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Mutations in the trafficking protein YIF1B cause a new post-natal neurodevelopmental syndrome associated with Golgi and primary cilium alterations

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Running title: *YIF1B*, a candidate gene for rare disease

Abstract

Human post-natal neurodevelopmental delay is often associated with cerebral alterations that could lead by themselves or associated with peripheral deficits, to premature death. Here, we report the clinical features of 10 patients from six independent families with mutations in the autosomal *YIF1B* gene encoding a ubiquitous protein involved in anterograde traffic from the endoplasmic reticulum (ER) to the cell membrane, and in Golgi apparatus (GA) morphology. The patients displayed global developmental delay, motor delay, visual deficits with brain MRI evidence of ventricle enlargement, myelination alterations and cerebellar atrophy. A closely similar profile was observed in the *Yif1b*-KO mouse model developed to identify the cellular alterations involved in the clinical defects. In the central nervous system, mice lacking *Yif1b* displayed neuronal reduction, altered myelination of the motor cortex, cerebellar atrophy, enlargement of the ventricles, and subcellular alterations of ER and GA compartments. Remarkably, although YIF1B was not detected in primary cilia, biallelic *YIF1B* mutations caused primary cilia abnormalities in skin fibroblasts from both patients and *Yif1b*-KO mice, and in ciliary architectural components in the *Yif1b*-KO brain. Consequently, our findings identify *YIF1B* as an essential gene in early post-natal development in human, and point out a new genetic target that should be tested in patients developing a neurodevelopmental delay during the first year of life. Thus, our work is the first description of a functional deficit linking Golgipathies and Ciliopathies, diseases so far associated exclusively to mutations in genes coding for proteins expressed within the primary cilium or related ultrastructures.

We therefore propose that these pathologies should be considered as belonging to a larger class of neurodevelopmental diseases depending on proteins involved in the trafficking of proteins towards specific cell membrane compartments.

Keywords: neurodevelopmental delay, ER, Golgi, primary cilium

Introduction

The main causes of infant mortality during the first year of life are fetal encephalopathy, seizures or brain alterations that develop after birth. Genetic studies have demonstrated that single gene defects are often responsible for these alterations. Among mutations that have been shown to impair neurodevelopmental processes, those affecting Golgi apparatus (GA) trafficking genes do not cause anomalies at birth, but alterations appear during early post-natal development (Passemard et al., 2017). Trafficking proteins are involved in multiple secretory pathways requiring a complex ballet of vesicular carriers organizing sorting, packing, routing and recycling through interactions with Rab GTPases, Rab effectors or Rab regulators. Mutations in genes coding for trafficking proteins have been shown to cause severe neurodevelopmental delay, white matter defects and intellectual disability in human (Mir et al., 2009; Seifert et al., 2011; Handley et al., 2013; Liegel et al., 2013; Feinstein et al., 2014; Shamseldin et al., 2016). We previously characterized, in rodent, YIF1B, a trafficking protein ortholog of YIF1P that was initially identified in *Saccharomyces cerevisiae* as interacting with YIP1P (a GA membrane protein able to bind Ras-like GTPases) and essential for secretion (Matern et al., 2000). YIF1B is localized in the intermediate compartment between the endoplasmic reticulum (ER) and the GA. It participates in the anterograde traffic from the ER to the cell membrane of the membrane-bound Vesicular Stomatitis Virus G Protein in Hela cells and in neurons. Its deletion in mouse neurons leads to GA disorganization (Alterio et al., 2015) and alteration of the dendritic targeting of a specific serotonergic receptor (Carrel et al., 2008). A recent study identified mutations in the *YIF1B* gene in patients displaying progressive encephalopathy with various degrees of mixed movement disorder, microcephaly and epilepsy (AlMuhaizea et al., 2020). In the current study, we also identified mutations of the *YIF1B* gene in six families comprising 10 affected patients with neurological deficits that could not be classified by clinicians as a specific pathology, but whose clinical manifestations were close to those observed in Golgipathies. We took advantage of the *Yif1b*-KO mouse model to investigate the cellular, subcellular and physiological alterations that could underlie clinical features in patients carrying *YIF1B* mutations. Our investigations demonstrate that *Yif1b* depletion in mice induces central nervous system alterations similar to those observed in patients. Moreover, investigating cellular and subcellular structural integrity in the *Yif1b*-KO mouse model allowed us to infer that some of the clinical features observed in patients could be attributed to neuronal degeneration associated with GA, ER and primary cilium alterations.

Materials and methods

Human subjects

Clinical and molecular data from 10 patients with *YIF1B* variants from different clinical centers in France, Saudi Arabia, Iran, Canada and Italy were collected through clinical or genetics network such as GeneMatcher (Sobreira et al., 2015). None of these patients were previously reported. Each referring clinician filled out a table with detailed birth parameters, developmental, neurological, behavioural and seizure medical history including electroencephalogram (EEG), electromyogram (EMG) and imaging data when available.

DNAs of all members of families one to six was studied by whole-exome analysis. Informed consent was obtained from each patient included in this study and this study protocol conformed to the ethical guidelines of the 1975 Declaration of Helsinki as reflected in a priori approval by the institution's human research committee (Comité de Protection des Personnes, Ile de France II, Approval ≠ 2015-03-03/DC 2014–2272, Paris, France).

Cell culture

Human and murine primary fibroblasts were successfully derived from skin biopsies and cell lines were isolated by selective trypsinization and proliferated at 37 °C, 5% CO_2 in Opti-MEM Glutamax I medium (Invitrogen, France) supplemented with 10% fetal bovine serum (FBS, Invitrogen), 1% ultroser G substitute serum (Pall, France), and 1% streptomycin/penicillin (Invitrogen). Fibroblasts between passage seven and nine were plated at 10^5 cells/well in 24-well plates. Patient lymphoblastic cell lines were grown in RPMI medium, 10% FBS, sodium pyruvate and penicillin/streptomycin.

Animals

Homozygous *Yif1b*-KO mice and control littermates were obtained from previously established colonies (Alterio et al., 2015). Experimental mice were housed with companions of the same gender and breeding mice in "trio" (two females with one male) in standard cages type L2 with Celle Bel MAXI-25 cellulose bedding and maintained under a 12 hr light/dark cycle with constant temperature (23.0 ± 1.0 °C) and humidity (60%), with food and water *ad libitum*. Experiments were performed during the light phase on 3-5 and 12 month-old male mice, and compared to matched controls. All efforts were made to reduce the number of animals used in these experiments. Experiments were performed in agreement with the institutional guidelines for use of animals and their care, in compliance with national and international laws and policies

(Council directives no. 87–848, October 19, 1987, Ministère de l'Agriculture et de la Forêt, Service Vétérinaire de la Santé et de la Protection Animale, permissions no.75-805 to JM). All protocols were approved by the ethical committee #C2EEA34 and were licensed by the Directorate General for Research and Innovation (French MESR) under protocol authorization #00717.06. Experimental design was done according to ARRIVE guidelines.

Antibodies

The following primary antibodies were used: mouse monoclonal anti-Arl13B antibody (Antibodies Inc, 1/500); anti-CTR433 antibody (1/1600) (Jasmin et al., 1989), anti-γ-tubulin antibody (Sigma, 1/20000); rabbit polyclonal anti-acetylated tubulin antibody (Sigma, 1/2000), anti-Calbindin D28K antibody (Sigma, 1/3000), anti-Iba1 antibody (Wako, 1/800), anti-pericentrin antibody (Abcam, 1/1000), anti-CEP83 (Sigma, 1/500), anti-CEP164 antibody (Sigma, 1/1000), anti-Myelin Basic Protein [(Colman et al., 1982), 1/200]; goat polyclonal anti-rootletin antibody (Santa-Cruz, 1/500). Fluorophore-labeled secondary donkey-antibodies used were Cy3-conjugated anti-rabbit antibody, Alexa 488-conjugated anti-goat antibody, Cy3-conjugated anti-mouse antibody (Jackson ImmunoResearch, 1/500).

Western blot analyses

Cultured fibroblasts were harvested and homogenized by sonication in sample buffer (Laemmli, 1970). Proteins (2 µg), quantified using the Pierce BCA Reducing Agent kit, were analyzed by SDS-PAGE and blotted to polyvinylidene difluoride (PVDF) membrane. Incubation with affinity-purified rabbit anti-YIF1B antiserum (1/1000) (Carrel et al., 2008) and mouse anti-actin monoclonal antibody (Sigma, 1/5000) was performed. Immunoreactivity was detected using a chemo-luminescence detection kit and a ChemiDoc Touch Imaging System (Bio-Rad).

Histochemical and immunostaining methods and quantitative analysis of labeling

Mice were deeply anaesthetized using pentobarbital (60 mg/kg,) and perfused by trans-cardiac puncture with 0.9% NaCl (warmed at 37 °C, 10 ml) followed by paraformaldehyde (4%, ice-cooled, 150 ml). Brains were removed, post-fixed at 4°C for 1 hr and rinsed in phosphate buffer 0.1 M. Free floating sections (40 µm) were obtained using a Leica vibratome VT1000E and frozen sections using a Leica cryostat CM3000.

Luxol fast blue/cresyl violet staining

Vibratome sections and frozen sections were incubated at 53 °C overnight in luxol fast blue 0.1%. Sections were rinsed in distilled water before differentiation in lithium carbonate solution

0.05% for 30 seconds. Slides were rinsed in distilled water and counterstained at 53 °C for 1 hr in cresyl violet 0.1%. Sections were dehydrated, cleared and mounted in a resinous medium.

Immunofluorescence on sections

Floating sections were blocked for 1 hr at room temperature with TBS, Tween-20 0.1% (T20) containing 5% normal donkey (v/v), 0.4% BSA (w/v) and 0.1% gelatin (w/v) before incubation overnight at 4 °C with primary antibodies diluted in TBS–T20 containing 0.4% BSA and 0.1% gelatin. After primary antibody incubations, the sections were washed three times (10 min each) in TBS–T20 containing 0.1% gelatin and then incubated for 1 hr at room temperature with complementary fluorophore-labeled secondary donkey antibodies. After PBS washes, sections were mounted in Fluoromount-G solution (CliniSciences). Immunofluorescence images were generated using a Leica TCS SP5 microscope (PL-APO 63X, NA1.4 oil, Imaging medium Leica).

Immunofluorescence on cells

Cultured fibroblasts were grown to 80% confluency and serum-starved for 48 hr. Cells were fixed with methanol 100% and processed for immunofluorescent staining as previously described (Alterio et al., 2015). Colored and fluorescence images were generated using a Zeiss photomicroscope (25X magnification, bright field illumination) and a Zeiss LSM700 confocal microscope respectively (40X magnification, Carl Zeiss) or a Leica TCS SP5, PL APO microscope (63X and 100X).

Two and three dimensional analysis of labeling

The ImageJ (2D analysis) and Volocity software (3D analysis) were used for image processing and quantification. Quantification of the fluorescent MBT immunostaining intensity was performed using ImageJ software. The ciliary length was quantified by maximal axis using Arl13B and acetylated tubulin labeling close to basal bodies labeled by g-tubulin and pericentrin respectively using the Volocity software.

Electron microscopy

For electron microscopy section, perfusion was performed using 2.5% glutaraldehyde and 2% paraformaldehyde. Brain and testis were removed and post-fixed in the same fixative at 4 °C for 4 hr and rinsed in phosphate buffer 0.1 M. Vibratome free floating coronal or sagittal sections taken throughout the cerebellum (150 µm-thick), the hippocampus and the testis (200 µm-thick) were maintained at 4°C in phosphate buffer saline 50 mM, pH 7.4. Tiny pieces of 2mm x 1mm from both cerebellar lobules 9 and 10, from the hippocampal CA1 region and from the seminiferous tubules were dissected and post-fixed for 30 min in 1% osmium tetroxide, rinsed

and incubated 1 hr in a solution of 1% uranyl acetate in water. After dehydration in a graded series of ethanol solutions, pieces of seminiferous tubules were flat-embedded in epoxy resin (Epon) and allowed to polymerize for 48 h at 60 °C. The ultrathin sections, cut with a Reichert ultramicrotome, were mounted on mesh grids, stained with lead citrate and analyzed on a JEOL 100 electron microscope equipped with a GATAN CCD camera.

Behavior

Spontaneous locomotion

Locomotor activity was measured using an actimeter, a computer-based photo-beam apparatus (Actisystem II, Panlab). Actimeter boxes (area: 30 x 15cm; height: 18cm; with grid floor) detected mouse movements by means of infrared light beams. Mice were placed in the boxes for 60 min and horizontal activity was monitored (in cm).

Motor coordination

In the Locotronic apparatus (Intellibio Innovation, France), the mice crossed a 124 x 28 x 20 cm flat ladder with bars (7 mm in diameter) set 1 cm apart. Infrared sensors situated above and below the bars monitored paw errors. Mice were habituated to learn to cross the ladder and then placed in the apparatus in three conditions, a first session with no traps, a second with traps (five withdrawing bars separated from 5-7 bars) and a third with no traps. During these tests, errors of hind legs were counted. For basal and learned motor coordination, each mouse was placed onto the horizontal rod of an accelerating Rotarod apparatus (model 7650, Ugo-Basile, Comerio, Italy), rotating at a speed increasing from 4 to 40 rpm over 5 min. The time required for the mouse to fall from the rod was recorded. Mice were acclimatized to the apparatus by placing them for 2 min on the rod at 5 rpm, 3 min before the first test. Motor coordination performance was evaluated the first day just after acclimatization session of each mouse (with a 20-min resting period between two successive training). Mice were trained three times a day on the two consecutive days. Motor coordination performance of each mouse was determined by the longer time to fall on the three trials.

Visual performances (optomotor response)

Mice were placed on a platform in the form of a grid (11.5 cm diameter, 19.0 cm above the bottom of the drum) surrounded by a motorized drum (29.0 cm diameter) that could be revolved clockwise or anti-clockwise at two revolutions per min, the optimal velocity for evoking an optokinetic response in the mouse. After 10 min of adaptation in the dark, vertical black and white stripes of a defined spatial frequency were presented to the animal. These stripes were rotated alternately clockwise and anti-clockwise, for 2 min in each direction with an interval of

30 s between the two rotations. Various spatial frequencies subtending 0.06, 0.13, 0.25 and 0.5 cpd (cycles/degree) were tested individually on different days in a random sequence. Animals were videotaped with a digital video camera (Sony, DCR-TRV24E) for subsequent scoring of head tracking movements. Tests were performed in scotopic conditions, using the night shot position of the camera. Head movements were scored only if the angular speed of the head corresponded to that of the drum rotation.

Retinal electrophysiology

Electroretinograms (ERG) were performed in male adult mice, dark-adapted for 12 hr prior to recording. They were anaesthetized by intra-peritoneal injection of ketamine/xylazine (10 %/7.5%, diluted in 0.9 % NaCl, 10 µl/g body weight). Corneas were anaesthetized with oxy-buprocaïne chlorhydrate (0.4%, Thea Lab, France) and pupils were dilated with tropicamide (0.5%, Thea Lab). Each animal was placed on a heating pad, eyelids were retracted to maintain eyes open during recording. A gold electrode was placed onto corneas with a drop of methyl-cellulose (Ocry-gel, France) while the neutral and reference electrodes were placed on the tail and head of animals respectively. Light stimulations were delivered in a Ganzfeld with flash intensity of 0.04-8 cds/m^2 for scotopic and 8 cds/m^2 for photopic conditions. The flicker ERG was also used to isolate cone responses at 10 Hz flash frequencies and 1 cds/m^2 intensity. Amplitudes of the scotopic a- and b-waves were measured at the maximum negative and positive peaks of the recordings with respect to the baseline before stimulation.

Statistical analysis

Statistical software used to perform analyses was Prism Graph Pad. The significance of the data obtained was determined by performing unpaired student's t-test, Mann Whitney test, Wilcoxon test, Two-way ANOVA followed by post-hoc tests. p values were considered significant when ≤0.05: **** $p \leq 0.0001$, *** $p \leq 0.001$, ** $p \leq 0.01$, * $p \leq 0.05$.

Results

Identification of mutations in the *YIF1B* gene in patients displaying neurodevelopmental delay

In 10 patients displaying severe neurodevelopmental delay, from six unrelated families, we have identified by whole exome sequencing, biallelic homozygous mutations (family one to

four, patients 1.1, 1.2, 2.1, 2.2, 3.1, 3.2, 4.1, 4.2) and two compound heterozygous mutations (family five and six, patients 5.1, 6.1) in the *YIF1B* gene (Fig.1A, Table S1, Fig.S1).

In the two affected sibs of family one (born from Somalian parents from the same tribe), the homozygous mutation leads to a stop codon at the glutamate residue in position 200 (p.Glu200*), resulting in a short truncated protein with only one transmembrane domain (Fig.1B, S1). In family 2, affected children (born from French parents from the same village with probable distant consanguinity) carried a homozygous variation in the splice donor site of intron 5. The splicing effects of this variant was identified by sequencing the Yif1b cDNA transcript in immortalized lymphocytes from this patient. The homozygous variation in the splice donor site of intron five produced a skipping of exon five which leads to a frameshift followed by a premature stop codon 17 amino acids downstream (p.Ala161Glyfs*18). Patients from family three (born from Saudi parents from the same tribe) carried a single homozygous nucleotide duplication producing a frameshift leading to a truncated protein 12 amino acids downstream (p.Ala60Cysfs*13) that lacks all transmembrane domains. In family four, the two affected sibs (born from Iranian parents, consanguineous) carried a homozygous variant in the cytosolic N-terminus of YIF1B (p.Lys123Gln). In the two other families, patients 5.1 and 6.1 carried two different variants inherited from each parent. The affected child 5.1 carried one variant identical to patient 2.1 (skipping of exon five confirmed also on DNA sequencing from grown immortalized lymphocytes, Fig.S1) and a frameshift (p.Met233Serfs*6) leading to a truncated protein lacking the three last transmembrane domains. The affected child 6.1 carried a premature stop codon (p.Tyr167*) on one allele leading to a short truncated protein with only one transmembrane domain and a frameshift leading to a truncated protein 35 amino acids downstream (p.Ala193Profs*36) on the other one (Fig.1B, Fig.S1).

YIF1B splice-site and point mutations identified in patients behave as loss of function alleles

We investigated the expression level of the YIF1B protein in cells isolated from patients. Western blot experiments were performed with a YIF1B antibody directed against the N-terminal cytoplasmic peptide of YIF1B, that recognizes all YIF1B variant proteins mutated and/or truncated downstream of the antigenic site (Carrel et al., 2008). In immortalized lymphocytes from patient 2.1, and 5.1, no YIF1B protein (full length or truncated, expected at 22 KDa was detected, whereas bands were obtained for control cells at the expected size (34 KDa) (Fig.1C). We also confirmed the lack of detection of YIF1B protein in fibroblasts cultured from the skin of patient 2.1 (Fig.1D). In fibroblasts from patient 4.2 that carries a missense point mutation

(Lys123Gln, Fig.S1), only small amounts of YIF1B were detected compared to the control (Fig.1D).

In conclusion, truncated YIF1B proteins lacking all transmembrane domains were not expressed in cells isolated from these patients and a missense mutation in the third transmembrane domain of the protein was enough to induce a strong decrease of its expression. These observations are similar to those we made on the stability of truncated rat YIF1B proteins. The constructs lacking all the transmembrane domains could not be expressed in COS Cells. Moreover, increasing the number of transmembrane domains also increased the level of expression of the Yif1b constructs (Al Awabdh et al., 2012).

YIF1B mutations in human cause a new post-natal developmental deficit syndrome

The detailed description of the clinical features of patients is presented in Table S1 and a summary of the most common clinical features in Table S2. All patient birth parameters are normal, including head circumference. All subjects display developmental deficit starting after birth. Patients with truncated proteins (from family one, two, three, five and six) never achieved or subsequently lost head control and were never able to sit, stand and walk. Patients with point mutations (4.1 and 4.2) displayed strong physical developmental delay. In all patients, severe cognitive impairment was observed and mental disability was evaluated in patients with point mutations. Motor alterations, such as spasticity, hypotonia and hypertonia were observed in all patients. Other neurological alterations were detected in patients including seizures, swallowing difficulties, dystonia and central hypoventilation. Among patients with *YIF1B* mutations leading presumably to the lack of YIF1B protein, some died prematurely (patients from family one and two) and some survived under mechanical ventilation and with an effective treatment for their seizures (patients 5.1 and 6.1). Patients with truncated YIF1B protein that lived with normal respiratory function did not have seizures or pharmaco-resistant-seizures (3.1 and 3.2).

Patients with truncated YIF1B protein had cerebral palsy and abnormalities in their brain structures as detected by brain MRI (Fig.1E), except for patients from family three. MRIs were obtained from four patients 1.1, 1.2, 5.1 and 6.1 at two developmental stages (except for patient 1.2 that died early). Patients showed delayed myelination and/or thin corpus callosum the first year of life (white arrows on parasagittal T1 images). Progressive cerebellar vermian and pons atrophies (white arrowheads), and increase of the cisterna magna size are observed (Fig.1E2, white star). Progressive parenchymal loss and a ventricular dilatation are detectable in all patients. The strongest phenotype is observed in patient 5.1 who was older than the other patients

at the time of the last MRI (Fig.1E2). Comparison of two MRI from the same patient (6.1) at the age of 9 and 40 weeks suggests that defects appeared after birth as no alteration was detected at 9 weeks (Fig.1E3).

To better understand the requirement for YIF1B at the cell, tissue and organism levels, we characterized the mice constitutively lacking the *Yif1b* gene (*Yif1b*-KO) (Alterio et al., 2015).

Yif1b-KO mice have deficits similar to those observed in patients carrying *YIF1B* mutations

Yif1b-KO mice did not present post-natal lethality. Therefore, we investigated phenotypes in these mutant mice that were related to clinical traits of patients that could be responsible for premature death in human such as hypoventilation and seizures (see supplementary methods). Ventilation in adult *Yif1b*-KO mice was evaluated using plethysmography under normal air and in hypercapnia. We demonstrated that *Yif1b* deletion has no impact on the ventilation of mice (Fig.S2A). Patients show seizures starting as early as 4 months of life but adult *Yif1b*-KO mice did not display spontaneous seizures. We then tested the sensitivity of the mutants in pharmacological induced kindling conditions and demonstrated that neither the latencies to present myoclonic-clonic or generalized seizures were decreased in *Yif1b*-KO after acute injection of pentylenetetrazole (Fig.S2B).

In mice, YIF1B did not seem to be essential for ventilation or in the equilibrium of excitatory/inhibitory balance in the cortex and the hippocampus, a function known to be altered in epilepsy, which suggests that some compensatory mechanism occurred in this species. We then investigated other phenotypic alterations in the *Yif1b*-KO adult mice that could model the defects observed in patients.

Patients carrying *YIF1B* mutations exhibit visual impairment (nystagmus, strabismus, optic atrophy and retinopathy, Table S1). In patients 3.1 and 5.1, lack of visual evoked potential was observed even with flash stimulation suggesting retinal dysfunction. Likewise, the patient from family 5 of the recently published study on patients carrying *YIF1B* mutations also exhibited cortical blindness (AlMuhaizea et al., 2020). Hence, we further explored visual functions in the *Yif1b*-KO mice. Mutant mice showed head movements demonstrating some visual perception but without the frequency-dependent increase observed in WT mice (Fig.2A). *Yif1b*-KO mice displayed visual performance deficits in optometer response. We then investigated retinal physiology in *Yif1b*-KO mice and showed that the visual impairment was caused by retinal dysfunc-

tion, as indicated by the electroretinogram amplitude decreases (Fig.2B, 2C). In scotopic condition, if the a-wave reduction and latency increase indicated an impaired phototransduction in photoreceptors (Fig.2C1), the further b-wave amplitude decrease (Fig.2C2) was consistent with the optic nerve atrophy in patients.

Since patients display several motor and locomotor deficits (Table S1 and Table S2), we explored these functions in *Yif1b*-KO mice. Basal locomotion was normal in *Yif1b*-KO mice (Fig.3A), however when fine locomotor coordination was explored by the locotronic test, a deficit in *Yif1b*-KO mice was observed with an increase of mistakes on the horizontal ladder (Fig.3B). Motor coordination behavior was assessed using the Rotarod test. Performances on the first day of Rotarod testing in *Yif1b*-KO were normal compared to WT (WT=85.86±8.552 sec, n=14; *Yif1b*-KO=91.25±9.022, n=12; student's t-test, $p>0.05$), however coordination learning was altered in *Yif1b*-KO mice performing three consecutive days (Fig.3C).

Yif1b-KO mice have anatomical cerebral defects similar to those observed in patients

As MRIs from patients revealed specific brain alterations (Table S1 and Table S2), we investigated the presence of similar defects in the adult brain *Yif1b*-KO mice.

Myelination was evaluated by the level of expression of Myelin Basic Protein (MBP) that is localized in the myelin sheath surrounding myelinated axons. We focused on motor cortex of *Yif1b*-KO as these mice displayed motor behavioral alterations (Fig.3) and demonstrated that MBP was reduced in layers I and V-VI (Fig.4A) consistent with delayed myelination observed in patients (Fig.1E). Anatomical analyses revealed that *Yif1b* depletion in mice showed a moderate dilatation of the ventricles (Fig.4B1, 4B2) as observed in patients (Fig.1E). Finally, in *Yif1b*-KO, cerebellar atrophy was systematically observed (Fig.4C1) as detected in patient brains (Fig.1E). In mice, this defect was associated with changes in the shape of the cerebellum (Fig.4C2) and the decrease of cerebellar weight (without brain hypoplasia) (Fig.4C3).

Cellular and subcellular defects underlying cerebellar atrophy in the *Yif1b*-KO brain

We investigated cellular changes that underlie cerebellar atrophy in the cerebellum of the *Yif1b*-KO mouse. A reduction of 17% of Purkinje cells (PC, identified with calbindin-immunofluorescent labelling) and the disorganization of the dendritic tree of remaining cells were observed, possibly accounting for the decreased molecular layer thickness (Fig.5A, see high magnification pictures). Interestingly, the decrease of PC in mutant mice was not statistically amplified

between 3 and 12 months-old (Fig.5A2). PC death was not due to apoptosis, as the number of apoptotic Purkinje cells, detected by TUNEL staining showed no difference between the cerebellum of WT and *Yif1b*-KO mice (Fig. S6). This indicates that necrotic mechanisms were responsible of the PC degeneration as further verified in our ultrastructural study. PC degeneration in 3-month-old *Yif1b*-KO mice was confirmed on semithin toluidine blue-stained cerebellum sections in which moderately electron dense PC cell bodies and dendrites were observed (Fig.5B). Since cerebellar glial cells and especially the microglia are sensitive to homeostatic microenvironmental disruptions during disease and neuronal degeneration (Cvetanovic et al., 2015; Tay et al., 2017), we performed Iba1 immunofluorescence to assess the microglial response in the cerebellum. We found not only an increase in the density of microglia cells in the cerebellar cortex of *Yif1b*-KO mice compared to their WT littermates (Fig.5C1,5C2) but also the presence of hypertrophic microglia in the PC and molecular layers, where they were sometimes grouped into foci (Fig.5C1), probably removing debris from degenerating PC. Although these microglial responses are generally good indications of their reactive function, it remains to assess their cytokine profiles and their interactions with surrounding cells to determine their exact roles in this degenerative process.

We then performed transmission electron microscopy analysis in PC layer in WT and *Yif1b*-KO mice. Ultrastructural examination of WT mice sections (Fig.6A,6C,6E) were comparable to those previously described (Palay and Chan-Palay, 1974). In sections from *Yif1b*-KO mice, evident signs of alterations encompassing the cell somata and the entire dendritic arbor were observed, such as an increase of the electron density of the cytoplasm (Fig.6B) and in the number of lysosome-like profiles (Fig.6B, 6D, arrows). An abnormal fragmentation of the GA (Fig.6D) with dilatation of cisterns (Fig.6B, 6D) was also often observed, as inferred from the CTR433-immunostained sections displaying a clearly disorganized PC Golgi complex (Fig.S3), and in agreement with our previously reported electron microscopic study on *Yif1b*-KO CA1-hippocampal neurons (Alterio et al., 2015). Morphological alterations were present in the PC dendrites of mutant mice, such as large autophagosome-like vacuoles (Fig.6F, arrowheads). In addition, consistent with the presence of some Golgian outposts-like profiles in the molecular layer (Fig.S3, arrowheads), stacks of Golgi cisterns were often seen in primary dendritic profiles of *Yif1b*-KO PC (Fig.S4). Considering that PC dendrites have been shown to have no Golgi outposts (Liu et al., 2017), GA was abnormally targeted to dendrites in mutant PC. The ER displayed the most diverse and notable aberrant configurations in *Yif1b*-KO PC, such as unusual elongated loose network of RER cisterns throughout the peripheral cytoplasm and

with less ordered arrays of RER in the perinuclear cytoplasm (Fig.6B). Frequently, abnormal wavy segments of smooth ER (SER) were continuous with normal rough ER (RER). Among alterations detected, RER cisterns were associated with clusters of abnormal dilated and very clear vesicles dispersed throughout the perinuclear cytoplasm, between RER cisterns and the nuclear envelope (Fig.7A, arrows). Normal RER cisterns sometimes were continuous with abnormal and compacted aggregates of smooth ER membranes which appeared as bulky stacks of ER cisterns attached to the neuronal membrane (Fig.7B), sometimes with round and compacted agglomerates of SER membranes (Fig.7C) or with concentric and compacted whorls of smooth ER membranes (Fig.7D). Such abnormal configurations of ER aggregates were constantly located in the basal pole near the emergence of the PC axon (Fig.S5).

Consistent with the reduced number of PC identified with calbindin-immunostaining in *Yif1b*-KO mice sections, scattered or groups of PC perikarya and dendrites in the molecular layer displayed ultrastructural features of advanced degeneration. Degenerating PC appeared shrunken with numerous dilated Golgi cisterns, abnormal darkening of highly condensed cytoplasm and nucleoplasm surrounded by swollen profiles of the Bergmann glia (Fig.7E). Ultrastructural features of apoptotic cell death, such as chromatin condensation and nuclear fragmentation (Dusart et al., 2006) were not observed in our study, suggesting that PC lacking *Yif1b* rather die by necrotic mechanisms. Consistent with this ultrastructural finding, no difference was observed in detection of apoptosis (see supplementary methods) between WT and *Yif1b*-KO mice (Fig.S6).

It has to be noted that evident signs of alterations of GA and ER were observed in other neuronal types, such as in pyramidal neurons of the CA1-hippocampal layer [(Alterio et al., 2015) and Fig.S7] and that these alterations did not impair the physiological properties of neurons, such as demonstrated by recordings in hippocampal neurons and remaining PC (see supplementary methods and Fig.S8).

Yif1b/*YIF1B* deletion affects the primary cilium

Breeding *Yif1b*-KO male mice lead us to uncover their infertility because of the lack of progeny of homozygous male *Yif1b*-KO mice (Fig.S9A) and the quasi absence of spermatozoa in their epididymis (see supplementary methods and Fig.S9B). The few remaining spermatozoa exhibited structural alterations of the flagella with disorganization of microtubule doublets or supernumerary flagella (Fig.S9C). Altogether, cerebellar alteration, male sterility, visual and olfactory deficits (see supplementary methods and Fig.S10) in *Yif1b*-KO mice resemble to some of

the phenotypes observed in ciliopathies. Cilium integrity strongly relies on traffic from the GA, and YIF1B is essential for proper anterograde traffic and GA integrity (Alterio et al., 2015). We thus investigated possible alterations of primary cilia in *Yif1b*-KO mice brains, focusing on the PC layer of the cerebellum that was strongly affected in the mutant (Fig.8). The immunolabeling for Arl13B (Fig.8A1) demonstrated that the lack of *Yif1b* induced an increase in the length of primary cilia (Fig.8A2). The axoneme of primary cilium in PC is short (Del Cerro and Snider, 1969) and was very rarely observed in our electron microscopy analysis of the cerebellar cortex. To characterize the ultrastructural defects of the primary cilium and ciliary related structures, we examined pyramidal cells in the hippocampus that displays numerous long primary cilia (Wang et al., 2011). We found in pyramidal neurons from CA1, alterations of structures involved in primary cilium anchorage, such as an increase in striated rootlet thickness extending from basal bodies and centrioles (Fig.8B1), an increase in the inter-basal body/centriole distance and a reduction in the length of basal bodies (Fig.8B2, 8B3). We then investigated the consequences of *YIF1B*/*Yif1b* deletion in primary cilia and basal body structures, in cultured fibroblasts of patients and mutant mice. Abundance of ciliated cells was significantly reduced in patient 2.1 as compared to controls (Fig.8C1). Moreover, cilia average length, based on either acetylated tubulin (Fig.8C2) or Arl13B stainings was one third shorter in patients 2.1, 4.2 and 5.1 fibroblasts compared to controls (Fig.8C3, 8C5). These patients are carrying truncating mutations that lead to undetectable levels of YIF1B (patient 2.1 and 5.1) or a point mutation leading to a highly reduced expression of YIF1B (patient 4.2). Rootletin volume was increased in patient cells (Fig.8C4, 8C5), suggesting changes in the rootlets that anchor primary cilia. However, CEP83 (Failler et al., 2014) and CEP164 (Schmidt et al., 2012) (components of the centriole and of distal appendages) immunostainings showed no change in basal body proteins (Fig.8C6, 8C7). Consistently, cilia of *Yif1b*-KO mouse cultured skin fibroblasts were shorter than controls (Fig.8D).

Discussion

Our work has unveiled ten individuals from six independent families with recessive mutations in the *YIF1B* gene. These patients displayed some variability in their clinical features, however common phenotypic profile including severe global developmental and motor delay, central hypoventilation, seizures, visual alterations and brain abnormalities were observed with mutations leading to a truncated protein or a point-mutated *YIF1B* protein. Recently, a case report also identified patients carrying mutations in the *YIF1B* gene (AlMuhaizea et al., 2020). Interestingly, one patient of 7 years –old (Family 5 of the case report) had a mutation similar to the

patients of family 1 from our study and was from the same Somalian origin. However, although the clinical features are similar, the patients identified in our study deceased at 15 months. This discrepancy between the effect of *YIF1B* mutation on survival, points out that lacking YIF1B has fatal consequences in human for the ability of feeding and the development of epilepsy. The patient with the p.glu200* mutation (Family 5 of the case report published by AlMuhaizea *et al.*, 2020) is still alive but is fed by tube and its epilepsy is pharmacologically controlled. Remarkably, two children from the same family died at 2 months and 6 months, the last one from pneumonia, and had hypotonia similar to our Family 1 siblings. Comparing cases of the two studies clearly demonstrated that mutations in the *YIF1B* gene presumably leading to the absence of YIF1B protein are not always developing epilepsy. This is even more prone by the fact that the same mutation led to epilepsy in one patient, whereas her sister is not affected (family 3 of this study).

In this study, we demonstrated that truncation of the YIF1B protein after the first transmembrane domain led to undetectable levels of protein. This suggests that truncated YIF1B protein was instable or degraded, as previously observed when we attempted to express truncated YIF1B proteins in COS cells (Al Awabdh et al., 2012). Furthermore, a point mutation in YIF1B protein led to a strong decrease of its expression (patient 5.1). Thus, we can postulate that the loss of function of YIF1B is responsible for defects observed in patients. In this context, *Yif1b*-KO mice that constitutively lack YIF1B were analyzed and we demonstrated that deletion of the *Yif1b* gene in adult mice recapitulated some defects observed in children carrying *YIF1B* mutations, such as enlargement of ventricle size, cerebellar dystrophy, locomotor alterations and visual deficits.

The clinical features of patients carrying *YIF1B* mutations did not fit with any previously described pathologies. However, some traits, such as severe neurodevelopmental delay, white matter defects and/or progressive cerebral atrophy and intellectual disability were similar to a class of illnesses recently classified as Golgipathies (Passemard et al., 2017). YIF1B is a trafficking protein that interacts with the small GTPase rab6 and Yip1A, another trafficking protein (Al Awabdh et al., 2012). Golgipathies include diseases in which mutated genes encoded GA proteins but also vesicular carriers involved in the sorting, packing, routing and recycling through interactions with Rab GTPases, Rab effectors or Rab regulators that orchestrate secretory pathways. Here, we also demonstrated that *Yif1b* deletion leads to disorganization of the ER in mice hippocampal neurons and cerebellar PC. Such alteration was also visualized after complete depletion of YIP1A expression (Dykstra et al., 2010), a protein directly interacting

with YIF1B (Al Awabdh et al., 2012). ER and GA disorganized morphologies has been also observed when the ER resident protein BCAP31 is mutated in patients and is associated with motor and intellectual disabilities, dystonia, cerebellar atrophy and poor myelination (Cacciagli et al., 2013).

We previously showed that YIF1B is involved in the anterograde traffic from ER to the cell membrane and more particularly to specific subcompartments of the neuronal cellular membrane, but also in GA morphology (Carrel et al., 2008; Al Awabdh et al., 2012; Alterio et al., 2015). In this study, we demonstrate that at the cellular level, YIF1B is also essential for ER morphology and primary cilium integrity and that its deletion leads to alteration of the myelination and to neuronal death. We will discuss how cellular alterations observed in the CNS of the *Yif1b*-KO mutant mice could participate to brain alterations observed in patients carrying *YIF1B* mutations.

Myelination in the brain relies on proliferation, migration and differentiation of oligodendrocyte progenitor cells in mature oligodendrocytes (OL) that are able to synthesize myelin sheaths to insulate axons. On one hand, the myelin protein plasmolipin (PLLP) assembles liquid-ordered lipids in the GA to allow their targeting to the cell membrane for generating new membranes necessary for myelination (Yaffe et al., 2015), demonstrating the crucial role of GA in myelination. On the other hand, several receptors have been proposed to be implicated as regulators in OL maturation necessary for myelination (Mogha et al., 2016). Alteration of anterograde traffic and GA disorganization (Alterio et al., 2015) or mis-targeting of specific receptors (Carrel et al., 2008) in *Yif1b* depleted OL could be responsible for altered myelination.

Patients carrying *YIF1B* mutations presented progressive cerebellar atrophy and progressive parenchymal reduction. However, the cellular mechanisms that led to these defects could not be studied in brains from deceased patients since none was ever analyzed by anatomo-pathology. Among the mechanisms that lead to cerebellar atrophy during post-natal development in patients, the reduced number of PC (like that observed in mutant mice) could be implicated. The cellular death could result from the defect of ER-GA transport. Indeed it has been shown that inhibition of ER-GA traffic was linked to neurodegeneration in a pathological model, such as ALS (Soo et al., 2015). On another hand, it has been demonstrated that the deletion of GM130, a GA protein, caused GA fragmentation, impaired secretory trafficking in PC, and resulted in PC cell death (Liu et al., 2017), suggesting that GA disorganization could also be implicated in neuronal death. GA defects could also contribute to the progressive parenchymal

reduction observed in the cortex of patients by altering dendritic organization. We recently demonstrated that the deletion of *Yif1b* in mice led to the decrease of the number of dendritic spines in the hippocampus (Martin et al., 2020) and here, we found abnormal localization of GA in the initial segment of PC dendrites in the cerebellum. Growing evidence indicates that the secretory pathway regulates dendritic growth and maintenance. Suppression of the ER-GA transport inhibits dendrite outgrowth in developing mammalian neurons (Horton et al., 2005; Ye et al., 2007) and deletion of GM130 in mice, that slows down the anterograde traffic, induces a reduction of dendritic size and arborization in the cerebellum (Liu et al., 2017). Furthermore, in Drosophila, Yif1 plays a key role in the dendrite pruning in ddaC sensory neurons during early metamorphosis (Wang et al., 2018). How accumulation of GA in the dendritic tree or the acceleration of the anterograde traffic could affect dendritic arborization, dendritogenesis and/or maintenance of dendritic spines should be further investigated in *Yif1b*-KO mice. However it suggests that these defects could account for the reduction of cerebral grey matter in patients carrying *YIF1B* mutations.

GA has been involved in the delivery of cargo proteins to the primary cilium (Madhivanan and Aguilar, 2014; Stoetzel et al., 2016), a tiny protrusion expressed at the surface of all cells which plays different functions depending on the developmental stage and the organ (Pampliega and Cuervo, 2016; Avalos et al., 2017). Mechanistically, defects observed in primary cilia in the *Yif1b*-KO mouse could be a direct consequence of the GA alteration and/or abnormal trafficking from the GA to the cilium. This is supported by the disorganization of the GA in the mutant but also by previous results showing that the YIF1B protein is a chaperone protein for rab-dependent trafficking along microtubules, a pathway that has been shown to be essential for protein sorting to the primary cilium and for ciliary length (Madhivanan and Aguilar, 2014). The more prominent effect of *Yif1b/YIF1B* deletion in mice and human is cerebellar atrophy. It has been demonstrated that PC and Bergmann cells have cilia that were proposed to be involved in cerebellar development (Di Pietro et al., 2017). PC need intact ciliary Sonic Hedgehog (Shh) signaling for their proper development and dendritogenesis (Dahmane and Ruiz i Altaba, 1999; Cheng et al., 2018). How primary cilium dysfunction in the cerebellum of *Yif1b*-KO mice affects this brain structure should be further explored, focusing on the kinetics of alterations of PC and Bergman glia cell network in correlation with defects in Shh signaling. In addition, primary cilium alterations could also be implicated in visual deficit, male sterility and olfaction alterations observed in *Yif1b* depleted condition, as these defects are reminiscent of ciliopathies.

In summary, *YIF1B* is essential for post-natal brain maturation in human. Deletion of *Yif1b* in the mouse affects brain integrity with milder consequences than in human, suggesting that mice can overcome the lack of YIF1B during post-natal development. *Yif1b* deletion in mice induces cell-dependent subcellular alterations in the GA, the ER and the primary cilium/basal body structures that lead to an altered myelination and neuronal survival. Although the implication of the GA in primary cilium genesis has been evoked for a long time, our study demonstrates a link between Golgipathies and Ciliopathies (Sorokin, 1962; Kim et al., 2014). The crosstalk or cascade of events between GA and ER damage and ciliary defects, ultimately resulting in neurodevelopmental dysfunction remain to be clarified.

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Competing interests

The authors declare no competing financial interests.

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Figure legends

Fig.1 *YIF1B* mutations in six independent families. A. Pedigrees of six families of patients (grey circle was a donor egg). B. Schematic representation of the human *YIF1B* gene, mRNA and protein with five transmembrane domains (TM1-5) and location of the mutations in red (with patient identification) on flat and secondary structure drawing. C. Western blot analysis of YIF1B protein in lymphocytes from patients (2.1, 5.1) and controls (Ctl, Ctl 5). D. Western blot analysis of the YIF1B protein in fibroblasts isolated from skin biopsies of patients (2.1, 4.2) and controls (Ctl, Ctl 4). ns, non specific. E. MRI from the patients**.** E1. Patients from family one with sagittal T1W, axial T1W and T2W images (DWI images showing there is no diffusion restriction). Images were taken at 24.3 weeks for patient 1.2 and at 25.9 and 60.1 weeks for patient 1.1 that survived longer. E2. Patient 5.1 with sagittal T1W and axial T2W at 43.3 and 119.3 weeks. E3. Patient 6.1 with sagittal and axial T1W images at 9 and 40 weeks. Note thin corpus callosum, small cerebellum and abnormal pons (small white arrows on T1W images), enlargement of the ventricle (white arrow on T1W image at 119.3 weeks), reduction

of white matter, and volume loss with enlargement of the lateral and third ventricles and prominence of the pericerebral fluid spaces and of the Sylvian fissures.

Fig.2. *Yif1b*-KO mice have visual deficits A. Optometric tests (WT, n=5; mutant, n=6). In scotopic conditions, two-way ANOVA indicated significant differences among spatial frequencies [F(3,27)=9.468; p<0.0001] and genotypes [F(1,27)=4.507; p=0.0407] with no significant optometric stripe x genotype interaction [F(3,27)=1.188, p=0.3279]. In photopic conditions, two-way ANOVA indicated significant differences among spatial frequency [F(3,27)=9.367; p=0.001] and genotypes [F(1,27)=5.617, p=0.0233] and no optometric stripe x genotype interaction [F(3,27)=2.142, p=0.1199]. B and C. Abnormal retinal activity of *Yif1b*-KO mice in photopic (B) and scotopic (C) conditions revealed by electroretinograms recording (WT, n=7; *Yif1b*-KO n=6). B. In photopic conditions, electroretinogram amplitude wave and flicker amplitude were significantly reduced in mice *Yif1b*-KO (student's t-test, p=0.0049 for wave amplitude and p=0.013 for flickers). C1, C2. In scotopic conditions, two-way ANOVA for a-wave indicated significant genotype x flash intensity interaction [F(3,44)=3.39, p=0.262] with a significant difference between genotype at flash intensities >0.04 cd.s/m^2 (C1) indicating with latency increase an impaired phototransduction in photoreceptors. Two-way ANOVA for b-wave indicated significant difference between genotypes [F(1,44)=0.0187, p<0.0001) and flash intensities [F(3,44)=0.0187) with no genotype x flash intensity interaction [F(3,44)=1.625, p=0.1971] (C2). Data are presented as mean ± SEM and data points are shown. *, p<0.05; **, p<0.01; ****, p<0.0001.

Fig.3. Locomotor and motor functions in *Yif1b*-KO mice. A. Normal basal locomotion of *Yif1b*-KO mice was observed compared to control littermates. Time course of basal locomotor activity was assessed by total photobeam breaks measured in 5-min intervals (WT, n=7; *Yif1b*-KO, n=5). B. Locotronic test revealed an alteration of fine motor coordination in *Yif1b*-KO (n=12) compared to WT (n=12). Two-way ANOVA indicated significant difference among sessions (L, test with regular ladder, M, missing bars in the ladder) [F(2,44)=5.463; p=0.0076] and genotypes [F(1,22)=4.78, p=0.0397] with no significant session x genotype interaction [F(2, 44) = 0.6814, p=0.5112]. C. Alteration in learning ability evaluated by the Rotarod test (WT, n=14; *Yif1b*-KO, n=13). No difference was observed at day one (WT=85.86±8.552 sec and *Yif1b*-KO=93.92±8.719 sec, student's t-test, p=0.5153). At day 3, two-way ANOVA indicated a significant effect of time x genotype interaction [F(2,50)=3.594, p=0.0348]. Bonferroni's post hoc test showed a significant impact of the mutation. Data are presented as mean ± SEM and data points are shown. *, p<0.05.

Fig.4. Histological and anatomical defects in *Yif1b*-KO brain. A. In the motor cortex, Myelin Basic Protein immunostaining revealed a decrease of myelination in *Yif1b*-KO compared to WT in layer I and layers V-VI [interaction layer x genotype, $F(3,42)=7.422$, $p=0.004$; $p<0.05$ and $p<0.0001$ in layer I and V-VI respectively, WT *vs* *Yif1b*-KO with Bonferroni]. B. Anatomical comparison of WT and *Yif1b*-KO mice brain shows the moderate enlargement of ventricles in *Yif1b*-KO compared to WT mice (enlargements of the inserts in lower micrographs) on horizontal brain slices from 10 months old mice (B1) and in Luxol Fast Blue-stained coronal sections (B2). C. Cerebellar gross anatomy and histological sections, cerebellum related-behavioral in WT and *Yif1b*-KO. C1. Upper view of the cerebellum shows reduction in size of *Yif1b*-KO compared to WT mice. C2. Change in the shape of cerebellar vermis revealed by Calbindin immunolabeling on parasagittal brain sections. C3. Quantification of cerebellum weight shows a decrease in *Yif1b*-KO (n=6) as compared to WT (n=7) (Mann Whitney test, $p=0.0082$). Data are presented as mean ± SEM and data points are shown. **, $p<0.01$, ****, $p<0.0001$. (F) fornix, (cc) corpus callosum, (LV) lateral ventricle, (3V) third ventricle. Scale bars = 100 µm for A, 1 mm for B1 and C2, 500 µm for B2 and C1.

Fig.5. Cellular alterations in the cerebellum of *Yif1b*-KO mice. A. Calbindin immunolabeling revealed fewer PC in *Yif1b*-KO cerebellum compared to WT as shown on representative (A1, 3 months-old mice) and quantified images (A2; WT, n=30; Yif1b-KO, n=25 from 6 and 5 animals respectively for 3 months-old animals, white and black bars; and WT, n=10; Yif1b-KO, n=10 from 2 animals of each genotype for 12 months-old animal, blue bars,; A Two-way ANOVA indicated significant difference between genotypes [$F(1,71)=20.8$, $p<0.0001$] with no significant difference between age [$F(1,71)=0.35$, $p=0.555$] or genotype x age interaction [$F(1,71) = 2.69$, $p=0.01055$].. Morevoer, altered dendritic arborizations of PC was observed in *Yif1b*-KO compared to WT (see enlarged pictures). B. Semi-thin parasagittal resin sections (1-2 µm) stained with toluidine blue from the cerebellar cortex in the region of vermis including lobules IX and X. The cerebellar cortical layers in WT mice exhibit a normal cyto-architecture with somata of PC containing a large pale nucleus surrounded by a slightly stained pericaryon and unstained dendritic profiles in the molecular layer, whereas the cerebellar cortex of *Yif1b*-KO mice shows two PC with moderately dense cell bodies (arrow) and darkening of proximal and distal dendritic processes (arrowhead) or grouped darkened PC, suggesting different stages of degeneration. C. Increased microglial activation revealed by density of microglia throughout the cerebellar cortex and foci of Iba1-positive cells [C1, representative images; C2, quantification (WT, n=16; *Yif1b*-KO, n=16; student's t-test, $p<0.0001$)]. Note the hypertrophic cell bodies

and shortened thick processes in the molecular and PC layers. Data are presented as mean ± SEM and data points are shown. **, p<0.01, ****, p<0.0001. (MoL) Molecular layer, (PCL) Purkinje cell layer, (GrL) Granular cell layer. Scale bars = 100 µm for A, 50 µm for B and C.

Fig.6. Transmission electron micrographs of Purkinje cells (PC) in the cerebellar cortex of WT and *Yif1b*-KO adult mice. A,C,E. Representative ultrastructure of cerebellar PC in the WT mice. Micrograph illustrating the normal arrangement of the cytoplasmic organelles centered on the wrinkled nucleus and the regular array of the rough endoplasmic reticulum cisterns in the perinuclear zone (A). The typical ultrastructural aspect of the Purkinje cells Golgi complex organized as a compact stack of flat and long cisterns (C). A representative PC dendritic process in the molecular layer showing a normal ultrastructure (E). B,D,F. Ultrastructure of the PC in the *Yif1b*-KO mice displaying morphological alterations in the perikaryon and the dendritic processes. The soma of a PC exhibiting irregular arrangement of elongated and anastomosed cisterns of RER, largely dispersed throughout the perikaryon and the perinuclear zone (B). Note also the presence of lysosomes (arrows) scattered all over the perikarion (B, D). Multiple short stacks of PC Golgi complexes displaying abnormal dilated saccules (D). A PC dendritic profile with two large autophagosome-like vacuoles (arrowheads) which are partially filled with masses of cytoplasmic material in a process of degradation (F). Scale bars are represented on the pictures. (N) nucleus, (RER) rough endoplasmic reticulum, (GA) Golgi apparatus, (D) dendrite.

Fig.7. Electron micrographs showing diverses RER ultrastructural abnormalities and degenerating dark Purkinje cells in *Yif1b*-KO adult mice. A. Abnormal clusters composed of two to three enlarged vesicles with a transparent lumen which appear systematically associated with RER cisterns (arrows) and very close to the nuclear envelope. B. Abnormal compact and thick stack of parallel endoplasmic reticulum cisterns with the lumen almost entirely obliterated and connected with normal cisterns of RER (arrows). The aberrant stack is located at the periphery of the PC and closely apposed to the cell membrane (*). C. The ultrastructure of a PC displaying two moderately compacted endoplasmic reticulum whorls of RER cisterns (arrows). D. Abnormal round endoplasmic reticulum agglomerate displaying a central crystalline arrangement of tubules. Note on their periphery the continuity with RER cisterns (arrows). E, F. Electron micrographs at low magnification of some degenerating darkened PC. The darkened cell bodies have an irregular pyknotic nucleus with homogenous nucleoplasm without obvious heterochromatin. The cytoplasm is very dense with the organelles tightly packed and displaying

accumulations of intensely stained endoplasmic reticulum membranes and dilated Golgi complexes scattered throughout the cytoplasm (arrows). Degenerating neurons are covered with enlarged clear astroglial profiles (arrowhead) with rare basket synapses (E). Moderate darkened shrunken PC dendritic profiles in molecular layer (F, arrows). Scale bars are represented on the pictures. (N) nucleus, (RER) rough endoplasmic reticulum, (SER) smooth endoplasmic reticulum, (Cyt) cytoplasm.

Fig.8. Impact of the loss of *Yif1b*/*YIF1B* on the primary cilium. A. Primary cilia in cerebellum sections of WT and *Yif1b*-KO mice. A1. Cerebellar sections immunolabeled with Arl13B, a ciliary marker. A2. Distribution of cilium length and ciliary length means were quantified in Purkinje cell layer of cerebellar lobules nine and 10 respectively (WT, n=185 and *Yif1b*-KO, n=269 cilium, eigth images of four animals per genotype). Cilia were significantly longer in *Yif1b*-KO compared to WT (11.4 % increase of mean; Mann Whitney test, p=0.0005). B. Ultrastructural analysis of CA1 pyramidal neurons of *Yif1b*-KO mice revealed alterations in primary cilia. Representative pictures showing close interaction of GA with rootlets and centrioles (B1), and the basal body ultrastructural morphology (B2). Quantification of the distance between centrioles (WT, n=8; *Yif1b*-KO, n= 6; Mann Whitney, p=0.0007) and of the basal body length in WT and *Yif1b*-KO neurons (WT, n=5; *Yif1b*-KO, n= 8; Mann Whitney, p=0.0042) (b3). C. Quantification of cilium components in cultured fibroblasts from control and patients skin. Percentage of ciliated cells (control, n=13 fields, 337 cells; patient 2.1, n=12 fields, 332 cells; t-test with Welch's correction, p<0.0001) (C1). Cilium length using acetylated tubulin staining (C2; control, n=284, patient 2.1, n=270) and Arl13B staining [C3; control ctl, ctl4 (unaffected sister), ctl5 (unaffected mother), n=70-80; patient 2.1, 3.1 and 5.1, n=70-80; student's t-test with Welch's correction or Mann Whitney test, p<0.0001]. Volume of rootletin (C4; controls, n=20, patient 2.1, n=20; student's t-test, p=0.0268; C5, representative pictures of Arl13B and rootletin immunostaining below graphs). Volume of CEP83 (C6) and CEP164 (C7), identified by labelling adjacent to cilia (control, n=20, patient 2.1, n=20). D. The quantification of cilium length was performed using Arl13B staining on fibroblasts isolated from mice skin (WT, n=70; *Yif1b*-KO, n=70, Mann Whitney test, p<0.0001). Scale bars: 20 µm in A, and 2 µm in B. Data are presented as mean ± SEM and data points are shown. *, p<0.05, **, p<0.01, ***, p<0.0001. Scale bar: 20 µm for A1. (Mol) Molecular layer, (PCL) Purkinje cell layer, (Grl) Granular cell layer, (GA), Golgi apparatus, (C) centriole, (R) rootlet.

Supplementary methods

Acute seizure induction by pentylenetetrazole (PTZ) and scoring

Before testing, mice were acclimated to the procedure room for at least 1 h. PTZ (Sigma Chemical Co., St. Louis, MO), made fresh daily, was dissolved in saline and filter sterilized, and it was administered intraperitoneally (i.p.) in a volume of 10 mL/kg body weight. Following an single PTZ injection, mice were monitored for 30 min and the time and severity of behavioral seizures scored and recorded by an observer blinded to genotype using a 5-point modified Racine scale (0-4: 0 = no behavioral change; 1 = hypoactivity; 2 = myoclonus; 3 = generalized convulsion with righting reflex; 4 = generalized convulsion with loss of righting reflex).

Plethysmographic recordings

Respiratory activity was measured by a barometric method, whole-body plethysmography with one recording chamber containing the animals, a reference chamber and a differential pressure transducer. The plethysmograph chambers were of 20 mL and the pressure difference between the two chambers was measured with the differential pressure transducer (Validyne, Northridge, CA, USA; DP 103–14) connected to a sine wave carrier demodulator (Validyne, CD15). After demodulation, the analog signal from the transducer was amplified, filtered, passed through an analog-to-digital converter (Labmaster TL-1, Axon Instruments, Foster City, CA, USA; sampling frequency 1 kHz) and then captured and stored on disk. Plethysmograms were calibrated during each recording session by injecting 2.5–5 µL of air in the recording chamber with a Hamilton (Reno, NV, USA) syringe. Ventilation at rest was initially measured in animals breathing room air (basal). The chamber was then flushed with gas mixture: 3%, 5% and 8% CO_2 (mixed with 20% O_2, balance N_2) for hypercapnia protocol and recovery was assessed with room air (post-hypercapnia). In each session the animals were challenged with room air 10 min, hypercapnic air 5 min and room air 10 min. Activity periods, which typically included exploratory movements, grooming, and sniffing, were detected based on large disturbances in the respiratory signal caused by the combined effects of positional changes and changes in breathing pattern. Temperature was maintained constant in the experimental room. A computer-assisted method was used to measure breathing variables over activity-free and apnea-free periods and ventilation (Ve) was calculated as $Ve = V_{Tnorm}/(Ti + Te)$ with $V_{Tnorm} = V_T$/animal body weight with V_T, tidal volume, Ti and Te, inspiration and expiration duration.

Sperm Analysis

Cauda epididymides were collected from mice. Epididymides were minced and placed into 500 µl of M2 medium (Sigma-Aldrich) at RT from 10 min to allow sperm to swim-out. Sperm concentration and mobility were assayed by using a haemocytometer under video-microscopy.

Spontaneous odor exploration and discrimination task

Mice were tested in a well-ventilated room using a Plexiglas testing box with a grid floor allowing the experimenter to expose an odorized Whatman paper filter at various locations without disturbing the animal. 10 µl of odorant solution (1% vol/vol in mineral oil, Sigma-Aldrich) dropped at the center of the paper was used as odorant source. Three different odors were used for this experiment: the habituation odor, pentanal (H), a similar odor with one more carbon atom in the molecular structure, hexanal (C+1) and an odor with three more carbon atoms, octanal (C+3). Grouped mice were handled daily for two weeks and individually habituated to the testing box for 20 min the two days before the experiment. On the day of the experiment, after 5 min period of habituation, the habituation odor was presented four consecutive times during 2 min (H1 to H4). Then, C+1 odor was presented for 2 min (discrimination 1) followed by another presentation of the habituation odor (H5) and finally mice were exposed to C+3 odor (discrimination 2). Each odor presentation was followed by a 3 min inter-trial interval. The whole experiment was videorecorded and the time spent actively sniffing (nose on the filter) was measured *a posteriori* for each 2 min-exposure by two independent experimenters blind to the animal genotype.

Terminal deoxynucleotidyl transferase-mediated digoxigenin dUTP Nick-end labeling (TUNEL) staining.

The ApopTag peroxidase apoptosis detection kit (Millipore Corporation -S7100, Germany) was employed to detect apoptotic cells in situ by labeling DNA strand breaks by the TUNEL method. We applied the procedure of the kit manual for tissue cryosections without quenching endogenous peroxidase. Briefly, brain sagittal cryosections of 12 µm thickness from WT (n=2) and *Yif1b*-KO (n=3) mice were fixed with 1.5% paraformaldehyde in PB for 10 min. Sections were rinsed in PBS (3 x 5 min) before incubation in the working strength TdT enzyme for 1 h at 37 °C and then treated with the working strength stop/wash buffer for 10 min at room temperature. Following incubation with the anti-digoxigenin peroxidase conjugate for 30 min at room temperature and rinsing in PBS, the color was developed for 5 min in Tris buffer containing 0.05% 3,3-diamino-benzidine tetrahydrochloride peroxidase substrate and 0.01% hydrogen

peroxide (Sigma). Finally, sections were counterstained by the Nissl staining protocol, dehydrated and covered with mounting media and coverslip.

Brain electrophysiology

Parasagittal (200 μm) slices of the cerebellar vermis were prepared using a vibroslicer (Leica VT1200S) in ice-cold bicarbonate buffered saline (BBS) solution containing the following: 125 mM NaCl, 2.5 mM KCl, 2 mM $CaCl_2$, 1 mM $MgCl_2$, 1.25 mM NaH_2PO_4, 26 mM $NaHCO_3$, and 10 mM glucose (saturated with 95% O2–5% CO2), pH 7.3. The slices were left to recover for 1 h at 34 °C and then stored at room temperature (20–24 °C) until use. For recordings, slices were perfused with the above solution (1–1.5 mL/min) at room temperature and observed using an upright microscope with DIC Nomarski optics and a 60X water immersion objective. Recording electrodes were pulled from borosilicate capillaries (approx. 2.5 MΩ with chloride-based internal solution). Electrophysiological recordings were performed with a Heka EPC-9 amplifier. Evoked IPSCs were recorded using a cesium-based internal solution to optimize voltage-clamp in Purkinje neurons with the following composition: 150 mM CsCl, 4.6 mM $MgCl_2$, 0.1 mM $CaCl_2$, 10 mM HEPES, 1 mM EGTA, 4 mM Na-ATP, and 0.4 mM Na-GTP. Holding potential was − 70 mV. After a control period longer than 5 min, long-term depression (LTD) was induced by a previously described pairing protocol (Casado et al., 2002). Two PF stimuli with an interstimulus interval of 10 ms were paired at 0.5 Hz for 2 min with a depolarization of the Purkinje neuron to 0 mV for 100 ms. Somatic depolarization preceded parallel fiber stimulation by 20 ms.

Transverse hippocampal slices (400 μm) were obtained as previously described (Potier et al, 2000) from mice anesthetized with halothane before decapitation. Slices were prepared in ice-cold artificial cerebrospinal fluid (aCSF) and placed in a holding chamber for at least 1 hr. The composition of aCSF was as follows (in mM): NaCl 124, KCl 3.5, $MgSO_4$ 1.5, $CaCl_2$ 2.3, $NaHCO_3$ 26.2, NaH_2PO_4 1.2, and glucose 11, pH 7.4. A single slice was transferred to the recording chamber at a time and continuously perfused with aCSF pre-gassed with 95% O_2 - 5% CO_2. Extracellular recordings were obtained at RT from the apical dendritic layer of the CA1 area using micropipettes filled with 2 M NaCl. Presynaptic fiber volleys (PFVs) and field excitatory postsynaptic potentials (fEPSPs) were evoked by electrical stimulation of the Schaffer collaterals and commissural fibers located in the *stratum radiatum*. The averaged slope of three successive PFVs and fEPSPs was measured using Win LTP software (Anderson and Collingridge, 2001). To evaluate the level of receptor activation and compare between groups, the

fEPSP/PFV ratio was calculated giving an index of synaptic efficacy (I_{SE}) that was plotted against increased stimulus intensities (300, 400 and 500 µA). In order to investigate LTP of synaptic transmission, a test stimulus was applied every 10 sec in a control medium and adjusted to obtain a fEPSP with a baseline slope of 0.1 V/sec. In one set of experiments, the averaged slope of three fEPSPs was thus measured for 15 min before the delivery of theta-burst stimulation (TBS), consisting of five trains of four 100 Hz pulses each, separated by 200 ms and delivered at the test intensity. This sequence was repeated three times with an interburst interval of 10 s. In a second set of recordings, a high frequency stimulation (HFS) was delivered as a conditioning stimulation, consisting of 1 train at 100 Hz pulses for 1 sec. In all experiments, testing with a single pulse was then resumed for 60 min after the delivery of the conditioning stimulation to determine the level of LTP.

Supplementary Table and Figure legends

Table S1. Clinical diagnosis of patients carrying mutation in the *YIF1B* gene. NA, not applicable, ND, not determined, SD, standard deviation.

Table S2. Summary of the most common clinical features of patients carrying *YIF1B* mutations. Clinical feature for patient 2.2 is partial, total number of patient in this table does not include him.

Fig.S1. The *YIF1B* gene consists of eight exons shown alternatively in black and blue. The reference YIF1B protein (NP_001034761.1) contains five transmembrane domains that are shown in bold characters. In mutated proteins from patients (1.1, 1.2, 2.1, 2.2, 3.1, 3.2, 5.1 and 6.1), additional amino acids and the premature stop codons (*) are indicated in red.

Fig.S2. A. Breathing pattern of *Yif1b*-KO mice were not different from WT littermates under room air, under hypercapnia challenge or during recovery with room air after hypercapnia (WT, n=4. *Yif1b*-KO, n=5). B. Comparison of pentylenetetrazole (PTZ)–induced convulsive seizure latency between *Yif1b*-KO and WT littermate revealed that *Yif1b*-KO were not more sensitive to the development of seizures induced by the drug (n=6 mice per genotype, Unpaired t test, p=0.8701). Data are presented as mean ± SEM and data points are shown.

Fig.S3. Immunostained GA with the anti-CTR433 antibody in sagittal sections of the cerebellar cortex from WT and *Yif1b*-KO mice. The CTR 433-positive GA in WT PC appears as an intricate network completely encircling the nuclei. CTR433-positive staining is also well depicted in other cells of molecular and granular layers. GA-stained network in the *Yif1b*-KO PC displaying altered arrangement. Note that some PC bodies completely lack GA in the basal pole (arrows) and multiple small CTR 433-positive profiles in the molecular layer (arrowheads) which are not seen in control mice. Scale bar: 10 µm. (MoL) Molecular layer, (PuL), Purkinje layer, (GrL) Granular layer.

Fig.S4. Electron micrographs of abnormal localization of GA in the dendrite profile segment of a PC in a *Yif1b*-KO mouse cerebellar molecular layer which is surrounded by Bergmann glia (BG) and parallel fibers. Note the presence of a large autophagosome-like vacuole (arrow) which is partially filled with masses of cytoplasmic material in process of degradation.

Fig.S5. Electron micrographs at low magnification of PC bodies in a *Yif1b*-KO mouse cerebellum with aberrant agglomerates of endoplasmic reticulum (ER) preferentially localized in the basal sector of the cytoplasm oriented to the granular cell (GrC) layer. A. A densely stacked ER (arrow) stuck against the basal membrane of the neuron. B, C. An abnormal round agglomerate of ER (arrows) is seen in the basal cytoplasm of PC.

Fig.S6. TUNEL detection of apoptotic neurons in the cerebellum of WT and *Yif1b*-KO mice. The apoptotic cells (brown) are shown in center of the drawn squares. It should be noted the very low density of apoptotic cells detected through the cerebellar sections which looks similar in the WT and the *Yif1b*-KO mice. Such apoptotic cells are unambiguously and preferentially localized in the granular (GrL) and the molecular (MoL) layers of WT and *Yif1b*-KO mice. Only one apoptotic cell was seen close to the Purkinje cell (PCL) layer, in all of the analyzed sections from the KO mice. Scale bar: 500 µm.

Fig.S7. Electron micrographs of aberrant ER aggregates in pyramidal CA1-hippocampal neurons of *Yif1b*-KO mice. A. An abnormal round ER aggregate which resembles those found in cerebellar Purkinje neurons. B. An aberrant striated compact stick-like formation of ER connected to an adjacent curved stack of ER (arrows) and multiple small round dense particles (arrowheads).

Fig.S8. Electrophysiological properties of PC and CA1 pyramidal neurons. A. Plasticity properties of Purkinje cells in slices of *Yif1b*-KO (n=6) and WT (n=5) cerebellum. LTD experiment

was performed by pairing a somatic depolarization of the PC with a stimulation of the parallel fiber. Stimulation periods are indicated by « p » on the time bar. Each point corresponds to the average of 10 evoked IPSCs. The amount of LTD is quantified by the percentage of the maximal current obtained in the control period. The LTD obtained in both conditions was statistically identical (*Yif1b*-KO: 55.1 ± 0.9, WT: 55.8 ± 1.4 %). B. Electrophysiological properties of CA1 pyramidal neurons. Comparison of input/output (I/O) curves showed that I_{SE} was not significantly altered in *Yif1b*-KO animals (33 slices) as compared to WT animals (16 slices) whatever the stimulation intensity. In slices from WT (n = 7) and *Yif1b*-KO mice (n = 11), HFS induced a significant increase in the fEPSP slope, when compared to baseline levels, that persisted until the end of the recording (stimulus effect, p = 0.001 and < 0.0001 for WT and *Yif1b*-KO mice respectively). Comparison of the mean LTP magnitude determined for the last 15 min of recordings, e.g. between 45 and 60 min after the conditioning stimulation, was similar in WT (40.4% ± 9.7) and *Yif1b*-KO (49.1% ± 9.6) mice (p = 0.53). In the same way, no change in the amplitude of synaptic plasticity (p = 0.81) was found when TBS-induced LTP induced in slices of WT mice (24.4% ± 7.1; n = 7) was compared to the potentiation induced in *Yif1b*-KO mice (27.2% ± 7.2; n = 14). These results indicated that *Yif1b* deletion does not impact basal glutamate synaptic transmission and either the threshold or the amplitude of synaptic plasticity in hippocampal CA1 networks. Data are presented as mean ± SEM.

Fig.S9. A. Genotype distribution of offspring of *Yif1b* mutants. B. Strong decrease of spermatozoa motility in *Yif1b*-KO epididymis (WT, n=8; *Yif1b*-KO, n=3; Mann Whitney test, p=0.0238). C. Microtubular organization in spermatozoa flagella from WT and *Yif1b*-KO mice testis, sectioned in transverse or longitudinal planes showing alterations in the number doublets, or loss of doublets or multiple flagella in *Yif1b*-KO. Data are presented as mean ± SEM and data points are shown. *, p<0.05.

Fig.S10. Olfactory performances in WT (n=20) and *Yif1b*-KO (n=16) mice using spontaneous odor exploration paradigm. Spontaneous discrimination between hexanal (H) and C1 or C3 using a habituation and / or dishabituation task. Odorant exposition: 2 min, ITI: 3 min. H(1)-H(4): repeated exposition to heptanal (1%); C1: hexanal (1%); C3: octanal (1%). Mice habituated normally to repeated presentation of the habituation odorant [$F(3,102)=6.579$; $P<0.001$]. Mice of both genotypes discriminated changes of odorant with responses differing according to the novel stimulus [stimulus effect: $F(1,34)=8.729$, $p<0.01$; stimulus x discrimination interaction: $F(1,34)=6.598$, $p<0.05$; $F(1,34)=11.969$, $p<0.01$ and $F(1,34)=18.387$ and $p<0.001$ for

C+1 and C+3 discriminations respectively]. Both genotypes similarly discriminated C+1 [discrimination effect: $F(1,34)=11.969$, $p<0.01$; WT: $p=0.033$ $z=-2.128$, *Yif1b*-KO: $p=0.0309$ $z=-2.158$, Wilcoxon test] whereas *Yif1b*-KO mice discriminated less C+3 than WT [genotype effect: $F(1,34)=6.91$, $p<0.05$, discrimination effect: $F(1,34)=18,387$, $p<0.05$, WT: $p<0.01$, $z=-2.987$, *Yif1b*-KO: $p<0.05$, $z=-2.172$; C+3 vs C+1 exploration: WT: ns, *Yif1b*-KO: $p<0.05$, $z=-2.43$, Wilcoxon test]. Data are presented as mean ± SEM and data points are shown. *, $p<0.05$ *vs* WT; # $p<0.05$, H4 *vs* C+1; § $p<0.05$, §§§ $p<0.001$, H5 *vs* C+3.