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Recessive PRDM13 mutations cause fatal perinatal brainstem dysfunction with cerebellar hypoplasia and disrupt Purkinje cells differentiation

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Title: Recessive *PRDM13* mutations cause severe brainstem dysfunction with perinatal lethality, cerebellar hypoplasia and disrupt Purkinje cell differentiation

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ABSTRACT

Pontocerebellar hypoplasias (PCH) are congenital disorders characterized by hypoplasia or early atrophy of the cerebellum and brainstem, leading to a very limited motor and cognitive development. Although over 20 genes have been shown to be mutated in PCH, a large proportion of affected individuals remains undiagnosed. We describe four families with children presenting with severe neonatal brainstem dysfunction, and pronounced deficits in cognitive and motor development, associated with four different bi-allelic mutations in *PRDM13*, including homozygous truncating variants in the most severely affected individuals. Brain MRI and foetopathological examination revealed a PCH-like phenotype, associated with major hypoplasia of inferior olive nuclei and dysplasia of the dentate nucleus. Notably, histopathological examinations highlighted a sparse and disorganized Purkinje cell layer in the cerebellum. *PRDM13* encodes a transcriptional repressor known to be critical for neuronal subtypes specification in the mouse retina and spinal cord, but had not been implicated, so far, in hindbrain development. snRNAseq data mining and in situ hybridization in human, show that *PRDM13* is expressed at early stages in the progenitors of the cerebellar ventricular zone, which gives rise to cerebellar GABAergic neurons, including Purkinje cells. We also show that loss-of-function of *prdm13* in zebrafish leads to a reduction in Purkinje cells numbers and a complete absence of the inferior olive nuclei. Altogether our data identified biallelic mutations in *PRDM13* as causing a olivopontocerebellar hypoplasia syndrome, and suggest that early deregulations of the transcriptional control of neuronal fate specification could contribute to a significant number of cases.

MAIN TEXT

INTRODUCTION

Cerebellar hypoplasia (CH) refers to reduced cerebellar volume with a (near-) normal shape based, in vivo, on neuroimaging. It is a relatively common finding associated with a wide range of clinical features [1-3]. Additional neuroimaging findings, such as abnormal size or shape of the pons or the medulla oblongata, are key to categorize CH and better predict the clinical outcome. Early steps of cerebellar development are shared with the brainstem explaining the frequent association of abnormal development of these structures in disorders such as Joubert syndrome [MIM:213300], Rhombencephalosynapsis[4], Pontine Tegmental Cap Dysplasia [MIM:614688], Diencephalic–Mesencephalic Junction Dysplasia [MIM:251280] or Pontocerebellar Hypoplasia (PCH [MIM:607596])[5]. PCH is described as a group of generally neurodegenerative disorders with prenatal onset, progressive features, and at least 13 subtypes, based on neuropathological, clinical and MRI criteria[6; 7]. The prognosis of PCH disorders is poor and most affected individuals die during infancy or childhood. Despite the current effort to identify new genes associated with PCH, a large number of affected individuals remains without genetic diagnosis[6]. PCH often results from defects in apparently ubiquitous cellular processes such as tRNA synthesis, mitochondrial, glycosylation, inositol or purine nucleotide metabolisms[5; 7; 8]. In parallel to the typical PCH presentation, mutations in *CASK* [MIM:300172], *CHMP1A* [MIM:164010], and *TBC1D23* [MIM: 617687] genes can lead to non-neurodegenerative disorders categorized respectively as PCH-like (or MICPCH [MIM: 300749]), PCH8[9] and PCH11[10] disorders. Few cases with mutations in *PTF1A* [MIM: 607194] can be classified in this category of non-neurodegenerative syndrome, with an imaging pattern similar to PCH[11], although these are characterized by an extremely severe impact on cerebellar development, associated with neonatal diabetes[12]. Interestingly, PTF1A is known to play a key role in

hindbrain neural stem cell fate specification; whether other cell fate determinants factors contribute to congenital cerebellar disorders remains to be further explored. Indeed, for many key hindbrain stem cell fate determinants (e.g., *ATOH1* [MIM: 601461], *SKOR2* [MIM: 617138], *EN2* [MIM: 131310]) there are no associated and clearly defined Mendelian disorders yet. A better knowledge of the clinical consequences associated with the loss of function of these key genes would improve our understanding of their roles in human cerebellar and whole brain development.

PRDM13 [MIM:616741], a known target of PTF1A, encodes a transcriptional regulator previously implicated in neuronal specification in the retina and spinal cord[13; 14 15; 16]. However, until recently, no role had been attributed to this factor during cerebellar development. Indeed, Whittaker et al. reported a variant of this gene in three individuals with a hypogonadotropic hypogonadism syndrome associated with cerebellar hypoplasia[17]. Here, we report a significantly different syndrome based on eight individuals from four families of different origins, associated with loss-of-function mutations of *PRDM13*. This syndrome associates perinatal lethality with severe brainstem dysfunctions (e.g. feeding and respiratory difficulties, central apnea, bradycardia), together with persistent cerebellar hypoplasia. We also clearly establish that *PRDM13* is critically needed for the cerebellar GABAergic lineage differentiation, including Purkinje cell as well as for olivary nuclei genesis.

MATERIAL AND METHODS

Patients' recruitment and investigation

Families 1 and 2 were recruited through the departments of genetics of the Pellegrin and the Necker Enfants Malades Hospitals, and the French reference center for cerebellar malformations and congenital diseases at Trousseau Hospital, France. Families 3 and 4 were identified through GeneMatcher[18]. Family 3 was referred to the clinical genetics department of university hospitals Bristol. Family 4 was recruited through the Genetic and Developmental Medicine Clinic Sultan Qaboos University Hospital, Oman. Written informed consents have been obtained both from all the participants and the legal representatives of all the children. Procedures followed were in accordance with the ethical standards of the national and institutional responsible committees.

Index cases of the four families have been analyzed using Whole-Exome Sequencing (WES). For family 1, trio WES (proband F.1-1 and both parents) used the Sure Select Human All Exon 58 Mb V6 protocol (Agilent Technologies) to prepare libraries that were sequenced with a HiSeq2500 system (Illumina), at the Imagine institute (Paris, France). Sequences were aligned to the reference human genome hg19 using the Burrows-Wheeler Aligner (BWA). Downstream processing was carried out with the Genome Analysis Toolkit (GATK), SAMtools and Picard. The annotation process was based on the latest release of the Ensembl database, gnomAD 2.1. Variants were annotated and analyzed using the Polyweb software interface designed by the Bioinformatics platform of Imagine-Université de Paris[19]. Family 2-1 proband was studied by WES performed in proband only, at Sorbonne Université, Trousseau Hospital (Paris, France), with the following details: SeqCap EZ MedExome capture kit (Roche) and sequence on Illumina NextSeq 500. The BaseSpace cloud computing platform with BWA, GATK Unified Genotyper and the VariantStudio v.3.0 provided by Illumina was used for analysis. Targeted Sanger sequencing was performed in proband, parents, and affected sibling of the family 2 to confirm the presence of the homozygous or heterozygous variants in affected children and parents, respectively. For family 3, WES was performed on both children and their healthy parents by the Exeter Genomics Laboratory (Exeter, UK). Libraries were prepared using the Twist Core Human Exome Kit (Twist Bioscience) and sequenced on the NextSeq 500 or NovaSeq seq platform (Illumina). Reads were aligned to human genome build hg19, and analyzed for sequence variants using a custom-developed analysis tool as previously described[20]. Family 4-3 proband was explored with clinical Exome at BGI Europe (Copenhagen, Denmark). Exome capture was performed with the Agilent SureSelectXT Human All Exon 50Mb Kit and sequencing was done on HiSeq2000TM (Illumina). Analysis was performed at Sultan Qaboos University hospital (Muscat, Oman). An in-house database of 1562 exomes was used to filter out common population specific variants[21]. Sanger sequencing was used to confirm the mutation in the affected individual. The DNA from parents and siblings was not available. Detailed clinical reports are included in **document S1**.

In situ hybridization (ISH) with human samples

Tissue collection, processing and ISH was performed at University College London/HDBR as previously described[22], and under the approval of the National Research Ethics Service (NRES). Briefly, tissues were fixed in 4% PFA /10% formalin overnight, embedded and sectioned at 6 microns. ISH was performed using RNAscope® 2.5 HD Detection Kit and commercially available probes (Advanced Cell Diagnostics) for *PRDM13* and *PPIB* as a positive control with the references 459851 and 313901, respectively.

scRNA-seq data retrieval

Data re-analyzed as part of this study were retrieved from the Gene Expression Omnibus (GEO) public database under accessions GSE120372 for mouse embryonic cerebellum[23] and GSE158142 for zebrafish brain scRNA-seq data[24] and from the Human Gene Expression During Development atlas (Descartes, https://descartes.brotmanbaty.org) for human cerebellum snRNA-seq data[25]. Mouse scRNAseq data corresponds to a single developmental time point, embryonic day 13.5 (E13.5; n = 9,353 cells). Zebrafish scRNA-seq data were retrieved as processed R objects (.rds files) for different development stages (24 hours post fertilization to 8 days post fertilization; n = 95,730 cells) and containing, among others, the assigned clusters information at different resolutions as shown in[24]. Human cerebellum snRNA-seq data were retrieved as sparse gene counts by cell (n = 1,091,999 cells) along with cell type assignations and gene annotations metadata.

Dataset	Species	Source	Number of single cells
Cerebellum	Mouse	GSE120372	9,353
Cerebellum – Purkinje neurons	Human	Descartes Atlas	280,377
Cerebellum – Inhibitory interneurons	Human	Descartes Atlas	129,890
Cerebellum – Granule neurons	Human	Descartes Atlas	312,674
Cerebellum – Astrocytes	Human	Descartes Atlas	268,809
Cerebellum – Vascular endothelial	Human	Descartes Atlas	7,349
Cerebellum – Unipolar brush	Human	Descartes Atlas	52,646
Cerebellum – Microglia	Human	Descartes Atlas	4,428
Cerebellum – Oligodendrocytes	Human	Descartes Atlas	16,104
Cerebellum – SLC24A4_PEX5L positive cells	Human	Descartes Atlas	19,722
Head – 24 hpf	Zebrafish	GEO GSE158142	9,105
Head – 36 hpf	Zebrafish	GEO GSE158142	10,719
Head – 48 hpf	Zebrafish	GEO GSE158142	15,558
Brain – 5 dpf	Zebrafish	GEO GSE158142	31,659
Brain – 8 dpf	Zebrafish	GEO GSE158142	28,689

Mouse and Human cerebellum data processing

Data were imported into R v4.0.3 creating a Seurat v4.0 object[26] from counts. Further data processing followed the standard Seurat workflow for data normalization, dimensionality reduction (principal component analysis, PCA) and graph-based clustering described in Butler *et al.*[27]. Differentially expressed genes for the identified cell subpopulations were determined using Wilcoxon rank sum tests on genes present in at least 20% of cells in the population of interest, only retaining positive gene markers. Testing was limited to genes which showed, on average, at least 0.2-fold difference (on a log-scale) between the different groups. Finally, genes displaying an adjusted *p*-value inferior to 5% ($P_{adj} < 0.05$) were retained.

Visualization

PRDM13, *SOX2* and *PTF1A* gene expression levels were represented over t-distributed stochastic neighbor embedding (t-SNE) projection plots of distinct single cells at different developmental stages for zebrafish and mouse datasets or over Uniform Manifold Approximation and Projection (UMAP) plots for human cerebellum data. Simultaneous visualization of the expression levels of two different genes was performed with the *FeaturePlot* Seurat function (blend = TRUE parameter).

Zebrafish lines and maintenance

Wild-type AB (ZFIN: ZDB-GENO-960809-7) and prdm13^{sa16464} (ZDB-ALT-130411-4933) zebrafish were used for the experiments. The *prdm13^{sa16464}* line was generated by the Zebrafish Mutation Project (ZMP)[28] and obtained from the EZRC center (Karlsruhe). Zebrafish were maintained using standard fish-keeping protocols and experimental protocols conformed to French and European ethical and animal welfare directives (project authorization from the Ministère de l'Enseignement Supérieur, de la Recherche et de l'Innovation to M.C.). Genotyping of individuals from the *prdm13^{sa16464}* line was performed by PCR using the following allele-specific primer pairs:

$^{5'}$GAGCGATACATTTGCCGG$^{3'}$ $^{5'}$CTCAGCAGGGGATATCTTCG$^{3'}$(WT allele)

$^{5'}$ATGGCCACAAACAACACAAA$^{3'}$ $^{5'}$GGTATTTGAATATTCTCCAACAAGAT$^{3'}$(sa16464 allele)

Fresh fin clips from adult breeders or tails from fixed larvae were digested with Proteinase K (200µg/mL) diluted in PCR buffer (10 mM Tris pH 8, 2 mM EDTA, 0.2% Triton X-100) for 3h at 55°C. Proteinase K was inactivated by a 5 min incubation at 95°C. PCR was performed with ReadyMix™ Taq PCR Reaction Mix (Sigma-Aldrich).

Zebrafish embryos and tissue fixation

Zebrafish embryos and larvae were euthanized with tricaine (0.2% in embryo medium) and fixed overnight at 4 °C in a 4 % solution of paraformaldehyde (PFA) in PBS. For ISH on larval or juvenile brains (>5dpf), brains were dissected out in cold PBS after fixation. Following fixation, samples were washed twice in PBS. Whole embryos and larvae were bleached with a peroxide-containing bleaching solution (5 % Formamide, 0.5X SSC, 3 % H202) until dark pigments are no longer visible. Samples were either stored in PBS at 4°C (for IHC) or dehydrated in serial dilutions of methanol/PBS and stored in 100 % methanol at -20 °C (for ISH).

RNA extraction

Dechorionated 24hpf zebrafish embryos were euthanized on ice with tricaine (0.2% in embryo medium) and homogenized with a pestle in Trizol solution (Thermofisher, 1 mL Trizol for 50 – 100 embryos). After a 5 min incubation at RT, chloroform was added (200µL per mL of Trizol). The Eppendorf tube was shaken for 15 seconds and centrifuged at 12000 g for 15 min. The upper phase was transferred to a fresh tube containing 500 µL Isopropanol and incubated at RT for 10 min. The tube was centrifuged at 12000 g at 4 °C for 10 min. After removal of supernatant, the pellet was washed with 70 % EtOH and dissolved in 44 µL RNAse-free water. Genomic DNA was removed using RNAse-free DNAse I treatment (NEB): 5 µL of 10x DNase buffer and 1 µL of RNase-free DNase was added to the 44 µL of RNA and the mix was incubated for 30 min at 37 °C. To stop the reaction, 1 µL of 0.25 mM EDTA was added and the tube was incubated at 65 °C for 10 min. RNA concentration was measured using a nanodrop and stored at -80 °C.

Cloning

A fragment of zebrafish *prdm13* transcript (Reference Sequence: NM_001326454.1) covering the full-length CDS was amplified by PCR with the AccuPrime™ Taq DNA Polymerase System (Invitrogen) using the following primers:

5'TGGATCCACAGAGCCAACCATGCAAACG3'

5'TTCTAGATTAGTGTGTCCGTAAAGTGG3'

The 1881nt-long PCR product was cloned inside the pCR™2.1-TOPO™ plasmid, using TOPO™ TA Cloning™ Kit (Invitrogen) according to the manufacturer's instructions. Clone sequences were verified by Sanger sequencing with universal M13 primers.

RNA probes synthesis

For *prdm13* RNA probe synthesis, a DNA matrix was first generated by PCR from pCR™2.1-*prdm13* plasmid using ReadyMix™ Taq PCR Reaction Mix (Sigma-Aldrich) and M13 primers. For *ptf1a* RNA probe synthesis, a plasmid containing the full-length *ptf1a* transcript (pBS-SK-*ptf1a*(FL)) was kindly donated by Pr Hibi (Nagoya University). 10 µg of plasmid DNA was linearized using EcoRI (NEB). DNA matrices were purified using PureLink™ PCR Micro Kit (Invitrogen) and eluted in 10 µL of RNAse-free water. To generate DIG-labeled probes, 1 µg linearized plasmid was used as a template for transcription reaction using T3 or T7 RNA polymerase and DIG RNA Labeling Kit (Roche), according to the manufacturer's instructions. Transcription reaction was carried out for 3 h at 37 °C and a final incubation with DNase I was performed to remove template DNA. Unincorporated nucleotides were removed with the ProbeQuant G-50 Micro Columns Kit (GE Healthcare). The RNA probe was stored at -80 °C.

Whole-mount ISH

In situ hybridization were carried out according to standard protocols[29]. Briefly embryos or dissected brains were rehydrated, washed with PBST and incubated in hybridization buffer at 65 °C for 3 h. Brains were then incubated overnight at 65 °C with DIG-labeled RNA probes diluted at 1/100 in hybridization buffer. Brains were washed in serial dilutions of hybridization buffer/2XSSC at 65 °C to remove excess probe. An immunohistochemistry with anti-DIG antibody coupled to alkaline phosphatase (Roche,

1/5000 dilution) was then performed. In situ signals were revealed either with NBT/BCIP (Roche) or Fast Red (Sigma) for fluorescent visualization. Following ISH, 2-months old juvenile brains were sectioned using a vibratome (Leica VT1200) and mounted on microscope slides.

Whole-mount IHC

For whole-mount IHC, zebrafish larvae were permeabilization in acetone at -20°C for 20 min. Samples were washed 3 times with PBST (0.1 % Triton in PBS). Antigen retrieval was then performed through an incubation in HistoVT One (Nacalai Tesque) buffer for 1 h at 65 °C. Larvae were washed three times for 5 min each with PBST, and once in PBS-DT (0.1% Triton + 1% DMSO + 1% BSA in PBS). Samples were incubated in Blocking Buffer (5% goat serum in PBS-DT) for at least 1h at RT, and then with primary antibodies (see antibody table) diluted in Blocking Buffer for 24h at 4 °C. This was followed by 6 washes with PBST for 15 min. Larvae were incubated overnight at 4 °C with secondary antibodies (see antibody table) diluted 1:1000 in PBS-DT. The brains were then washed six times for 15 min in PBST. Brains were mounted in a glycerol solution (80% glycerol/PBS) for confocal imaging.

Antibodies

Antibody name	Source	Identifier
Sheep Anti-Digoxigenin Fab fragments Antibody, AP Conjugated	Sigma-Aldrich	Cat# 11093274910; RRID: AB_514497
Mouse monoclonal (IgG1) Anti-SOX2 antibody [20G5]	Abcam	Cat# ab171380; RRID: AB_2732072
Mouse monoclonal (IgG2b) Anti-HuC/D, Clone 16A11	Thermo Fisher Scientific	Cat# A-21271; RRID:AB_221448
Rabbit Anti-Calbindin D-28k (for zebrafish)	Swant	Cat# CB-38a; RRID:AB_10000340
Mouse monoclonal Anti-Calbindin-D-28K, Clone CB-955 (for human)	Sigma-Aldrich	Cat# C9848, RRID:AB_476894
Mouse monoclonal (IgG2a) anti-PCNA Antibody, clone PC10	Santa Cruz Biotechnology	Cat# sc-56; RRID:AB_628110
Mouse polyclonal (IgG1) LHX1 antibody (4F2)	DSHB	Cat# 4F2-s; RRID:AB_531784
Mouse monoclonal (IgG1) anti-Parvalbumin	Millipore	Cat# MAB1572; RRID:AB_11211313
Goat anti-Mouse IgG1 488	Thermo Fisher Scientific	Cat# A-21121; RRID:AB_2535764

Goat anti-Mouse IgG2a-633	Thermo Fisher Scientific	Cat# A-21136, RRID:AB_2535775
Goat anti-Mouse IgG2b-633	Thermo Fisher Scientific	Cat# A-21146, RRID:AB_2535782
Goat anti-Rabbit-546	Thermo Fisher Scientific	Cat# A-11010, RRID:AB_2534077

Image acquisition

Brightfield images were acquired with a Stereomicroscope (Leica M165FC) equipped with a color digital camera (Leica DFC450C). Fluorescent images were acquired on confocal microscopes (LSM700), using either a 20X air objective (Plan Apochromat 20x/0.8 M27) or a 40X oil objective (Plan-Apochromat 40x/1.3 Oil M27). 3D renderings were generated using the Imaris software (version 9.7.2, Bitplane). The 3D image was cropped to feature only the pallium as our region of interest and contrast was adjusted for each channel. For Sox2 immunostaining signals, background was subtracted using the remove outliers function of the ImageJ image analysis software (version 1.53c; http://fiji.sc).

Quantification and Statistical Analyses

3D Images were cropped and then segmented manually using semi-automatic detection with the Imaris spots function followed by manual curation. The whole cerebellum was segmented to quantify PARV+ Purkinje cells and CALB+ Eurydendroid cells. Data are presented as mean ± 95% confidence interval. Statistical analyses and graphical plots were done using GraphPad Prism version 9.2.0 for Windows (GraphPad Software, San Diego, California USA, www.graphpad.com). Deviation from Mendelian ratios was tested using Chi-square tests and pairwise comparisons were carried out with two-tailed unpaired t-tests.

RESULTS

Identification of biallelic *PRDM13* pathogenic variants in families with brainstem dysfunction and cerebellar hypoplasia

In a collaborative effort to identify additional mechanisms underlying PCH and PCH-like disorders [5; 19], we recruited a French family of North-African origin with distant consanguinity (Family 1) where three siblings presented with cerebellar vermis hypoplasia and brainstem dysgenesis (**Figure 1A**). For the proband (F.1-1) and sibling F.1-6, fetal brain MRI showed severe cerebellar hypoplasia with abnormal foliation (**Figure 1B**, **Clinical table**, **Supplementary document S1**) and intact supratentorial structures. At birth, they presented with profound hypotonia and feeding difficulties without sucking abilities. Several episodes of desaturation and altered consciousness related to a potential central origin required oxygenation therapy. The affected child F.1-1 died at the age of 25 months without reaching any developmental milestones and her sibling F.1-6 died at the age of 4 months. During the fourth pregnancy, similar manifestations as the first pregnancy were detected by close ultrasound follow-up and parents opted for a medical termination at 24 weeks of gestation (WG). Fetal pathological examination of F.1-4 further documented the disrupted development of the cerebellum and the brainstem (**Figure 2A, B** ; see below), consistent with a type of PCH. Using whole exome sequencing (WES), we identified a homozygous single base pair deletion in the *PRDM13* gene in the three affected children. This variant, c.839del (p.(Ala280Glyfs*21)), is predicted to cause a frameshift followed by premature termination after the first zinc finger domain (**Figure 1D**). *PRDM13* encodes a transcription factor with key functions in inhibitory neurons differentiation [13; 14]. *Prdm13* knockout mice display dorsal-ventral cell specification defects in the fetal neural tube and die neonatally of unknown reasons[16]. Interestingly, a homozygous *Prdm13* deletion that removes the C-terminal part of the protein after the first zinc finger domain reflects a null phenotype[16]. Based on these first observations, we hypothesized that *PRDM13* loss of function could cause the neurodevelopmental disorder observed in Family 1. In our effort to replicate this finding, we identified five other affected individuals from three additional families (**Figure 1, Clinical table**) with highly comparable clinical phenotypes including cerebellar hypoplasia, brainstem dysfunction and a diagnosis consistent with a form of PCH (individuals F.2-1, F.2-2, F.4-3; **Figure 1B, C**). To have a broad view of the phenotypical consequences resulting from *PRDM13* mutations, we used a genotype-first approach[18] which led to the identification of families 3 and 4. WES

was systematically used to uncover the genetic variants suspected to cause their disorder. For each family, the homozygous variant located in *PRDM13* is the only one compliant with autosomal recessive Mendelian inheritance and/or the most likely to disrupt gene function (**Supplementary information S1**). Affected individuals of families 2, 3 and 4 were homozygous for variants c.844del (p.(Val282Serfs*19)), for c.1856A>T (p.(His619Leu)), and for c.800del (p.(Gly267Aspfs*34)), respectively (GenBank: NM_021620.3, NP_067633.2; **Clinical table**). All variants are either absent or encountered (at the heterozygous state) with a frequency lower than 0.001% in gnomAD (v2.1.1). The variant identified in family 3 is the only missense variant identified (p.(His619Leu)), affecting a fully conserved histidine (up to *S. cerevisiae*, not shown) with a CADD score of 32. This histidine residue (**Figure 1D bottom-right**, in red) is one of the invariant residues of the Cys2-His2 binding motif[30]. Consequently, the functionality of the third zinc finger domain is abrogated by the p.(His619Leu) variant.

Family 2 is also a French family of North-African origin with consanguinity. They had three pregnancy attempts; the second pregnancy resulted in a fetus who was interrupted for presenting with cerebellar and brainstem hypoplasia (fetus F.2-1, **Figure 1B**) and the third one resulted in a newborn who died at 22 days with a similar condition and profound hypotonia, inability to eat and respiratory distress (infant F2.2, **Figure 1C**). Family 3 is from the UK, from Pakistani origin, with two affected girls with a milder presentation. The F.3-1 elder sister presented with neonatal hypotonia, bradycardia and respiratory distress. Brain MRI identified a non-progressive form of PCH. She had recurrent episodes with symptoms of dysautonomia associated with somnolence and then unresponsiveness. Similarly, F.3-2 presented with hypotonia, neonatal autonomic problems, including temperature instability and bradycardia. She has an apparently less severe condition compared to her sister, without severe episodes of altered consciousness and autonomic instability, but she presented with developmental delay, slow weight gain and cerebellar hemispheres hypoplasia with preserved brainstem on MRI.

Family 4 is a consanguineous family from Oman, with a single affected individual presenting with microcephaly, feeding difficulty, apnea, hypoventilation and who died at 16 months. Brain MRI showed a dysplasia and a reduced size of the cerebellar vermis and the hemispheres (**Figure 1C**).

Truncations of *PRDM13* are associated with early disruption of cerebellar and brainstem development

Neuropathological examination was performed for fetuses F.1-4, F.2-1 and the infant F.2-2, covering the stages 24 GW, 31 GW and 34 GW + 3 postnatal weeks (equivalent to 37 GW) (**Figure 2A-F**). These three cases displayed features distinctive of PCH[31] with unremarkable supra-tentorial structures: i) reduced brainstem and cerebellum over total brain weight (3.4% for F.2-1 at 31 GW (-5.2 SD) and 3.7% for F.2-2 at 37 GW (-3.6 SD), **Table S1**), ii) transverse diameter of the cerebellum below the norm at 31 GW (<5th percentile) and 37 GW (<5th percentile), iii) delayed/abnormal cerebellar foliation. At the histological level, the three individuals also presented comparable cerebellar and brainstem anomalies. Cerebellar vermis thus showed a hypoplastic but relatively preserved structure at 24 GW (**Figure 2A, B, S, T**) and a severe hypoplasia with abnormal foliation at 31 GW and 37 GW (**Figure 2C-F, U, V**). The dentate nuclei were dysplastic and fragmented for the three cases (**Figure 2M-R**). At the level of the brainstem, olivary nuclei were fragmented or extremely hypoplastic (**Figure 2G-L**) and pons nuclei appeared smaller (not shown). By contrast, pyramidal tracts were preserved. Histological examination of the cerebellar cortex, with a immunostaining against CALB to label Purkinje cells, revealed the presence of a disorganized Purkinje cell layer at 24 GW and 31 GW (**Figure 2W-Z**) and a decreased density of these cells at 31 GW and 37 GW (**Figure 2Y-AB**). Additionally, multiple Purkinje cells heterotopia are detected in the white-matter (**Figure 2T**, top-left inset) for all the affected fetuses and infant.

***PRDM13* is expressed in neural stem cells of the hindbrain in mouse and human embryos**

During mouse development, Prdm13 was shown to be necessary to balance neuronal fates in the dorsal neural tube and in the retina[13; 15; 16] but no defined role of this factor was described during hindbrain development. Interestingly, the expression of *Prdm13* in the neural tube is under the control of Ptf1a[13]. Ptf1a is a bHLH (basic Helix-Loop-Helix) proneural transcriptional factor and determines the fate of progenitors of the cerebellar ventricular zone (VZ)[32-34], which give rise to all cerebellar GABAergic neurons (Purkinje cells and inhibitory interneurons) and astrocytes. In addition, Ptf1a is necessary for the development of several brainstem nuclei, including the inferior olivary nucleus (ION) [35; 36]. In order

to better appreciate the expression of *PRDM13* during central nervous system development, we explored available transcriptomic datasets and performed *in situ* hybridization experiments. Analyzing a published single-cell RNA-sequencing (scRNAseq) dataset from the mouse embryonic cerebellum at E13.5[23], we observed that *Prdm13* is expressed in cell clusters corresponding to cerebellar progenitors of the VZ (**Figure S1A**) and co-expressing *Ptf1a* (**Figure S1B-C**). This confirmed a previous report showing expression of *Prdm13* in the mouse cerebellar VZ at E11.5 [13]. To further demonstrate the link between *PRDM13* mutations and hindbrain developmental anomalies, we examined its expression during human brain development. We first exploited publicly available single-nuclei RNA-sequencing (snRNAseq) datasets from fetal cerebellum samples (9-21 post-conception weeks (PCW)) [25] and observed an enriched expression of *PRDM13* in specific cell clusters (**Figure 3A**). These clusters are located at the crossroad between cell clusters annotated as fully differentiated cells that are Purkinje cells, inhibitory interneurons and astrocytes. These clusters also show an enriched expression of both *PTF1A* and the neural stem cell marker *SOX2*, suggesting they might correspond to VZ progenitors (**Figure 3A**). To get a comprehensive picture of the spatio-temporal pattern of *PRDM13* expression during human brain development, we performed *in situ* hybridization on sections of human embryonic and fetal samples using the highly-sensitive and specific RNAscope technology. Considering the early expression of *PRDM13* seen in the embryonic mouse cerebellum, we studied *PRDM13* expression over an extended temporal window from Carnegie Stage 13 (CS13, corresponding to ~32 days post-conception (dpc)) to 15 PCW. At CS13, we detected specific expression of *PRDM13* in the dorsal neural tube, similarly to the described expression pattern in mice (**Figure 3B, B''**). We also detected *PRDM13* transcripts in dorsal ventricular domains of the hindbrain (**Figure 3B, B'**). These expression domains are maintained at CS21 (~51 dpc) (**Figure 3C, C''; 3D, D''**). At this stage, additional expression sites become apparent, and, notably, a strong expression is detected in the cerebellum, at the level of the ventricular zone (**Figure 3C, C' and 3D, D'**). This cerebellar expression of *PRDM13* is quite transient as it faints already at 10 PCW (**Figure S1D**); at this stage *PRDM13* expression is maintained only in scattered cells of the subventricular zone (**Figure S1D'**). Expression of *PRDM13* was no longer detected in the cerebellum at 15 PCW (**Figure S1E**). We also detected expression of *PRDM13* in the inner layer of the developing retina (**Figure S1F, F'**), consistent with the described role of Prdm13 in the

development of amacrine cells in mice[15]. Altogether, our data show that *PRDM13* is expressed in human progenitors of the cerebellum and hindbrain at an early neurogenic phase. This result further argues for the implication of this gene in olivopontocerebellar hypoplasia, and points to a pathological mechanism involving neuronal fate misspecification. Consistent with the absence of obvious supratentorial anomalies in the individuals investigated in this study, no expression of *PRDM13* was detected in the forebrain and the midbrain, except for the hypothalamus (**Figure S1G, G'**).

Homozygous disruption of *prdm13* causes hindbrain developmental defects in zebrafish

To validate the implication of *PRDM13* in hindbrain development, we took advantage of the zebrafish. Its cerebellar development relies on homologous transcription factors[37] and its genome includes a unique ortholog of *PRDM13*. To further validate this model, we first analyzed the expression of *prdm13* during zebrafish brain development, using *in situ* hybridization. At 24 hours post-fertilization (hfp), we detected specific *prdm13* expression in rhombomeres 2-7, with a higher intensity in the ventricular zone and in HuC-positive differentiating neurons of rhombomere 7 (r7) (**Figure 4A, B-right panel**). This expression in the developing hindbrain, particularly enriched in r7, was also confirmed by the analysis of available scRNAseq data from 24hpf zebrafish embryos[24] (**Figure S2A, B**). Of note, dorsal progenitors from r7, expressing *ptf1a*, were recently shown to give rise to neurons of inferior olivary nuclei[35; 38]. Additionally, scRNAseq data revealed expression of zebrafish *prdm13* in pharyngeal arch cells at this stage (**Figure S2A, B**). At 48hpf, we could detect expression of *prdm13* in the cerebellar primordium located in the dorsal part of r1 (**Figure 4C**). The expression of *prdm13* in the cerebellar VZ is maintained at larval stages (**Figure 4E**) and is very similar to the expression of *ptf1a* (**Figure 4F**). *prdm13* transcripts are located in VZ neural progenitors expressing Sox2 and PCNA, and also at least transiently in differentiating cells exiting the VZ (**Figure 4C and 4G**). Analysis of scRNAseq data from 5dpf larval brain also highlighted the expression of *prdm13* in cell clusters annotated as "neural progenitors" and co-expressing *ptf1a* and *sox2* (**Figure 4H and Figure S2E**). The expression of *prdm13* in the cerebellar and hindbrain progenitors is maintained in the 2-month-old juvenile zebrafish brain (**Figure S2F, F'**), which is probably related to the maintained neurogenic activity of these regions beyond developmental stages in zebrafish. As in human embryos, we also observed additional expression sites for *prdm13* in

the developing eye and hypothalamus (**Figure 4H and S2E**). Altogether, our results demonstrate that *prdm13* expression pattern is highly conserved across vertebrates, and is consistent with an early role in hindbrain neurogenesis. It also validated the zebrafish as an appropriate model for functional studies of *PRDM13*-related neurodevelopmental anomalies.

We thus analyzed the phenotype of a zebrafish homozygous mutant for *prdm13*, available from the Zebrafish Mutation Project (*sa16464*)[28]. The mutant allele is a point mutation that creates a premature stop codon and leads to a truncation of the Zn fingers domains, comparable to the mutations identified in families 1, 2 and 4 (**Figure 5A**). Homozygous mutant embryos *prdm13*$^{sa1646/\,sa16464}$ (hereafter referred to as *prdm13*$^{-/-}$), obtained from a cross between heterozygous *prdm13*$^{sa16464/+}$ fish (*prdm13*$^{+/-}$), survive at least until 7 days post-fertilization (dpf), when larval lethality begins to be observed. Genotyping individual larvae demonstrated a distortion of the Mendelian distribution at 9dpf (Chi square test; p= 0.0002), due to the death of *prdm13*$^{-/-}$ mutant larvae (**Figure 5B**). Although *prdm13*$^{-/-}$ larvae have a roughly normal morphology, we noted that they present an abnormal body curvature and lower jaw morphology (**Figure 5C**). We then explored their brain phenotype, more particularly the cerebellum and posterior hindbrain. We fluorescently labelled cerebellar Purkinje cells with an antibody against Pvalb7, a validated marker in this species[39], and quantified these cells in 3D reconstructions (**Figure 5D**). We could show that the number of Purkinje cells was significantly reduced in *prdm13*$^{-/-}$ larvae, when compared to wild type *prdm13*$^{+/+}$ larvae (Figure 5E; n=5 (*prdm13*$^{-/-}$) and n=6 (*prdm13*$^{+/+}$); unpaired t-test p=0.011). In contrast, other cell populations such as CALB+ Eurydendroid cells (EC), functionally equivalent of deep cerebellar nuclei in mammals[37], were not affected (**Figure 5F**; n=3 (*prdm13*$^{-/-}$) and n=3 (*prdm13*$^{+/+}$); unpaired t-test p=0.7854). We also labelled ION neurons with an antibody against LHX1 in wild-type and mutant larvae (**Figure 5G**) and observed a total absence of these neurons in *prdm13*$^{-/-}$ larvae. Altogether these data indicate that homozygous disruption of *prdm13* in zebrafish leads to a late lethality in larvae, associated with a specific reduction in Purkinje cell numbers and an absence of ION neurons (**Figure 5H**). This phenotype shares striking similarities with the clinical features and neuropathological findings in affected child and fetuses with biallelic *PRDM13* mutations, further corroborating their causative role in the pathology.

DISCUSSION

Several PRDM transcription factors are already known to be involved in central nervous system development and diseases. *PRDM8* [MIM:616639] regulates cadherin-11 to ensure proper neural circuit formation[40] and is mutated in progressive myoclonic epilepsy-10 (EPM10 [MIM:616640]). *PRDM12* [MIM:616458] plays a role in sensory neuron perception and is mutated in hereditary sensory and autonomic neuropathy type VIII (HSAN8 [MIM:616488]). *PRDM15* [MIM:617692] is mutated in syndromes with neurodevelopmental defects and a progressive nephropathy[41]. All these genes play a critical role in the development as illustrated by the embryonic lethality or severe neurodevelopmental phenotypes of their respective KO mouse mutants. Likewise, the knockout of *Prdm13* in mouse, as well as a deletion of its three last Zn finger domains, cause neonatal death, although the cause of this death has not been investigated[16]. However, until recently, the only congenital disorders implicating *PRDM13* were macular dystrophies. Tandem duplications and single-nucleotide variants in the non-coding region of *PRDM13* have thus been reported to cause North Carolina Macular Dystrophies (NCMD [MIM: 136550] and other similar autosomal dominant retinal dystrophies[42; 43]. The implication of *PRDM13* in these pathologies is likely linked to the highly specific expression of *PRDM13* in the inner layer of the embryonic human retina that we report in this study and with the role of *Prdm13* in the specification of a subset of amacrine cells demonstrated in mice[15]. The precise molecular mechanism underlying these *PRDM13* associated retinopathies is unknown, but is suspected to result from an up-regulation of *PRDM13*[44]. This is compatible with our current findings, as we demonstrate that loss-of-function variants cause a very different clinical phenotype. Visual acuity could not be assessed in most of the affected individuals with truncating variants as they died early during infancy and eye histological examination was not performed. The eldest sister from family 3, carrying a missense mutation and presenting with milder clinical features had a normal fundus exam although she presented with high myopia. As we show that *PRDM13* is expressed in multiple brain regions that are not affected in NCMD, it is also possible that NCMD-causing variants lead to eye-specific deregulations of *PRDM13* expression.

During the preparation of this manuscript, Whittaker et al. [17] reported a recessive *PRDM13* mutation associated with hypogonadotropic hypogonadism syndrome. The three affected individuals are adult or adolescent presenting with scoliosis, sexual development defects and moderate intellectual deficit. Two of them also present with cerebellar hypoplasia. The authors of this study hypothesized that the mutation identified, a deletion encompassing a splice acceptor site, leads to a truncation of the four Zn finger domains and cause the phenotype. This would be at odds with the lethal phenotype observed in the mouse mutant with a deletion of the Zn finger domains [16]. Then, using a different *Prdm13* mouse mutant, deleted for the PR domain, Whittaker et al. demonstrated a role for *Prdm13* in cerebellar development but without finding any locomotor or Purkinje cell differentiation defect.

Here, we present multiple important differences with the study of Whittaker al. First, we report a significantly different syndrome based on eight individuals from four families of different origins. This syndrome associates perinatal lethality with brainstem dysfunctions (e.g. respiratory and swallowing defects) and/or severe brainstem malformation, together with persistent cerebellar hypoplasia. Consistently with the previously published mouse data, and further demonstrating the critical requirement of the Zn finger domains, three out of the four families carry mutations of *PRDM13* that truncate these domains and the last is predicted to disrupt one of them. Using a zebrafish mutant lacking the four Zn finger domains, we further validated the critical need for *PRDM13* during brainstem development in vertebrate. Secondly, we demonstrate that the *PRDM13* gene shows a strong and spatio-temporally restricted expression pattern during early human hindbrain development, explaining why Whittaker et al. can barely detect *PRDM13* expression in CS23-stage human fetus. Thirdly, correlations of human and zebrafish data from samples with truncation of *PRDM13* demonstrate the requirement of this gene for Purkinje cell differentiation. It can be speculated that the clinical differences observed between the two studies come from the specific mutation identified by Whittaker et al. that might alter splicing but still allows for the production of some functional transcripts. However, the exact consequence of this splicing mutation on *PRDM13* transcripts remains to be assessed. The partial overlap with our clinical findings, with the presence of cerebellar hypoplasia and global developmental delay, suggests that this variant might be a hypomorphic allele. We note that affected individuals carrying missense variants (i.e. family 3) also present with milder clinical phenotypes in our study. The

presence of the three other functional Zn fingers might be sufficient to maintain the functionality of the protein to a certain degree. However, unlike the affected individuals from the aforementioned study, they do not present scoliosis although postural kyphosis has been detected in the child F.3-1. Clinical follow-up of the very young affected children from family 3 will help to determine if impaired sexual development, hypogonadism, and progressive scoliosis are consistent clinical features associated with partial loss-function of *PRDM13*.

Among the clinical features in affected individuals with *PRDM13* mutation, some variable features are observed (e.g. cardiac malformation in family 1, see clinical table) and could involve recessive variants other than *PRDM13* mutations[45]. Differently, epilepsy or seizures have been observed in several members of the families 3 and 4. Epilepsy is also present in typical PCH cases that are generally associated with mutations in ubiquitously expressed genes (e.g. genes involved in tRNA processing)[7]. Here, *PRDM13* has a very restricted expression pattern and a direct impact of its mutations in tissues where it is not detected, such as the cerebral cortex, is unlikely. However, a protective role of the cerebellum, through the inhibition of aberrant discharges that lead to cortical seizures, has been suggested[46] and so an indirect contribution of the cerebellar malformation to this epilepsy phenotype could be speculated.

Using a meta-analysis of scRNA-seq data as well as a detailed expression study performed during zebrafish hindbrain development, we could define original molecular hypotheses to understand *PRDM13* role in brainstem, and cerebellar Purkinje cells development. Several lines of evidence support the idea that these defects result from cell-autonomous neuronal misspecification. Indeed, we show that *prdm13* is expressed, together with *ptf1a*, in progenitors of the cerebellar VZ and dorsal ventricular zone of rhombomere 7. These Ptf1a+ progenitor domains have been demonstrated to generate respectively Purkinje cells and ION neurons[32; 35; 36; 38; 47]. Besides, Ptf1a is required cell autonomously for proper identity specification in neuronal precursors in the retina, spinal cord, cerebellum and posterior hindbrain[32; 35; 36; 48; 49]. In the dorsal spinal cord, Ptf1a loss-of-function thus induces a loss of GABAergic neurons[49]. Part of Ptf1a function is mediated by the transcriptional activation of its direct target *Prdm13*, which in turn inhibits the expression of determinants of the alternative glutamatergic fate, such as Tlx1/3 ad Lmx1b[13; 16]. Accordingly, *Prdm13* loss-of-function phenotype in the spinal cord resembles the one

observed in *Ptf1a*-null mutants, albeit less severe, as it affects mostly early-born neurons[16]. In the zebrafish cerebellum, we show that the phenotype of *prdm13* loss-of-function mutant is comparable but milder to *ptf1a* mutant[38]. This is also the case in humans, as *PTF1A* loss-of-function leads to a much more severe cerebellar hypoplasia than the one we observed in presence of *PRDM13* mutations. Altogether these data are in line with the hypothesis that, in the developing cerebellum and ION, PRDM13 plays a cell autonomous role in neural progenitors fate specification, downstream of PTF1A.

Our findings also suggest that *PRDM13* loss-of-function could impact cerebellar development both directly and indirectly, by impairing the development of key afferent pathways of the cerebellum. Indeed, inferior olivary nuclei are the origins of major cerebellar inputs that influence Purkinje cells maturation[50]. Interestingly, lesions, abnormalities of size or in neurotransmitter within key nuclei of the olivo-cerebellar complex have been observed in sudden infant death syndrome (SIDS) [51-53]. Therefore, a defect in these structures could be directly involved in the respiratory symptoms observed in SIDS. Alternatively, other brainstem nuclei can also be affected. Indeed, in mice, *Ptf1a* loss-of-function also affects the neuronal composition of brainstem nuclei involved in somatosensory and viscerosensory control circuits[36]. Among these nuclei, *Ptf1a* is required for the proper development of the nucleus of the solitary tract that is a major sensory nucleus of the dorsal medulla oblongata receiving cardiorespiratory afferent inputs among others. Considering the likely involvement of *Prdm13* in these developmental processes, its disruption may also impair the development of these nervous centers, and thereby explain the various brainstem dysfunctions shared by affected individuals with biallelic *PRDM13* mutations.

Like the cerebral cortex, the cerebellum is a brain region that has undergone during evolution a strong expansion and complexification in the human lineage. Recent studies in human early embryos showed that cerebellar germinal zones present some human-specific features[54]. Notably, as in the cerebral cortex, an expanded subventricular proliferation zone is detected above the VZ in the developing human cerebellum. However, little is known about the diversity of human VZ/SVZ progenitors and the molecular mechanisms involved in this expansion. Our study highlights how defects in the

transcriptional regulation of these early human-tailored neurogenic steps can contribute to severe neurodevelopmental disorders of the brainstem and cerebellum. Exploring further this early transcriptional network in a human context[55] will be of high interest to understand these pathologies.

DESCRIPTION OF SUPPLEMENTAL DATA

Supplemental data include a supplemental document S1, table S1 and supplemental figures S1 and S2.

COMPETING INTERESTS STATEMENT

The authors declare no competing interests.

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DATA AND CODE AVAILABILITY

This study did not generate datasets or code.

WEB RESOURCES

CADD, https://cadd.gs.washington.edu/snv

gnomAD, https://gnomad.broadinstitute.org

Online Mendelian Inheritance in Man, https://www.omim.org

ESP database, https://evs.gs.washington.edu/EVS/

dbSNP database, https://www.ncbi.nlm.nih.gov/snp/?cmd=search

Human single-cell RNA-seq dataset, Descartes, https://descartes.brotmanbaty.org

Gene Expression Omnibus database, https://www.ncbi.nlm.nih.gov/geo/

Ensembl genome assembly GRCh37, http://grch37.ensembl.org/Homo_sapiens/Info/Index

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FIGURE LEGENDS

Figure 1: Biallelic Mutations in *PRDM13* in affected children and fetuses with posterior fossa anomalies. (A) Families with predicted effect of *PRDM13* variants and pedigrees. All pathogenic variants are homozygous in affected individuals and segregated as recessive trait. (B) T2-weighted fetal magnetic resonance images of control at 26 WG, and case F.2-1, F.1-1, F.1-6 at 26 WG, 30 WG and 33 WG, respectively. Sagittal views are shown on top, the bottom parts show coronal views for control, F.2-1, F.1-6 and axial views for F.1-1. The imaging shows reduced cerebellar vermis volume (yellow arrowhead) with abnormal or incomplete foliation, and mild tapering of medulla oblongata for F.1-6. (C) Sagittal T1-weighted (top, for control, F.4-3), Sagittal T2-weighted (top, for F.2-2) and coronal T2-weighted (bottom) brain magnetic resonance images of control, individuals F.4-3 and F.2-2. For both children, a small and dysmorphic vermis is visible on the sagittal slices and small hemispheres are detected on coronal views.

(D) Identified *PRDM13* mutations. Bottom-right, modelling of the third zinc finger domain based on ZFP568 structure (PDB 5V3J). The mutated histidine residue (in red) is part of the Cys2-His2 binding motif. Blue, zinc ion.

Figure 2: Neuropathological examination of the fetuses F.1-4, F.2-1 and child F.2-2

Posterior view (**B**) and basal view (**A**, **C-F**) of the brain showing cerebellar hypoplasia in affected cases compared to stage-matched controls. Scale bar: 10 mm. Cerebella are outlined with white dashed lines. (**B**) An incomplete covering of the 4th ventricle by the vermis is visible for the fetus F.1-4. (**G-L**) Cross sections of the medulla stained with hematoxylin and eosin (HE) showing a fragmentation or extreme hypoplasia (arrows) of the olivary nuclei in affected cases. Pyramidal tracts are clearly visible (*) and inferior olivary nuclei are noted (arrowhead) in controls. Scale bar: 500 µm. Horizontal (**M**) and sagittal (**N-R**) HE-stained sections showing a dysplasia and fragmentation of the dentate nucleus (arrows) in affected cases. Arrowheads in controls point to the dentate nucleus. Scale bar: 500 µm. (**S-V**) Sagittal HE-stained sections at the level of the vermis showing delayed lobulation with absence of tertiary foliation in the cerebellum of F.2-2 case, and hypoplasia severely affecting the anterior vermis (**T, V**). Primary fissures are indicated with arrowheads. Scale bar: 1mm. Purkinje cell cluster heterotopia are visible in the white matter (**T**, top-left magnification). **W-AB**. Immunostaining of Purkinje cells using calbindin and showing a decreased number and delayed maturation of Purkinje cells in the cerebellar cortex of affected cases. Scale bar: 50µm

Figure 3: *PRDM13* expression in neural progenitors in the developing cerebellum and brainstem

A. Single-nucleus RNA-seq (sn-RNA-seq) data from human fetal cerebellum (9-21 PCW). snRNA-seq datasets were retrieved from the Human Gene Expression During Development atlas[25] (Descartes, https://descartes.brotmanbaty.org) and analyzed using the Seurat package. Cells are visualized on Uniform Manifold Approximation and Projection (UMAP) plots. In the first panel, cells are color-coded according to cell annotations from the Descartes atlas (PC: Purkinje cells; UBC: unipolar brush cells; GC: granule cells; IN: interneurons; Astro: Astrocytes; OPC: oligodendrocytes precursors; µglia: microglia; SP: SLC24A4_PEX5L_positive cells; Endo: vascular endothelial cells). In the three other panels, cells are colored in graded intensities, reflecting the expression levels of *PRDM13*, *PTF1A* and *SOX2*. **B-D**. Detection of *PRDM13* transcripts (red) by RNAscope in situ hybridization on coronal (**B**, **C**) or sagittal (**D**) sections through human embryos at Carnegie Stage 13 (**B**) and 21 (**C**, **D**). **B'**, **B"**, **C'**, **C"**, **D'**, **D"** are higher magnifications of the regions outlined by dotted squares in B, C and D

respectively. At CS13, specific expression of *PRDM13* is detected in the dorsal VZ of the caudal part of the hindbrain (Hb, **B'**) and in the dorsal VZ of the caudal neural tube (NT, **B''**). At CS21, *PRDM13* starts to be expressed in the primordium of the cerebellum (Cb, **C**, **C'**), while being maintained in the medulla oblongata (MO, **C**, **D**, **D''**) and spinal cord (SC, **C**, **C''**). In the cerebellum (**D'**), *PRDM13* is more specifically detected in the ventricular zone (VZ, black arrowheads), while it is absent from the rhombic lip (RL, white arrows). Scale bars: 100µm (B) and 500 µm (C, D).

Figure 4: *prdm13* expression during zebrafish hindbrain development.

A. Lateral view of a 3D reconstruction of the zebrafish hindbrain at 24hpf showing the expression of *prdm13* (ISH, magenta) with a whole-mount immunostaining for the neuronal marker HuC (green). Cell nuclei are counterstained with DAPI (blue). A dotted line contours the hindbrain region. Cb: cerebellum; rh1-7: rhombomere 1-7; ov: otic vesicle. Scale bar: 50µm. **B**. Transverse section through the 3D reconstruction shown in A, at the level of rhombomere 3 (left panel) and rhombomere 7 (right panel). Scale bar: 50 µm **C**. Optical z-plane showing *prdm13* expression (magenta) together with an immunostaining for Sox2 (blue) and HuC (green) in the cerebellum at 48hpf. Cell nuclei are counterstained with DAPI (grey). At this stage, *prdm13* starts to be expressed in the cerebellar primordium, corresponding to the dorsal part of the first rhombomere, adjacent to the Midbrain-Hindbrain Boundary (MHB). OT: Optic Tectum. Scale bar: 20µm. **D**. Schematic representation of the larval zebrafish brain at 6 days post-fertilization (dpf), with a magnification on the larval cerebellum. Like in mammals, the zebrafish cerebellum develops from two disctinct neurogenic progenitor pools: the rhombic lip (RL, green) and the ventricular zone (VZ, purple). The VZ gives rise to all the GABAergic neuronal populations, including Purkinje neurons, which migrate and align to form the Purkinje cell layer (PCL). **E-F.** Whole-mount ISH on zebrafish larval brains at 6dpf showing the expression of *prdm13* (**E**) and *ptf1a* (**F**) in blue. Note the expression of *prdm13* and *ptf1a* in the cerebellar VZ (black arrowheads) and dorsal hindbrain (black arrows). Cb: cerebellum; OT: Optic Tectum; Tel: Telencephalon; Hyp: Hypothalamus; MO: Medulla Oblongata. Scale bar: 100µm. **G.** Lateral view of a 3D reconstruction of the zebrafish cerebellum at 6 dpf showing the expression of *prdm13* (ISH, magenta) with a whole-mount immunostaining for the proliferation marker PCNA

(green). Cell nuclei are counterstained with DAPI (blue). Dotted lines outline the cerebellar primordium. *prdm13* transcripts are detected in proliferating progenitors of the VZ (white arrowheads). Scale bar: 10µm. **H.** t-distributed stochastic neighbor embedding (t-SNE) projection plots of distinct single-cell populations obtained from the zebrafish brain at 5dpf. The zebrafish single-cell RNA-seq (sc-RNA-seq) dataset was retrieved from the Gene Expression Omnibus (GEO) public database under accession GSE158142 [24]. In the first panel, cells are color-coded according to cluster annotations from the original publication[24]. In the three other panels, cells are colored in graded intensities, reflecting the expression levels of *prdm13, ptf1a* and *sox2*. Clusters in which *prdm13* expression is enriched are circled with dotted lines. *prdm13* expression is enriched in clusters 13, 25, 30 and 38, corresponding to neural progenitors (NPC) and that are also enriched for *ptf1a* and *sox2*. Expression of *prdm13* is also detected in clusters 1, 34 and 35, which are annotated as hypothalamic clusters (Hyp); 21, which corresponds to amacrine cells (AC) and 45, which correspond to Müller glia (MG).

Figure 5: Homozygous *prdm13* disruption causes reduction in Purkinje neurons numbers and loss of inferior olive nucleus neurons in zebrafish.

A. Zebrafish mutant allele *sa16484*. The mutant allele is a nonsense single-nucleotide mutation, leading to a truncation of the protein at the level of the first Zn Finger domain. **B.** Proportion of embryos of each genotype inside the progeny of a cross between two heterozygous fish from the *prdm13* ^{sa16484} line at different stages. The number of embryos genotyped per stage is indicated underneath each bar. At 9dpf the proportions differ significantly from the expected ratios (Chi square test; p= 0.0002). **C.** Photographs of wild-type and homozygous mutant larvae from the *prdm13* ^{sa16484} line at 7dpf. **C'** and **C''** are higher magnification of the head regions of a wild-type (**C'**) and homozygous mutant (**C''**) larva. Homozygous *prdm13* mutant larvae display an abnormal body curvature and lower jaw morphology (arrow in C''). Scale bar: 500µm **D**. 3D-reconstructions of the hindbrain in dorsal view, from a wild-type (top panels) and a homozygous mutant (bottom panels) larva at 6dpf. Larval brains were immunostained for Parvalbumin (PARV, green), a marker of Purkinje cells (PC), and Calbindin (CALB, magenta), marker of some eurydendroid cells (EC). The panels on the right show a higher magnification of the cerebellum; the last ones show the distribution of PC (green) and EC (magenta) cells, as determined by 3D-image

segmentation of the cerebellar region. Scale bar: 50µm **E-F.** Total number of PARV+ PC (**E**) and CALB+ EC (**F**) in wild-type and mutant *prdm13* larvae. Data are presented as mean ± 95% confidence interval. The number of PC is significantly reduced in mutant larvae (n=5 (*prdm13*$^{+/+}$) and n=6 (*prdm13*$^{-/-}$); unpaired t-test p=0.011), while no significant changes in EC numbers are observed (n=3 (*prdm13*$^{-/-}$) and n=3 (*prdm13*$^{+/+}$); unpaired t-test p=0.7854). **G.** Representative images of 3D-reconstructions of the hindbrain from a wild-type (top panels) and a homozygous mutant (bottom panels) larva at 7dpf. Larval brains were immunostained for LHX1 (green), which labels inferior olivary nucleus projection neurons (white arrow). The left and middle panels are lateral views and the right panels are ventral views centered on the inferior olivary nuclei (ION). Note the absence of ION neurons in mutant larvae (4 larvae were immunostained for each genotype). Scale bar: 20µm **H**. Schematic representation of the phenotype of *prdm13* mutant larvae, which present a reduction in the number of Purkinje cells (red) and an absence of ION projection neurons (green), which normally send climbing fibers onto PC.

Table 1 : Clinical table

	FAMILY 1			FAMILY 2		FAMILY 3		FAMILY 4
Patient/case identifier	F.1-1	F.1-4 (fetus)	F.1-6	F.2-1 (fetus)	F.2-2	F.3-1	F.3-2	F.4-3
Gender (M/F)	F	F	M	F	M	F	F	M
Age at last examination, at death or fetal age.	7 mo, died at 25 mo	24 GW	Birth, died at 4 mo	31 GW	2 days, died at 22 days	4 y 6 mo	11 mo	12 mo, died at 16 mo
GENETIC DATA								
Ethnic origin	Tunisia			Algeria		Pakistan		Arab
Consanguinity (yes/no)	yes			yes		no (parents unsure)		yes
gDNA (GRCh38/hg38)	Chr6(GRCh38):g.99613474del			Chr6(GRCh38):g.99613479del		Chr6(GRCh38):g.99614491A>T		Chr6(GRCh38):g.99613435del
cDNA (NM_021620.3)	c.839del			c.844del		c.1856A>T		c.800del
Protein	p.(Ala280Glyfs*21)			p.(Val282Serfs*19)		p.(His619Leu)		p.(Gly267Aspfs*34)
CADD-PHRED score	N/A			N/A		32		NA
PRE-NATAL FINDINGS								
Prenatal growth retardation	-	-	-	-	-	+	+	-
Cerebellar vermis hypoplasia	+	+	+	+	+	Not reported	Not reported	Not reported
GROWTH								
Delivery (wks)	39	24	37	N/A	34	39	37	40
Neonatal symptoms	Profound hypotonia	N/A	Profound hypotonia	N/A	Respiratory distress, axial hypotonia	Temperature, HR and BP fluctuations; Seizures	Temperature, HR and BP fluctuations	Respiratory distress, recurrent apnea
Likely autonomic symptoms	Swallowing defect, desaturation, respiratory distress, bradypnea	N/A	Swallowing defect, respiratory distress, no cough reflex, bradycardia	N/A	Swallowing defect, respiratory distress, recurrent apnea, bradycardia	Temperature, HR and BP fluctuations, respiratory distress,	Temperature, HR and BP fluctuations,	Respiratory distress, recurrent apnea
Weight at last examination (kg)	7 (-0.8 SD)	N/A	N/K	N/A	N/K	12.2 at 4y6mo (< -2 SD)	5.0 at 7.7mo (-3 SD)	5.2 (-4.5 SD)

HC at birth (cm)	N/A	N/A	N/K	N/A	33.5 (+1.3SD)	N/K	N/K	34 (-1SD)
HC at last examination (cm)	42 (-0.9SD)	N/A	N/K	N/A	N/K	46.4 at 4y6m (-2.7SD)	38.3 at 33w5d (-4.7SD)	38 (-7SD)
Presence of dysphagia	+, Nasogastric tube feeding	N/A	+, Nasogastric tube feeding	N/A	+, Nasogastric tube feeding	-	-	+, Nasogastric tube feeding
DEVELOPMENTAL HISTORY								
Motor (normal/delayed/absent)	Absent	N/A	Absent	N/A	Absent	Delayed	Delayed	Absent
Global developmental delay (severity)	Severe / Profound	N/A	N/A	N/A	N/A	Severe	Mild	Severe
NEUROLOGICAL FEATURES								
Axial hypotonia	+	N/A	+	N/A	+	+	+	+
Distal hypertonia	+	N/A	+	N/A	+	+	+	+
Spastic tetraplegia	+	N/A	N/K	N/A	N/K	-	-	+
Deep tendon reflexes	Normal	N/A	N/K	N/A	N/K	Reduced	Normal	Reduced
EPILEPSY								
Seizure	-	N/A	-	N/A	-	Neonatal period, focal seizures (on levetiracetam)	One seizure, on levetiracetam since.	Neonatal
BRAIN MRI								
Normal supratentorial brain	+	+	+	+	+	+	+	+
Cerebellar hemisphere hypoplasia	+	-	-	+	+	+	+	+
Cerebellar vemis hypoplasia	+	+	+	+	+	+	-	+
Brainstem hypoplasia	+	+	+	+	-	+	-	+
ASSOCIATED CLINICAL FEATURES								
Ophthamological findings	Abnormal ocular movement, suspicion of papillary edema or hypoplasia	N/A	Bilateral retinal hemorrhages	N/A	No eye tracking	Nystagmus with horizontal gaze, short sighted	-	-
Facial dysmorphism	Microretrognathia	-	-	-	Hypertelorism, epicanthic fold	-	-	Hypertrichosis, low anterior hairline, upslanting palpebral fissures, epicanthic folds
Oral cavity findings	Posterior cleft palate	Posterior cleft palate	-	-	-	-	-	-
Cardiac abnormalities	-	Trabecular ventricular septal defect	Ostium secundum atrial septal defect, patent ductus arteriosus	-	N/A	Sinus rhythm with occasional marked sinus arrhythmia	-	-
Gastrointestinal abnormalities	-	-	Gastroesophageal reflux	-	-	-	-	Recurrent vomiting, gastroesophageal reflux
Respiratory system	Oxygen therapy required (Nasal canula)	-	Oxygen therapy, base of the tongue ptosis, no cough reflex	-	Apnea, Oxygen therapy required in the context of prematurity	Respiratory distress	-	Poor respiratory effort, ventilation dependence
Other		Mild extremity malpositions	Mild feet malpositions			Postural kyphosis	Episodic dystonia	

Abbreviations: BP= Blood Pressure; EEG= Electroencephalography; HC= Head Circumference ; HR= Heart Rate; mo= month; N/A= Not applicable; N/K=not known; y= year; SD=standard deviation.