, these data provide insights into the critical role of NEDD4L in the regulation of mTOR pathways and their contributions in cortical development.

Development of the human cerebral cortex requires coordinated spatial and temporal regulation of interdependent developmental processes that include proliferation, migration and layering, as well as differentiation of distinct neuronal populations1,2. Disruption of any of these processes can result in a wide range of developmental disorders. Many of these disorders are classified within the group of malformations of cortical development (MCD) that includes lissencephaly, pachygyria, polymicrogyria (PMG), microcephaly and PNH3–5. MCD are often associated with severe intellectual disability and epilepsy, and their evolving classification is based on the developmental process thought to be affected first in combination with the underlying disrupted genes and biological pathways3.

Within the group of MCD associated with neuronal migration abnormalities, PNH represents about 31% of MCD (R.G., unpublished data). Anatomically, PNH is characterized by bilateral ectopic nodules of gray matter lining the lateral ventricles. Clinical presentations in patients with PNH are heterogeneous, although seizures and learning difficulties are the most common clinical features5. So far, mutations in the X-linked FLNA gene (filamin A)6, encoding a widely expressed 280-kDa actin-binding phosphoprotein, account

1Institut de Génétique et de Biologie Moléculaire et Cellulaire, Illkirch, France. 2CNRS U7104, Illkirch, France. 3INSERM U964, Illkirch, France. 4Université de Strasbourg, Illkirch, France. 5Institut Cochin, INSERM U1016, CNRS U8104, Paris Descartes University, Paris, France. 6Manchester Centre for Genomic Medicine, Central Manchester University Hospitals NHS Trust, Manchester Academic Health Science Centre, Manchester, UK. 7NIHR Biomedical Research Centre, Wellcome Trust Centre for Human Genetics, University of Oxford, Oxford, UK. 8Service de Généétique Médicale, University Hospital of Nantes, Nantes, France. 9Unité de Neuropédiatrie et d’Epileptologie Infantile, University Hospital of Montpellier, Montpellier, France. 10Epilepsy Genetics Program, Division of Epilepsy and Clinical Neurophysiology, Department of Neurology, Boston Children’s Hospital, Boston, Massachusetts, USA. 11A list of members and affiliations appears at http://dx.doi.org/10.1101/049056. 12Groupe interdisciplinaire de Génoprotéomique Appliquée-Neurosciences, University of Liège, Liège, Belgium. 13Hôpital Maison Blanche, University Hospital of Reims, Reims, France. 14Pediatric Neurology Unit, A. Meyer Children’s Hospital, University of Florence, Florence, Italy. 15Centre National de Génotypage, Institut de Génomique, CEA, Evry, France. 16Institut Imagine, Bioinformatics Platform, Paris Descartes University, Paris, France. 17Institute of Molecular Pathology, Vienna, Austria. 18Laboratoire de Biochimie et Génétique Moléculaire, Hôpital Cochin, Paris, France. 19Department of Clinical Genetics, Oxford University Hospitals NHS Trust, Oxford, UK. 20Institut Imagine, INSERM U1163, Paris Descartes University, Hôpital Necker–Enfants Malades, Paris, France. 21Service de Diagnostic Génétique, Hôpital Civil de Strasbourg, Hôpitaux Universitaires de Strasbourg, Strasbourg, France. 22These authors contributed equally to this work. Correspondence should be addressed to J.C. (chelly@igbmc.fr).

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for about 50% of bilateral PNH and the associated Ehler–Danlos conditions affecting mostly females. Compelling studies suggested that FLNA-related PNH results from the combination of defects affecting the polarized radial glia scaffold and its adhesion to the neuroependymal membrane, neural progenitor proliferation and neuronal migration. Other mechanisms contributing to PNH development were proposed following the identification of rare familial forms of PNH associated with recessive mutations in ARFGEF2, which encodes the ADP–ribosylation factor guanine exchange factor 2 (GEF2). Finally, other genetic forms of PNH have been mapped through array comparative genomic hybridization (aCGH) studies, but only one potential causal gene, C6orf70 (ERMARD) located at 6q27, has been identified.

Here we provide evidence implicating the E3 ubiquitin ligase gene NEDD4L in the development of PNH. We report the identification of missense mutations in NEDD4L in patients with PNH, bilateral syndactyly, cleft palate and neurodevelopmental delay. We further show that PNH-related NEDD4L mutants disrupt neurodevelopmental processes, likely through dysregulation of the mTOR and AKT signaling pathways.

RESULTS
Patients with PNH analyzed by whole-exome sequencing and mutations in NEDD4L

We used clinical and brain magnetic resonance imaging (MRI) data from patients with MCD referred for genetic investigations and selected 15 patient–parent trios for analysis by whole-exome sequencing. All families had a single affected patient with bilateral PNH. For the whole-exome sequencing approach, we applied a previously described experimental workflow to detect and prioritize sequence variants and validate significant findings. We then analyzed filtered exome data and searched for recurrence of de novo mutations in the same gene in unrelated patients. We identified de novo missense changes in NEDD4L in two patients with bilateral contiguous PNH, bilateral syndactyly of the second and third toes, and neurodevelopmental delay (P158 and BRC217; Fig. 1a, b, Table 1 and Supplementary Note). After validation of the mutations by Sanger sequencing (Supplementary Fig. 1a, b), we screened NEDD4L (coding exons and their flanking sequences) in a cohort of 96 patients with MCD and identified the c.2677G>A mutation (already detected in BRC217) in one additional patient (P347; Fig. 1c, Table 1 and Supplementary Fig. 1c). Interestingly, the phenotype of this patient was also characterized by bilateral PNH, bilateral syndactyly of the second and third toes, and neurodevelopmental delay (Table 1 and Supplementary Note). As the family of patient P347 consisted of two affected children (P347 and her brother), a healthy girl and healthy parents (Supplementary Fig. 1c), we tested the segregation of the variant in all members of the family. We found the variant to be present in a heterozygous state in the affected brother but absent from the DNA of the healthy sister and father. For the mother, however, sequencing traces consistently showed an imbalance in the height of the peaks corresponding to normal and variant alleles (Supplementary Fig. 1c). Altogether, these results were suggestive of germline and somatic mosaicism of a NEDD4L variant in the mother. To confirm this and evaluate the level of somatic mosaicism, we analyzed the mother’s DNA by digital droplet PCR and found that the frequency of the

Figure 1 Mutations in NEDD4L cause PNH and syndactyly. (a–e) Photographs and representative sections of brain MRIs for affected individuals illustrating frequent toe syndactyly and constant PNH. For each patient, two axial sections, or one axial and one coronal section, show confluent nodules of heterotopia lining the lateral ventricles (arrowheads). Sagittal sections show thin (c) or dysmorphic (a and c, II–3) corpus callosum. In d, in addition to PNH (arrowheads), the MRI section shows frontal PMG (white arrows). MRIs were performed at the age of 8 years (a), 12 months (b), 9 and 12 months (c), 7 months (d) and 8 months (e). MRIs were not available for patient Pnh31124 with the c.2082G>T; p.Gln694His mutation. Written consent was obtained to publish patient photographs. (f) Linear representation of the NEDD4L polypeptide showing the positions in the HECT domain of the heterozygous PNH-associated mutations. NEDD4L protein is characterized by an N-terminal C2 domain known to bind Ca²⁺ and phospholipids, two to four WW protein–protein interaction domains responsible for recognition of the substrate, and the C-terminal catalytic HECT domain.

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Table 1 Summary of clinical and neuroimaging features of patients harboring NEDD4L mutations

| Patients with PNH | BRC217 | Pnh31124 | DDDP110533 |
|------------------|--------|----------|-------------|
| P158 c.2690G>A   | p.Arg97Gln | p.Glu893Lys | c.2036A>G |
| P347 c.2677G>A   | p.Glu93Lys | p.Glu893Lys | Tyr679Cys |

| Inheritance | De novo | Maternal mosaicism | Maternal mosaicism | De novo | De novo | De novo | De novo |
|-------------|---------|--------------------|--------------------|---------|---------|---------|---------|
| Sex         | M       | F                  | M                  | F       | F       | M       | F       |
| Birth (GW)  | 40      | 41                 | 41                 | 41      | 38      | Full term | 40      |
| Birth weight | 3,750   | 3,160              | NA                 | 3,120   | 3,360   | NA                  | 3,000   |
| Syndactyly  | +       | +                  | +                  | +       | +       | +       | +       |
| Hypotonia   | + (at birth) | NA                | NA                 | ++ (2 months) | + (axial hypotonia at 34 months) | + (unable to sit or walk at 6 years) |
| Age at last examination | 6 years | 12 years | 2 years | 4 months | 4 years | 8 months | 6.2 years |
| HC (cm)     | 54 (+1.5 s.d.) | 50 (--2.4 s.d.) | 49.8 (+0.7 s.d.) | 39 (--1.2 s.d.) | 48 (--1.2 s.d.) | 43 (--1 s.d.) | 49.7 (--2.32 s.d.) |
| Height (cm) | 1.08 (--1.5 s.d.) | 1.25 (--3.5 s.d.) | 0.88 (+0.8 s.d.) | 61 (M) | 100 (+2 s.d.) | 68 (--0.5 s.d.) | NA |
| Weight (kg) | 16.5 (--1.5 s.d.) | 25 (--2.5 s.d.) | 10.9 (+0.7 s.d.) | 5.29 (--1.1 s.d.) | 14 (--0.8 s.d.) | 7.6 (--0.5 s.d.) | 20 (--0.51 s.d.) |
| Developmental delay | + | + (severe) | + (severe) | + | + | + | + (severe) |
| Seizures    | --      | + (late onset)     | +                  | --      | --      | --      | + (IS at 5 months) | + |
| Brain MRI findings | At 8 years | At 9 months | At 2 months | At 12 months | At 7 months | At 8 months | At 8 months |
| PNH         | + (bilateral) | + (bilateral) | + (bilateral) | + (bilateral) | + (bilateral) | + (bilateral) | + (bilateral) |
| CC anomalies | --      | --                  | --                 | Dysmorphic | Dysmorphic | PMG | -- |
| Cortex anomalies | --     | Cerebral atrophy    | Frontal cortical dysplasia | --     | --     | -- |
| Cerebellum anomalies | --      | Myopia              | Hearing impairment, Strabismus, Hypothalamic dysfunction, Arthrogryposis, Cryptorchidism | --      | --      | -- |
| Other       | --      | Optic atrophy       | Hearing impairment, Congenital hearing impairment, Arthrogryposis, Cryptorchidism | --      | --      | -- |

PNH, periventricular nodular heterotopia; PMG, polymicrogyria; CC, corpus callosum; GW, gestational week; HC, head circumference; IS, infantile spasm; NA, not available; +, present; −, absent. Transcript NM_001144967.2 (ENST00000400345) and protein Q96PU5 were used for annotation of nucleotide and protein changes, respectively.

mutated allele was around 16% (Supplementary Table 1). In view of these molecular data, the mother was reexamined and neurological, cognitive and behavioral evaluations were found to be normal.

Shortly after these initial findings, we reinforced the implication of NEDD4L by the identification of additional de novo mutations in three unrelated patients with PNH (Table 1). The first patient (PNC: Fig. 1d, Table 1 and Supplementary Fig. 1d) was identified through targeted screening of an MCD-related panel of genes in which we included NEDD4L. The second and third patients—DDDP110533 (Fig. 1e, Table 1 and Supplementary Fig. 1e) and Pnh31124 (Table 1 and Supplementary Fig. 1f)—were identified through data sharing of trios exome sequenced and analyzed as part of the Deciphering Developmental Disorders and Epilepsy Consortium studies. For all three patients, brain MRI results also showed the presence of PNH as associated with syndactyly and neurodevelopmental delay (Fig. 1d–e, Table 1 and Supplementary Note).

NEDD4L15 (also known as NEDD4-2) encodes a member of the NEDD4 family of HECT-type E3 ubiquitin ligases known to regulate the turnover and function of a number of proteins involved in fundamental cellular pathways and processes16–19. Interestingly, all mutations associated with PNH mapped to the HECT domain (Fig. 1f), and relevance of their pathogenic effect was suggested by the high degree of conservation of the affected residues (Supplementary Fig. 2a), bioinformatics predictions (from the MutationTester tool) and structural modeling of the HECT domain (Supplementary Fig. 2b–j).

Functional effects of wild-type NEDD4L and NEDD4L mutants

We first analyzed the expression of Nedd4l during mouse brain development. As illustrated in Supplementary Figure 3a (in situ hybridization at embryonic day (E) 15), Nedd4l transcripts were homogenously distributed in the cortical plate, ventricular zone and ganglionic eminences. Moreover, analysis of Nedd4l expression by real-time qPCR in developing mouse cortex from E12.5 to E18.5 showed a peak of expression at E16.5 (Supplementary Fig. 3b), a developmental stage characterized by both proliferation and migration.

To assess the cellular consequences of PNH-related mutations, we transfected N2A cells with cDNA constructs for wild-type NEDD4L and three different mutants and compared expression levels and localization by immunofluorescence (Fig. 2). We found that wild-type NEDD4L was highly expressed (Fig. 2a and Supplementary Fig. 4), and its localization was in line with what was previously reported20. In contrast, PNH-related mutant proteins were hardly detectable. Indeed, 48 h after transfection, we only detected a faint signal, comparable to the background signal of cells transfected with control empty vector (Fig. 2a). Similar results were observed upon transfection of primary cultured mouse neurons (Supplementary Fig. 4). We also confirmed the lack of mutant NEDD4L expression by immunoblotting using protein extracts from transfected N2A cells (Fig. 2b). In view of these results, we hypothesized that PNH-associated mutants are unstable and examined NEDD4L protein expression in transfected cells treated with the proteasome inhibitor MG132. We observed by immunofluorescence and immunoblot experiments high levels of expression of PNH-related NEDD4L mutants (Fig. 2a,b).

To further ascertain that the instability of NEDD4L mutant proteins represents a disease-relevant phenotype, we tested the expression of three control variants reported in the ClinVar and/or Exome Aggregation Consortium (ExAC) databases. Referring to NM_001144967.2 (ENST00000400345) and protein Q96PU5 were used for annotation of nucleotide and protein changes, respectively.

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cotransfected reporter GFP as a normalizer. We found that wild-type NEDD4L transcript levels were approximately 1.5- to 2-fold higher than mutant levels (Supplementary Fig. 6), a range of difference that does not explain the almost complete absence of the mutant proteins.

Because all the mutations map to the catalytic HECT domain of the NEDD4L E3 ligase and lead to protein instability, we sought to assess the consequences of disease-causing variants for enzymatic activity and self-ubiquitination. To this end, we focused on the Arg897Gln mutant and compared by two different approaches the ubiquitination ability of wild-type NEDD4L and the mutant.

First, to test specifically for NEDD4L ubiquitination, we transfected N2A cells with cDNA constructs encoding wild-type (WT) or mutant NEDD4L. For each construct, cultured cells were treated with either DMSO or MG132. NEDD4L immunostaining shows a cytoplasmic distribution with enrichment in the periphery of N2A cells for wild-type NEDD4L, whereas the Gln694His, Glu893Lys and Arg897Gln mutants are not detectable. Scale bar, 50 µm. (b) Immunoblots using protein extracts from N2A cells transfected with constructs encoding wild-type and mutant NEDD4L and cultured in the presence of either DMSO or MG132. Blots show lack of expression of PNH-associated mutants, while transfection for wild-type NEDD4L led to a high level of expression for NEDD4L protein. Note that PNH-associated NEDD4L mutants become detectable upon treatment of N2A cells with MG132.
exhibited ubiquitination activity as illustrated by the high-molecular-mass smear detected by antibody to NEDD4L (Supplementary Fig. 7b).

To further confirm and better visualize the ubiquitination activity of the NEDD4L mutant, we performed another series of immunoprecipitation and in vitro assays and immunoblot analysis in which we loaded four times less protein products to the assay with wild-type NEDD4L than to the one with mutant NEDD4L (Fig. 3).

Although accurate comparison of wild-type and mutant NEDD4L ubiquitination activities is difficult, both immunoprecipitation (Fig. 3a) and in vitro ubiquitination (Fig. 3b) approaches showed that mutant NEDD4L induced a high-molecular-mass smear detected with antibodies to V5 and NEDD4L.

These results suggest that the ubiquitination activity of mutant NEDD4L is preserved and possibly enhanced if we take into account the substantial level of ubiquitination activity despite the instability of the protein.

At first sight, the fact that PNH-related NEDD4L mutants are unstable led us to consider a haploinsufficiency mechanism. However, previously reported findings and our data made this hypothesis questionable. For instance, postmitotic neurons deficient for both Nedd4-1 and Nedd4l (analyzed in conditional double-knockout mice) migrate properly to the cortical plate21. Also, neuronal migration disorders and PNH are not among the features associated with haploinsufficiency resulting from heterozygous copy number variations (CNVs) reported in DECIPHER databases. Moreover, one stop-gain and three frameshift mutations were reported in the ExAC data set from which individuals affected by severe pediatric disease have been excluded. Finally, we used an in utero RNA interference

Second, we compared the ubiquitination ability of immunopurified V5-tagged NEDD4L (wild type and mutant) in an in vitro assay using recombinant E1 and E2 (Ubch7) enzymes with or without ubiquitin. In the presence of ubiquitin, both wild-type and mutant NEDD4L

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Figure 3 Ubiquitination activity of mutant NEDD4L. (a) Immunoprecipitation assay (IP; using antibody to V5 to precipitate tagged NEDD4L), with precipitates analyzed by immunoblotting (IB) using antibodies to ubiquitin and V5 to detect ubiquitinated NEDD4L (FT, flow through). (b) Analysis of NEDD4L ubiquitination activity in an in vitro assay using wild-type and mutant NEDD4L immunopurified from the lysates of transfected N2A cells and incubated with ATP, E1 enzyme and E2 (Ubch7) enzyme with (+) or without (−) ubiquitin (Ub). Reaction mixtures were analyzed by immunoblotting with antibodies to V5 and NEDD4L. Note that, because of the instability of mutant NEDD4L and the resulting imbalance in the amounts of wild-type and mutant NEDD4L and corresponding immunoblot signals, as shown in Supplementary Figure 7, immunoblot analysis was performed using four times less reaction mixture for wild-type NEDD4L than for mutant NEDD4L.
approach to analyze the consequences of Nedd4l downregulation on cortical neuronal migration in mice and found no significant difference between embryo brains transfected with short hairpin RNA (shRNA) targeting Nedd4l and scrambled shRNA control (Supplementary Fig. 8a,b).

In view of these convergent arguments, we then sought to study the consequences of PNH-related mutations for neurodevelopmental processes. We used an in utero electroporation (IUEP) approach and compared the consequences of expression of wild-type NEDD4L and mutants (Glu893Lys and Arg897Gln) for neuronal positioning, neuronal progenitor proliferation and terminal translocation. To assess effects on projection neuron positioning, we electroporated NEDD4L constructs in combination with Tomato reporter construct into progenitor cells located in the ventricular zone of E14.5 mouse neocortices and analyzed embryo brains 4 d later (E18.5). In E18.5 brain sections, we observed that neurons electroporated with the empty vector reached the cortical plate (Fig. 4a,b). However, IUEP of wild-type NEDD4L and PNH-related mutants induced significant arrest of cells within the ventricular zone and subventricular zone and intermediate zone with corresponding depletion in the cortical plate (Fig. 4a,b). We also assessed the effect on neuron positioning at postnatal day (P) 2 and highlighted differences that paralleled those observed at E18.5. Whereas neurons electroporated with empty vector were mainly located in superficial layers II–IV of the cortical plate, we found that neurons electroporated with constructs for wild-type NEDD4L and PNH-related mutants were abnormally distributed in the white matter and in layers V and VI of the cortical plate (Supplementary Fig. 9a,b).

As our cellular data suggested that PNH-related NEDD4L mutants are unstable, we also assessed whether the same effect could be observed in vivo in electroporated migrating neuronal cells. We found that cells electroporated with constructs for wild-type NEDD4L exhibited strong cytoplasmic immunolabeling (Fig. 4c and Supplementary Fig. 9c), consistent with a high level of NEDD4L expression. However, in neurons electroporated with mutant constructs, we only detected a faint signal that could correspond to endogenously expressed NEDD4L (Fig. 4c and Supplementary Fig. 9c).

To study terminal translocation 22–25, we conducted IUEP of the different constructs at E14.5 and collected brains 6 d later (P2) from the pups. We then assessed the distribution of electroporated neurons with a leading process adhering to the extracellular matrix (ECM) in the region previously defined as the primitive cortical zone (PCZ)24, and in the upper and lower Cux1 regions corresponding to two equal parts of the remaining Cux1-positive layer (Supplementary Fig. 10). We found that expression of wild-type and mutant NEDD4L led to an abnormal distribution of neuronal cells with a deviating enrichment in the lower Cux1 region (Supplementary Fig. 10), suggesting that terminal translocation was disrupted.

To test the consequences of the NEDD4L mutations on apical and basal progenitor proliferation, we performed IUEP at E14.5 and collected embryos 48 h later for immunohistochemistry using antibodies against PH3, Pax6, Tbr2 and Ki67. We found a significantly higher percentage of Tomato-positive and PH3-positive co-labeled cells expressing wild-type protein or HECT-domain mutants in the ventricular zone when compared to Tomato-positive cells expressing the control empty vector (Fig. 4d and Supplementary Fig. 9d). These results suggested an increased mitotic index of apical progenitors electroporated with wild-type NEDD4L and PNH-related mutants. We also quantified electroporated Pax6-positive and Tbr2-positive cells in the ventricular zone and subventricular zone and found an increased number of these cell populations only in brains electroporated with PNH-related mutant constructs (Fig. 4e and Supplementary Fig. 9c).

To assess the pool of proliferating cells, we evaluated cells positive for both Tomato and Ki67 in the ventricular zone and subventricular zone; in both regions, we did not observe any differences following electroporation with control empty vector and cDNA constructs encoding wild-type and mutant NEDD4L (Supplementary Fig. 9g).

We also performed immunohistochemistry against the Cux1 marker at E18.5 and observed that arrested neurons electroporated with wild-type NEDD4L or PNH-related mutants expressed Cux1, indicating that misplaced neurons in deep layers of the cortex are differentiated and fated for upper layers (Supplementary Fig. 9b).

Finally, we labeled brain slices (E16.5 and E18.5) with anti-Cux1 antibodies to cleaved caspase-3 to evaluate cell death rate and found no difference following electroporation with the different constructs (data not shown).

Pathways disrupted by wild-type and mutant NEDD4L causing PNH
Although the best known target of NEDD4L is the epithelial sodium channel (ENaC), shown to be involved in Liddle syndrome, a hereditary hypertension caused by elevated ENaC activity26, compelling evidence demonstrating crucial regulatory roles of NEDD4L in developmental processes has recently emerged19,27. In this study, we sought to investigate the consequences of wild-type and mutant NEDD4L on mTOR-dependent pathways for the following main reasons.

Figure 5 Wild-type NEDD4L and PNH-related mutants induce deregulation of mTORC1 and AKT activities. (a,b) Representative immunoblots using protein extracts from N2A cells transfected with empty vector or constructs for wild-type and mutant NEDD4L, showing the effect of wild-type and mutant NEDD4L on the levels of phosphorylated S6 (p-S6; reflecting mTORC1 activity) (a) and phosphorylated Akt (p-Akt; Thr308/Ser473) (b). (c) Histograms of densitometric measurements illustrating S6 and Akt phosphorylation. Data represent means ± s.e.m. from three independent experiments (Online Methods and Supplementary Table 2). *P < 0.05, **P < 0.01, ***P < 0.001, ****P < 0.0001.
First, in view of the association of MCD and distal-limb abnormalities observed in patients described in this study, we wondered whether these developmental abnormalities represent a particular condition in the wide range of developmental brain and body disorders caused by dysfunctions of the interdependent phosphatidylinositol 3-kinase (PI3K)–AKT–mTORC1 signaling pathways. Second, among the many identified signaling pathways that modulate neuronal migration, the recently reported insights linking neuronal migration deficit in tuberous sclerosis complex (TSC) pathology with a cascade involving mTOR signaling, E3 ubiquitin ligase Cul5 expression and Dab1 expression also led us to consider a potential effect of NEDD4L on mTORC1, Akt and Dab1 signaling pathways. Third, NEDD4L was recently identified as a critical player in regulation of the crosstalk between PI3K–mTORC2 and TGF-β–activin–Smad2–Smad3 (Smad2/3) signaling pathways.

Therefore, we first tested by cellular and immunoblot assays the effect of wild-type and mutant NEDD4L on mTORC1, Akt and Smad2/3 signaling activities. Following transfection of N2A cells with constructs for wild-type NEDD4L and PNH-associated mutants, we observed an increased level of phosphorylated S6 (Ser240/Ser244) that reflects elevated mTORC1 signaling activity (Supplementary Fig. 5a,c). In contrast, for Akt, only PNH-associated NEDD4L mutants were associated with significantly increased levels of Akt forms phosphorylated at Thr308 and Ser473 (Fig. 5b,c).

To reinforce the relevance and specificity of NEDD4L mutant expression to the above highlighted findings, we tested the effect of the three control variants on S6 and Akt phosphorylation and found that overexpression of the three NEDD4L variants was associated with a phosphorylation pattern that was similar to the one with wild-type NEDD4L (Supplementary Fig. 5b). For Smad2/3 activity, we tested the effect of wild-type and Arg897Gln NEDD4L under the basal condition and upon activation of the TGF-β pathway by activin A on the expression levels of Smad2 phosphorylated at Ser465/Ser467, Smad3 phosphorylated at Ser423/Ser425 and Akt phosphorylated at Ser473. Under the basal condition, we found that overexpression of wild-type NEDD4L had no effect on the activation level of Smad2/3, whereas expression of mutant NEDD4L was associated with a substantial increase in the levels of phosphorylated Smad2/3 (Supplementary Fig. 11). Upon activation by activin A of the TGF-β pathway, we found that overexpression of wild-type NEDD4L was associated with an increase in the levels of phosphorylated Akt and Smad2/3, whereas expression of the NEDD4L mutant was associated with a substantial decrease in the levels of phosphorylated Akt and stable levels of phosphorylated Smad2/3 (Supplementary Fig. 11). Collectively, these results suggested that expression of the NEDD4L mutant leads to disruption of the regulated crosstalk between Akt and Smad2/3 signaling in activin–TGF-β pathways.

To assess the in vivo contributions of these pathways to the developmental defects described above, we used an IUEP approach in combination with treatment with rapamycin (a well-known mTORC1 inhibitor) on pregnant mice and analyzed the effect on projection neuron positioning. Interestingly, in E18.5 brain sections from embryos electroporated at E14.5 with wild-type NEDD4L and subjected to DMSO or rapamycin treatment
Our findings demonstrate the critical role of NEDD4L in the regulation of processes involved in cortex development and implicated in the PNH syndrome. In the absence of NEDD4L, we observed a distinct pattern of Dab1 distribution. It is particularly important to note that the overexpression of wild-type NEDD4L, which is predicted to lead to conformational changes that favor transition from the inactive state to the open, active state and trigger autoubiquitination and proteasomal degradation, can rescue the neuronal position defect resulting from IUEP with constitutive activation due to mutations in the HECT domain of NEDD4L in PNH. We report the identification of one transmitted and five de novo mutations in seven patients (two siblings and five unrelated patients) with a common distinguishable phenotype characterized by PNH, intellectual disability, cleft palate and syndactyly (in six of seven patients). All mutations associated with this phenotype are located in the region of NEDD4L encoding the HECT domain of the E3 ubiquitin ligase NEDD4L, and one of these mutations, c.2677G>A, p.Glu893Lys, is a recurrent mutation that was found in three unrelated families (Table 1).

One of the intriguing effects of the PNH-related mutations was the likely increase in sensitivity of the corresponding mutants to autoubiquitination and degradation. To explain this sensitivity, we propose the hypothesis illustrated by the model in Figure 7a. Mutations causing amino acid changes in the HECT domain could lead to conformational changes and constitutive activation of catalytic function, which in turn could trigger autoubiquitination of NEDD4L mutant variants and their degradation. This hypothesis is based on previously reported studies suggesting that catalytic activity, including autoubiquitination of HECT E3 ligases, is dependent on NEDD4L conformation.

With the model of constitutive activation due to mutations in the HECT domain, one could expect not only autoubiquitination but also aberrant ubiquitination of other NEDD4L substrates, whereas a conventional loss-of-function mechanism is expected to lead to a deficit in ubiquitination activity. Moreover, despite autoubiquitination and degradation, the predicted state of constitutive activation for NEDD4L mutants could mimic the increased activity resulting from the overexpression of wild-type NEDD4L. This model could therefore reconcile, at least partially, the apparent discrepancy regarding the observed similar neurodevelopmental defects resulting from IUEP of stable wild-type NEDD4L and the unstable NEDD4L mutants.

Another finding that was observed with wild-type and mutant NEDD4L corresponds to a positioning defect of postmitotic neuronal cells. In the misplaced neuronal cells overexpressing wild-type NEDD4L, we observed a distinct pattern of Dab1 distribution. It is worth mentioning that this finding is in line with previously reported studies that demonstrated association between excess nucloeytoplasmic Dab1 in the cytoplasm of postmitotic neurons and its inhibitory

DISCUSSION

Our findings demonstrate the critical role of NEDD4L in the regulation of processes involved in cortex development and implicated in the PNH syndrome. In the absence of NEDD4L, we observed a distinct pattern of Dab1 distribution. It is particularly important to note that the overexpression of wild-type NEDD4L, which is predicted to lead to conformational changes that favor transition from the inactive state to the open, active state and trigger autoubiquitination and proteasomal degradation, can rescue the neuronal position defect resulting from IUEP with constitutive activation due to mutations in the HECT domain of NEDD4L in PNH. We report the identification of one transmitted and five de novo mutations in seven patients (two siblings and five unrelated patients) with a common distinguishable phenotype characterized by PNH, intellectual disability, cleft palate and syndactyly (in six of seven patients). All mutations associated with this phenotype are located in the region of NEDD4L encoding the HECT domain of the E3 ubiquitin ligase NEDD4L, and one of these mutations, c.2677G>A, p.Glu893Lys, is a recurrent mutation that was found in three unrelated families (Table 1).

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Figure 7 Models depicting the consequences of PNH-related mutations for NEDD4L stability and PI3K–Akt–mTOR and TGF–Smad pathways. (a) Wild-type NEDD4L (left) is shown in its closed and inactive conformation, and mutant NEDD4L (right) is shown with an alteration in the HECT domain predicted to lead to conformational changes that favor transition from the inactive state to the open, active state and trigger autoubiquitination and proteasomal degradation. (b) Overview of observed deregulations under basal conditions resulting from an excess of wild-type NEDD4L (left) and expression of PNH-related NEDD4L mutants (right). Red contours indicate deregulated proteins, and dotted arrows depict indirect and poorly understood relationships between components of signaling pathways.
effect on neuronal migration. In arrested neuronal cells electroporated with NEDD4L mutants, the unchanged distribution of Dab1 suggests that the neuronal migration defect caused by NEDD4L mutants could be mediated by a mechanism divergent from the one disrupted by overexpression of wild-type NEDD4L. In favor of this view is evidence suggesting that excess wild-type NEDD4L is associated with the disruption of signaling pathways regulated by mTORC1 and Dab1. In contrast, PNH-related mutants might act through deregulation of mTORC1, Akt, mTORC2 and TGF-β–Smad2/3 pathway activities (Fig. 7b). Although further investigations are required to define the exact mechanisms by which NEDD4L overexpression and variants affect these pathways, these hypotheses are supported by a recent study showing that NEDD4L catalyzes ubiquitination of PIK3CA and regulates PI3K–AKT signaling and by our rapamycin-based experiments. Indeed, the consistent rescue of the neuronal position defect and restoration of the cellular distribution of Dab1 by rapamycin treatment suggest that the migration and positioning defects induced by overexpression of wild-type NEDD4L could be mediated by mTORC1 pathway deregulation.

For PNH-related mutants, in addition to the increased activity of mTORC1, the deregulation of Akt and Smad2/3 activities under basal conditions and upon TGF-β activation provides an interesting entry point to understand further the role of NEDD4L in the regulation of neurodevelopmental processes underlying cortical development and the established implication of PI3K–AKT signaling pathways in a large spectrum of neurodevelopmental syndromes caused by activating mutations in AKT3, PIK3R2 and PIK3C, some of which are associated with phenotypic features of MCD. As a whole and to the best of our knowledge, we have for the first time shown that mutations in NEDD4L altering the HECT domain are associated with PNH and that excess NEDD4L is likely to be deleterious for brain development and functioning. This latter finding therefore provides a basis for a better understanding of phenotypes associated with duplications encompassing NEDD4L. In this study, we also reported evidence highlighting the disruptive consequences of NEDD4L mutations on the AKT–mTOR and TGF-β–Smad2/3 signaling pathways. Moreover, our study identified a potential novel disease-causing molecular mechanism, in which missense mutations might lead to a constitutively active state and loss of the mutant protein, but with functional consequences that are different from constitutive haploinsufficiency.

URLs. Exome Aggregation Consortium (ExAC), http://exac.broadinstitute.org/; MutationTester, http://www.mutationtaster.org/; DECIPHER, https://decipher.sanger.ac.uk/; Epi4K Consortium, http://www.ep4k.org/; Deciphering Developmental Disorders Study, http://www.dduk.org/; SubRVIS, http://www.subrvis.org/; Phytre web portal, http://www.sbg.bio.ic.ac.uk/phytre2/.

METHODS
Methods and any associated references are available in the online version of the paper.

Accession codes. Human NEDD4L (NM_001144967), mouse Nedd4l (NM_001114386), ClinVar accessions: SCV000267106–SCV000267109.

Note: Any Supplementary Information and Source Data files are available in the online version of the paper.

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AUTHOR CONTRIBUTIONS
L.B., H.J., E.L.I. and M.-V.H. conceived and designed the experiments, performed the experiments, performed statistical analysis and analyzed the data related to cellular, IUER and functional studies. S.S. provided technical assistance and performed ubiquitination experiments. N.D. provided technical assistance, performed expression and genetic studies, and prepared reagents. J.C.–S., K.A.M., B.L., U.W.L., A.P., N.B.W., R.G., D.K., P.H.J., E.L.I. and M.-V.H. performed expression studies during brain development. L.N. and J.G. performed expression studies during brain development. L.N. and J.G. contributed reagents and material, as well as critical suggestions for functional studies. T.S., the DDD study, A.B., R.O., C.M., P.N. and J.-F.D. contributed to the generation of whole-exome material, as well as critical suggestions for functional studies. The authors declare no competing financial interests.

COMPETING FINANCIAL INTERESTS
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ONLINE METHODS

Subjects, whole-exome sequencing and variant validation. Blood or DNA samples from affected individuals and their parents and informed consent were obtained from all participants in accordance with site-specific institutional review boards. Written consent was obtained to publish patient photographs. For the selected 15 patients with PNH in association with developmental delay and/or epilepsy, 3 of the 15 patients also had PMG. For all patients, mutations in known PNH-related genes (FNLA, ARFGEF2 and C6orf70) and pathogenic copy number variations (CNVs) had previously been excluded. DNA processing, library generation, exome enrichment and whole-exome sequencing in trios comprising affected subjects and their parents were performed and data were analyzed at the French National Centre for Genotyping (CNG, Evry, France), the Paris Descartes Bioinformatics platform and the Sanger Sequencing Centre as previously described13,14,38. Available genomic databases (dbSNP, 1000 Genomes Project, Exome Variant Server, Exome Aggregation Consortium and a local Paris Descartes Bioinformatics platform database) were used to filter exome variants and exclude variants with a frequency greater than 1%. De novo variants were analyzed by PCR and direct Sanger sequencing using DNA from patients and their parents. In the family with two affected siblings and suspected maternal somatic mosaicism, confirmation and estimation of the percentage of cells bearing the variant was performed by the droplet digital PCR approach (QX100 Droplet Digital PCR System, Bio-Rad Life Science Research) using DNA extracted from the peripheral blood of all members of the family (the two patients, the parents and the unaffected individual) and primers specific to the variant and wild-type sequences. Data were analyzed with QuantaSoft v.1.4 software (Bio-Rad Life Science Research).

Protein modeling. Amino acid substitutions were plotted onto the solved protein structure for the catalytic domain of the human NEDD4-like E3 ligase using the Phyre web portal. Models were built by homology modeling using Research Collaboratory for Structural Bioinformatics Protein Data Bank (PDB) code 2ONL. The images in Supplementary Figure 2 were rendered using Chimeras.

Cloning and plasmid constructs. Human untagged NEDD4L cDNA (NM_001144967.1) cloned into pCMV6-Entry vector (SC326303) was purchased from Origene. Mutations were introduced by site-directed mutagenesis using the QuikChange Site-Directed Mutagenesis kit (Agilent Technologies). Wild-type and mutated cDNAs encoding human NEDD4L were then inserted into the multiple-cloning site of psiSIRE vector under the control of the CAG promoter, pCDA3-nV5. For IUEP experiments, psiSIRE-NEDD4L vectors were electroporated in combination with a pCAGGS-IRES-Tomato vector to visualize electroporated cells.

For RNA interference (RNAi) experiments, a 29-mer sequence targeting mouse Nedd4l mRNA and a HuSH 29-mer non-effective shRNA scrambled cassette, both commercially designed and provided by Origene in the p-GFP-V-RS vector (TGS05433), were inserted into psiSIRE vector under the control of the U6 promoter. The Nedd4l shRNA directed against the coding sequence was checked for specificity in sequence databases. Because of the very low level of expression of NEDD4L, efficiency experiments were conducted using cotransfection with wild-type human NEDD4L cDNA and shRNA constructs in N2A cells and immunoblot analysis.

In situ hybridization. Mouse Nedd4l sense and antisense probes (nucleotides 462 to 1,470 of transcript NM_00114386) were synthesized using T7 RNA polymerase (Roche) from pET2.1-Nedd4l (nucleotides 462 to 1,470) and pET2.1-Nedd4l (nucleotides 1,470 to 462) plasmids. Non-radioactive RNA in situ hybridization on frozen brain sections was performed as previously described39.

qRT–PCR. Total RNA was prepared from the brains of mouse embryos at different time points of development and from cultured transfected cells with TRlZol reagent (Thermo Fisher Scientific), and cDNA samples were synthesized with SuperScript II Reverse Transcriptase (Invitrogen). qRT–PCR was performed in a LightCycler PCR instrument (Roche) using SYBR Green Master Mix (Roche). For transfection-based experiments, we used GFP as a normalizer (that is, systematically cotransfected as a reporter). For qRT–PCR of transcripts expressed from transfected cDNA constructs, RNA samples were treated with Turbo DNase (Ambion, Life Technology) to avoid amplification from plasmid DNA. Also, to ensure that we amplified transcripts expressed from transfected constructs, for each sample real-time qPCR (in triplicate) was performed using the cDNA reaction products obtained with or without reverse transcriptase and primers specific to NEDD4L and GFP.

Cell culture, transfections and immunofluorescence. Mouse neuroblastoma N2A cells were cultured in DMEM (Gibco) supplemented with 5% FCS and transfected using Lipofectamine 2000 (Invitrogen). MG132 (Calbiochem) was dissolved in DMSO solution buffer. DMSO was used as a control vehicle. Cells were treated with MG132 at a 10 μM concentration for 15 h before the end of culture. Expression of transfected genes was analyzed 48 h after transfection by immunocytochemistry and immunoblotting.

For primary cultures of neuronal cells, embryonic mouse cortical neurons (E17) were electroporated using the Amaxa mouse Nucleofector kit (Lonza) and maintained in Neurobasal medium supplemented with 2% B27, 1% glutamine and 1% penicillin-streptomycin. Cells were fixed in 4% paraformaldehyde 96 h after electroporation. Immunocytochemistry was performed according to standard procedures using antibody to Nedd4l (13690-1-AP, rabbit, Proteintech; 1:200 dilution) as the primary antibody and donkey anti-rabbit IgG 647 (A-21208, Life Technologies; 1:800 dilution) as the secondary antibody. Results were observed and photographed using a TCS SP5 confocal microscope (Leica Microsystems).

Immunoblotting. Cells were lysed in RIPA buffer (50 mM Tris–HCl, pH 7.7, 0.15 M NaCl, 1 mM EDTA and 1% Triton X-100) supplemented with protease inhibitors (Roche) and phosphatases inhibitors (Sigma-Aldrich). Protein concentration was measured using Bio-Rad protein assay reagent. Samples were denatured at 95 °C for 10 min in loading buffer and then resolved by SDS–PAGE and transferred onto nitrocellulose membranes. Membranes were blocked in 5% nonfat milk in TBS buffer with 0.1% Tween and then immunoblotted using the following primary antibodies at the specified concentrations: Nedd4l (13690-1-AP, rabbit, Proteintech; 1:1,000 dilution), actin (mouse, IGBMC; 1:1,000 dilution), Akt-pSer473 (4060, rabbit, Cell Signaling Technology; 1:1,000 dilution), Akt-pThr308 (2965, rabbit, Cell Signaling Technology; 1:1,000 dilution), Akt (pan) (4691, rabbit, Cell Signaling Technology; 1:1,000 dilution). S6-pSer236/242 (2211, rabbit, Cell Signaling Technology; 1:1,000 dilution), S6 (2217, rabbit, Cell Signaling Technology; 1:1,000 dilution), ubiquitin (sc-8017, mouse, Santa Cruz Biotechnology; 1:250 dilution) and V5 (R96025, mouse, Invitrogen; 1:5,000 dilution). All immunoblot experiments consisted of at least three independent replicates.

Immunohistochemistry. Mouse embryo brains were fixed by incubation overnight at 4 °C in 4% paraformaldehyde in 0.1 M phosphate buffer, pH 7.4. Brains were placed in a solution of 4% low-melting agarose (Bio-Rad) and cut into coronal sections (80 m) using a vibrating-blade microtome (Leica VT1000S, Leica Microsystems). Sections were maintained in 0.01% azide in PBS buffer. For immunodetection, sections were blocked with 1x PBS with 2% normal donkey serum (Jackson ImmunoResearch) and 0.3% Triton X-100 (PBS-T-NGS) for 30 min at room temperature. Primary antibodies were diluted in PBS-T-NGS and incubated with sections overnight at 4 °C. The following primary antibodies were used: Dab1 (AB5840, rabbit, Millipore; 1:500 dilution), Cux1 (sc-13024, rabbit, Santa Cruz Biotechnology; 1:1,000 dilution), KIs6 (IHC-00375, rabbit, Bethyl Laboratories; 1:250 dilution), NEDD4L (13690-1-AP, rabbit, Proteintech; 1:300 dilution), NeuN (MAB377, mouse, Millipore; 1:100 dilution), Pax6 (PAB-278P, rabbit, Covance; 1:200 dilution), Ph3 (06-570, rabbit, Millipore; 1:500 dilution) and TR2R (14-4875, rat, ebioscience; 1:200 dilution). After washes in 1x PBS, sections were incubated with Alexa Fluor–conjugated secondary antibodies (A-31573, donkey anti-rabbit IgG 647; A-21208, donkey anti-rabbit IgG 488; A-21206, donkey anti-rabbit IgG 647; A-31571, donkey anti-mouse IgG 647; all from Life Technologies) diluted 1:500 in PBS-T for 1 h at room temperature. Sections were washed and then mounted with Fluoromount-G mounting medium (Interchim). All images were acquired using a TCS SP8 confocal microscope (Leica Microsystems), and positionning analysis was achieved with ImageJ software (NIH) and proliferation analysis was performed with LAS AF software (Leica Microsystems). Graphs
were generated in GraphPad Prism 6 (GraphPad), and images were assembled with Adobe Photoshop 13.0.1 (Adobe Systems).

**Ubiquitination assays.** For immunoprecipitation assays, transfected cells were lysed with RIPA buffer supplemented with protease inhibitors (Roche), MG132 (25 μM) and PR-619 (20 mM) and protein extracts were incubated with anti-V5 agarose beads (A7345, Sigma) for 2 h at 4 °C under constant rotation in RIPA buffer. Immunoprecipitated proteins were eluted in Laemmli SDS buffer at 95 °C and subjected to SDS–PAGE. For in vitro ubiquitination assays, immunopurified NEDD4L from transfected N2A cells was incubated in reaction mixtures containing 200 nM E1 ubiquitin-activating enzyme (BostonBiochem), 400 nM E2 ubiquitin-conjugating enzyme (UbchH7; BostonBiochem), 400 μM ubiquitin (Sigma) and 2 mM ATP in reaction buffer (25 mM Tris-HCl (pH 7.5), 50 mM NaCl, 0.1 μM DTT and 4 mM MgCl₂). Reactions were incubated for 1 h at 30 °C and analyzed by immunoblotting with antibodies to ubiquitin, V5 and NEDD4L.

**In utero electroporation.** In utero electroporation was performed as described previously enlist using Swiss mice (Janvier). Animal experimentations were performed at the IGBMC animal facilities. The study has Animal Experimentation Research Ethics Committee approval (2014-059). Briefly, timed pregnant mice (E14.5) were anaesthetized with isoflurane (2 l per min of oxygen, 4% isoflurane during sleep and 2% isoflurane during surgery; Minerve). The uterine horns were exposed, and a lateral ventricle of each embryo was injected using pulled-glass capillaries with Fast Green (2 μg/ml; Sigma) combined with a final concentration of 1 μg/ml of DNA constructs prepared with the EndoFree plasmid purification kit (Macherey Nagel). The expression vector pCAGGS-Tomato was systematically co-electroporated, and fluorescent Tomato protein was used to visualize electroporated cells. Plasmids were further electroporated into the neuronal progenitors adjacent to the ventricle by delivering five electric pulses at 50 V for 50 ms at 950-ms intervals using a CU21EDIT electroporator (Sonidel). After electroporation, embryos were placed back in the abdominal cavity and development was allowed to continue until E16, E18 or P2. Embryo or pup brains were dissected and fixed in 4% paraformaldehyde in PBS overnight.

**Rapamycin treatment.** Rapamycin Ready-Made Solution (2.5 mg/ml in DMSO; Sigma) was previously diluted in PBS and then injected intraperitoneally at a concentration of 0.5 mg/kg daily from E15.5 to E17.5 into pregnant females electroporated at E14.5. Embryos were collected at E18.5 for analysis.

**Statistics.** All statistics were calculated with GraphPad Prism 6. Final counts are presented as the mean percentages ± s.e.m. One- or two-way ANOVA was performed for multiple comparisons followed by Dunnett’s or Sidak’s post-hoc tests, respectively, whereas unpaired two-tailed Student’s t tests were used for dual comparisons. P < 0.05 was considered significant: *P < 0.05, **P < 0.01, ***P < 0.001, ****P < 0.0001. On the basis of previous IUEP experiments performed in our laboratory, we considered that at least three embryos per condition would be necessary. After histological examination, only brains with comparable electroporated regions and efficiencies were retained for quantification. Data distribution was not tested but was assumed to be normal. Blinding was not applied for data collection and analysis. Statistical details are included in Supplementary Table 2.

**Exome sequencing data deposition.** Exome sequencing data have been deposited in the database of Genotypes and Phenotypes (dbGaP) under study accession phs000653.v1.p1 for the Phh31124 trio (proband EPGP012746) and the European Genome-phenome Archive (EGA) under accession EGAD00001001848 for the DDDP110533 trios. For the other patients analyzed by the whole-exome sequencing approach, no consent was obtained from the patients to deposit the data in a repository.