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## *CDK5RAP2* primary microcephaly is associated with hypothalamic, retinal and cochlear developmental defects.

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## ABBREVIATIONS

aRGc: apical radial glial cell

CC: corpus callosum

ERG: electroretinogram

EVA: vestibular aqueduct enlargement

HPE: holoprosencephaly

IHA: interhypothalamic adhesion

ID: intellectual disability

IQ: Intelligence Quotient

IUGR: intrauterine growth retardation

FSIQ: full scale intellectual quotient

MCPH: autosomal recessive primary microcephaly

NGS: next generation sequencing

OSVZ: subventricular zone

PCM: pericentriolar material

PM: primary microcephaly

SD: standard deviation

SD-OCT: spectral domain optical coherence tomography

SNHL: sensorineural hearing loss

SVZ: subventricular zone

VZ: ventricular zone

WG: week of gestation

WISC IV: Wechsler Intelligence Scale for Children-IV

WPPSI III: Wechsler Preschool and Primary Scale of Intelligence

## ABSTRACT

**Background:** Primary hereditary microcephaly (MCPH) comprises a large group of autosomal recessive disorders mainly affecting cortical development and resulting in a congenital impairment of brain growth. Despite the identification of more than 25 causal genes so far, it remains a challenge to distinguish between different MCPH forms at the clinical level.

**Methods:** 7 patients with newly identified mutations in *CDK5RAP2* (MCPH3) were investigated by performing prospective, extensive and systematic clinical, MRI, psychomotor, neurosensory and cognitive examinations under similar conditions.

**Results:** All patients displayed neurosensory defects in addition to microcephaly. Small cochlea with incomplete partition type II was found in all cases and was associated with progressive deafness in 4 of them. Furthermore, the CDK5RAP2 protein was specifically identified in the developing cochlea from human fetal tissues. Microphthalmia was also present in all patients along with retinal pigmentation changes and lipofuscin deposits. Finally, hypothalamic anomalies consisting of interhypothalamic adhesions, a congenital midline defect usually associated with holoprosencephaly, was detected in 5 cases.

**Conclusion:** This is the first report indicating that *CDK5RAP2* not only governs brain size but also plays a role in ocular and cochlear development and is necessary for hypothalamic nuclear separation at the midline. Our data indicate that CDK5RAP2 should be considered as a potential gene associated with deafness and forme fruste of holoprosencephaly. These children should be given neurosensory follow-up to prevent additional comorbidities and allow them reaching their full educational potential.

## INTRODUCTION

Primary microcephaly (PM) is a rare neuro-developmental disorder characterized by congenital microcephaly, intellectual disability of variable severity (ID) and occasional epilepsy that may reflect cortical mantle defects [1–3].

Clinically, PM includes isolated PM (MicroCephaly Primary Hereditary (MCPH), PM with short stature (Seckel syndrome, microcephalic primordial dwarfism), and syndromic PM (associated with non-neurodevelopmental manifestations). MCPH and Seckel syndrome may be further subdivided by the presence of cortical malformations and/or chorioretinopathy. Although this classification helps managing the differential diagnosis of the patients, it does not reflect underlying mechanisms. Most PMs show autosomal recessive inheritance (for review, see [1, 3]), although dominant forms have occasionally been described [4–6] and a mitochondrial origin has been proposed [7]. To date, more than 100 genes have been implicated in PM (for review, see [8]).

The cyclin-dependent kinase 5 (CDK5) regulatory subunit associated protein-encoding gene (*CDK5RAP2*; OMIM 608201) is among the first genes initially identified in PM (MCPH3, OMIM 604804) [9]. *CDK5RAP2* regulates CDK5 activity, required for centrosome cohesion [10], centriole duplication and attachment of the centrosome to the spindle pole [11], and plays a role in microtubule plus-end dynamics [12]. In addition, it is essential for DNA damage response [13]. In the developing mouse neocortex, *Cdk5rap2* is crucial for the maintenance of apical neural progenitors [14, 15] as its deficiency leads to spindle orientation defects, premature cell cycle exit and increased cell death during neurogenesis, resulting in a reduction in the final neuron number [15]. During human neurogenesis, *CDK5RAP2* is widely expressed in the ventricular and subventricular zones at week of gestation WG12, WG18, and in the superficial layers of the neocortex until term [16].

Variants in *CDK5RAP2* were first identified in association with PM in 2005 [9]. Since then, 35 patients from 17 families have been described, essentially through single case or mutation reports, and with limited clinical details [9, 17–29]. Most mutations reported are nonsense or frameshift variants (i.e. null variants), except for three siblings carrying missense variants [22] (Figure 1 and Supplementary Table 1). Patients with pathogenic variants in *CDK5RAP2* display heterogeneous phenotypes: the first 11 patients reported are microcephalic individuals (OFC from -4 to -9 SD during childhood) with normal stature and mild-to-moderate ID [9, 18, 26]. Three infants were reported with PM and mildly short stature (from -2.5 to -4 SD) that corrected with age. Three additional patients from 2 pedigrees were reported with Seckel-like microcephalic dwarfism [29]. Feeding difficulties [9, 20, 24, 26], skin areas of hypo- [9] and/or hyperpigmentation [21, 29], seizures [9] and deafness [9, 21, 26, 29] have been reported. *CDK5RAP2* variants have also been reported in patients with corpus callosum agenesis without microcephaly [22]. One patient developed acute lymphoblastic leukemia [9]. This heterogeneity is not supported by any genotype/phenotype correlation and the possibility of additional recessive or *de novo* mutation has not been ruled out.

Despite this range of presentations, MRI data are lacking. Brain MRIs performed in *CDK5RAP2* patients have failed to provide specific information. When available, data only indicate a reduced brain size and a qualitative impression of gyral simplification [23, 26, 28, 29], similar to reports in other MCPH [30]. Hypoplasia, agenesis or dysplasia of the corpus callosum have been occasionally reported [21, 22, 24]. As MRI data have not been reported for the majority of patients, the possibility that *CDK5RAP2* patients have specific brain malformations cannot be ruled out.

Furthermore, no systematic neuropsychological assessment of the patients has been published. Mild to moderate ID was reported in the first cases [9, 20, 31]. However, this finding has been challenged by more recent studies using neuropsychological assessments [26, 28] surprisingly showing that individuals with *CDK5RAP2* variants have a normal/borderline IQ. Hence, the severe reduction in brain volume in patients with *CDK5RAP2* variants does not appear to result in an equally severe impact on cognitive functions. There is thus an urgent need for reliable data on cognitive prognosis in MCPH as a prerequisite for genetic counseling, adequate educational therapy and medical follow-up.

Here, we took advantage of a cohort of 7 new *CDK5RAP2* patients, the largest series reported so far, to revisit the phenotype associated with *CDK5RAP2* by performing prospective, extensive and systematic clinical, MRI, psychomotor, neurosensory and cognitive examinations under similar conditions. We report 4 novel and 4 very recently described *CDK5RAP2* mutations [7] and show that, in addition to PM, all patients have skin pigmentation changes as well as congenital neurosensory defects, including microphthalmia and retinal pigmentation defects, and a unique cochlear malformation. In agreement with this latter finding, immuno-labeling on human fetal tissues identifies the *CDK5RAP2* protein in the developing cochlea, in addition to the fetal brain. Moreover, 5 patients exhibit a hypothalamic anomaly. Our data indicate that the *CDK5RAP2*-related phenotype is wider but more homogeneous than previously thought, and that ophthalmic and auditory investigations should be systematically included in the clinical follow-up of these children.

## MATERIAL and METHODS

### 1. Next Generation Sequencing or Targeted sequencing

Homozygosity mapping (patients 1 & 2) was performed by SNP array analysis (SurePrint G3 Human Genome CGH + SNP Microarray kit, Agilent Technologies). Coding exons and splice junctions of *CDK5RAP2* were sequenced by standard bidirectional Sanger methods. Mutations in patients 3 to 5 were identified by targeted Next Generation Sequencing. *CDK5RAP2* was included on a microcephaly gene panel for screening by multiplex PCR enrichment on a microfluidic support (Access-Array, Fluidigm Corporation) and 2×150bp sequencing with an Illumina MiSeq system. Sequencing reads were mapped to the UCSC Genome Browser (hg19) with MiSeq software, the Burrows-Wheeler Aligner, and the Genome Analysis Toolkit, and panel exons were 99% covered. Variants were filtered against MAF (>0.005), dbSNP, 1000 Genomes, the NHLBI ESP Exome Variant Server, and an in-house dataset. Rare variants were annotated for functional features of coding nucleotides with publically available databases, Polyphen2, SIFT, Mutation taster and Align GVGD. Confirmation of mutations identified by NGS and their familial segregation were carried out (Sanger sequencing). Patients 6 and 7 were part of a whole-exome sequencing study in a larger microcephaly cohort. Methods, sequencing results and few clinical hallmarks of these two patients were reported elsewhere [7].

### 2. Clinical, neuroimaging and neurosensory assessment of the patients

Seven patients carrying *CDK5RAP2* variants identified in Paris or Zurich genetic departments were recruited between 2014 and 2018. Inclusion criteria were: OFC  $\leq$  -2 SD at birth (WHO growth charts ([www.who.int/childgrowth/standards](http://www.who.int/childgrowth/standards))), no environmental cause of PM. Parents provided informed

consent for children's participation in a protocol approved by the ethics committee of Paris and was registered at ClinicalTrials.gov, Identifier:NCT01565005.

The protocol included a detailed clinical and neurological examination and the following exams/assessments:

**Cerebral MRI**, included coronal T2-weighted and T1-weighted 3D sequences with millimeter slices (1.5 Tesla Philips scanner).

**Cognitive assessment:** Intellectual abilities were assessed using the international Wechsler scales, according to age (WISC IV: 6 to 16 years, n=5, patients 1, 4-7; WPPSI III: 3 to 7 years 7 months, n=2, patients 2 and 3). Full Scale IQ (FSIQ) was calculated from four scores for WISC IV: a Verbal Comprehension Index (VCI), a Perceptual Organization Index (POI), a Working Memory Index (WMI) and a Processing Speed Index (PSI). The scores are independent and involve specific cognitive abilities such as lexical stock, general knowledge, verbal comprehension and verbal reasoning (VCI); visuomotor and visuospatial skills to examine a problem, organize thoughts and find solutions using cubes/pictures (POI); short-term memory, concentration abilities, mental manipulation, planning abilities, cognitive flexibility, arithmetic reasoning (WMI), performance speed in graphic realization and visual discrimination (PSI). FISIQ was calculated from three scores for WPPSI III (verbal IQ, performance IQ, and processing speed quotient). Only the non-verbal reasoning scale (POI) has been calculated for patient 3 due to language barrier.

#### **Neurosensory assessment**

*Ophthalmological assessment* included visual acuity measurement with Snellen optotypes axial length measurement, bio-microscopy of the anterior segment and ocular fundus examination. Fundus auto-fluorescence imaging, full field scotopic and photopic electroretinograms (ERGs), a pattern-ERG (pERG) and electro-oculography (EOG) were performed in all patients. Two patients underwent optical coherence tomography (3D-OCT).

*Audiological assessment* included pure tone and speech audiometry.

*Temporal bone high-resolution CT* included helical acquisition of submillimeter slices with multiplanar reconstructions (Philips Brilliance 64-CT).

### 3. Statistical analysis

The four scores required to calculate the FSIQ were compared to search for specific learning disorders (one-way ANOVA and Bonferroni post-hoc test).  $p < 0.05$  indicated significance.

### 4. Immunohistology in human foetal cochlea and brain

Human fetal temporal bone (1) and brains (2) specimen were obtained from morphologically normal miscarried fetuses (WG12 for (1), WG8 and WG12 for (2)), with the informed written consent of the parents in accordance with French law. Specimen were fixed, paraffin-embedded and processed to obtain 7-8  $\mu\text{m}$  sections. Immunohistochemistry was performed using antibodies against  $\gamma$ -Tubulin (ab11316, Abcam), Myosin VI (M0691, Sigma), CDK5RAP2 (A300-554A, Bethyl). Images were acquired using an Confocal Leica SP8.

## RESULTS



## 1. Molecular findings

All *CDK5RAP2* mutations are depicted in Figure 1, Table 1 and Supplementary Table 1.

Table 1

| Family Subject                                  | 1 patient 1                        | 1 patient 2                        | 2 patient 3  | 4 patient 4                    | 4 patient 5                    | 5 patient 6  | 6 patient 7   |
|---|------------------------------------|------------------------------------|--|--------------------------------|--------------------------------|--|---|
| Gender  | M                                  | F                                  | F  | F                              | M                              | M  | F   |
| CDK5RAP2 variant                                | c.564_565dup<br>(p.Lys189Argfs*15) | c.564_565dup<br>(p.Lys189Argfs*15) | c.5495dup,<br>(p.Leu1832Phefs*3)<br>;<br>c.217-220del,<br>(p.Glu73Thrfs*4) | c.1303dup<br>(p.Thr435Asnfs*4) | c.1303dup<br>(p.Thr435Asnfs*4) | c.558_559<br>del,<br>(p.Glu186<br>Aspfs*32)<br>;<br>c.4441C>T<br>,<br>(p.Arg148<br>1*) | c.<br>3928G>T,<br>(p.Glu1310<br>*)<br>;<br>c.4546G>T,<br>(p.Glu1516<br>*) |
| Ethnic origin                                   | Tunisia                            | Tunisia                            | Serbia   | Morocco                        | Morocco                        | Italian /<br>Romanian  | Swiss/<br>Austrian  |
| Consanguinity                                   | yes                                | yes                                | yes  | yes                            | yes                            | no   | no  |
| mother HC (cm, SD)                              | 56 (+1)                            | 56 (+1)                            | 54.5 (0)   | Not available                  | Not available                  | 53 cm  | Not available   |
| father HC (cm, SD)                              | 60 (+3.5)                          | 60 (+3.5)                          | 57.5 (+1.5)  | Not available                  | Not available                  | 58.5 cm  | Not available   |
| pregnancy duration (weeks of ammenorrhea)       | 38                                 | 40                                 | 39   | 39                             | 38                             | 41   | 39  |
| prenatally diagnosed microcephaly (ultrasounds) | 3rd trimester                      | 2nd trimester                      | non reported microcephaly  | non reported microcephaly      | 3rd trimester                  | 2nd trimester  | 3rd trimester   |
| fetal cranial MRI                               | Not performed                      | Not performed                      | Not performed  | Not performed                  | Not performed                  | Not available  | Not performed   |
| Weight at birth (kg, SD)                        | 2.490 (-2)                         | 3.160 (-0.5)                       | 2.965 (-1)   | 2.260 (-2,5)                   | 1.520 (-3,5)                   | 2.900 (-1,5)   | 2.005 (-3)  |
| Length at birth (cm, SD)                        | 45 (-3.5)                          | 45 (-3)                            | 47 (-2,5)  | 45 (-3)                        | 41 (-5,5)                      | 46 (-3)  | 44 (-3)   |
| HC at birth (SD)                                | 31 (-2,2)                          | 30.5 (-2.5)                        | 30.5 (-2,5)  | 29.5 (-3)                      | 26.5 (-5)                      | 30.5 (-2,5)  | 28 (-4)   |

|   |                                       |              |             |             |   |                 |             |
|---|---------------------------------------|--------------|-------------|-------------|---|-----------------|-------------|
| age at last examination (years)           | 10                                    | 4.5          | 4.5         | 11          | 10                                      | 8               | 14,5        |
| Weight at last examination (kg, SD)       | 52.5 (+5,5)                           | 12,5 (-2)    | 19.5 (+2)   | 48.3 (+3)   | 21.5 (-2)                               | 26 (+0,5)       | 34,3 (-3)   |
| length at last examination (m, SD)        | 1.34 (0)                              | 0.94 (-2)    | 1.07 (+1)   | 1.44 (+0,5) | 1.27 (-1.5)                             | 118 (-1,5)      | 146,4 (-2)  |
| HC at last examination (cm, SD)           | 48.5 (-3,4)                           | 42 (-6,6)    | 42.5 (-6,2) | 47.5 (-4)   | 42 (-7,7)                               | 42.8 cm (-7,2)  | 43.2 (-7.3) |
| Development                               |                                       |              |             |             |   |                 |             |
| age of walking (months)                   | 10.5                                  | 16           | 14          | 11          | 18                                      | 12              | 18          |
| mastication (if delayed, age in months)   | Delayed (24)                          | Delayed (24) | Normal      | Normal      | Delayed (24)                            | Delayed (30-36) | Normal      |
| age of first comprehensive words (months) | 18                                    | 18           | 30          | 24          | 54                                      | 18              | 21          |
| age of first sentences (years)            | 4.5                                   | 2            | 4.5         | 3           | unvested, severe articulation disorders | 8               | 3.5         |
| Language articulation (normal, delayed)   | Normal                                | Normal       | Delayed     | Normal      | Delayed                                 | Delayed (30 mo) | Delayed     |
| Social interaction (normal/delayed )      | Normal                                | Normal       | Normal      | Normal      | Normal                                  | Normal          | Normal      |
| Years in kinder garden                    | 3y                                    | 0            | 1y          |             |   |                 |             |
| Elementary school                         | first year in normal class, then home |              |             |             |   |                 |             |

|   |           |     |     |                      |                      |  |                 |
|---|-----------|-----|-----|----------------------|----------------------|--|-----------------|
|   | schooling |     |     |                      |                      |  |                 |
| Seizures                                      | 0         | 0   | 0   | 0                    | 0                    | 0  | 0               |
| Neurological Findings                         |           |     |     |                      |                      |  |                 |
| Hypertonia within the first two years of life | 0         | 0   | 0   | 0                    | 0                    | yes  | yes             |
| Hypotonia within the first two years of life  | 0         | 0   | 0   | 0                    | 0                    | no   | axial hypotonia |
| pyramidal syndrome                            | 0         | 0   | 0   | 0                    | 0                    | no   | 0               |
| inferior limb spasticity                      | 0         | 0   | 0   | 0                    | 0                    | yes  | Yes             |
| Ataxia  | 0         | 0   | 0   | 0                    | 0                    | uncoordinated movements  | Yes             |
| Behaviour                                     |           |     |     |                      |                      |  |                 |
| hyperactivity                                 | yes       | yes | yes | before 7-8 years old | before 7-8 years old | Quiet behaviour, but hyperactivity episodes (but repetitive movements) | no              |
| Tic disorders                                 | no        | no  | no  | no                   | 0                    |  | no              |
| agressiveness                                 | no        | no  | no  | no                   | 0                    | episodes of aggressiveness   | no              |

|   |   |   |  |   |   |  |                         |
|---|---|---|--|---|---|--|-------------------------|
| Intellectual abilities                        |   |   |  |   |   |  |                         |
| total IQ                                      | 62  | 67  |  | 40  | 56  | 52   | 46                      |
| sensorineural anomalies                       |   |   |  |   |   |  |                         |
| congenital hearing loss (uni/bilateral)       | no  | no  | no   | no  | no  | 0  | no                      |
| progressive hearing loss (uni/bilateral), age | bilateral, 9y   | no  | Unilateral, 7y                                   | no  | bilateral, 7y   | Bilateral, 10 y  | no                      |
| hearing (auditory threshold)                  | Right: severe deafness (70-80 dB)<br>Left: moderate deafness (20-50 dB) | normal Oto-acoustic emissions within the first months of life | Right : severe deafness (70dB),<br>Left : normal | Right: normal (15-20 dB), Left: normal (15-25 dB) | Right: severe deafness (80-90 dB), Left: severe deafness (60-70 dB) | Right: severe deafness (60-80 dB)<br>Left: moderate to severe deafness (40-60/60-80 dB)<br>bilateral | Right and left : normal |
| hearing aid (uni/bilateral)                   | unilateral  | no  | unilateral                                       | no  | bilateral   |  | no                      |
| retinal hypopigmentation (uni/bilateral)      | bilateral   | bilateral   | bilateral  | bilateral   | bilateral   | ?  | yes                     |
| retinal hyperpigmentation (uni/bilateral)     | bilateral   | bilateral   | bilateral  | bilateral   | bilateral   | ?  | no                      |
| ERG   | normal  | normal  | normal   | normal  | normal  | Not done   | normal                  |
| skin anomalies                                |   |   |  |   |   |  |                         |

|                           |     |     |     |     |     |   |     |
|---------------------------|-----|-----|-----|-----|-----|---|-----|
| area of hypopigmentation  | yes | yes | yes | no  | no  | no  | yes |
| area of hyperpigmentation | yes | yes | no  | yes | yes | yes<br>(Several café-au-lait spots on trunk, arms and legs) | yes |

CC: corpus callosum, dB: decibel, ERG: electroretinogramm, IQ: intellectual quotient, HC: head circumference, SD: standard deviation.

Homozygosity mapping performed by SNP array analysis revealed a common homozygous locus including *CDK5RAP2* in patients 1 and 2 and subsequent Sanger sequencing of *CDK5RAP2* revealed a homozygous frameshift duplication of 2bp in exon 7, leading to a premature stop codon (c.564\_565dup, [p.(Lys189Argfs\*15)] [GenBank: NM\_018249.5]; Chr9(GRCh37):g.123,298,747\_123,298,748dup). Both parents were heterozygous carriers. Gene panel analysis was performed in patients 3 to 5. Patient 3 was found to carry “assumed compound-heterozygous” *CDK5RAP2* variants, as the parents were not available for testing: one 4bp deletion in exon 4 (c.217\_220del, [p.(Glu73Thrfs\*4)] [GenBank:NM\_018249.5]; Chr9(GRCh37):g.123313156\_123313159del) and a 1bp duplication in exon 36 (c.5495dup, [p.(Leu1832Phefs\*3)] [GenBank:NM\_018249.5]; Chr9(GRCh37):g.123156873dup). These 2 variants are predicted to induce a frameshift leading to null alleles. Patients 4 and 5 displayed a homozygous pathogenic mutation in exon 12. This single nucleotide duplication creates a premature stop codon 4 codons downstream (c.1303dup, [p.(Thr435Asnfs\*4)] [GenBank: NM\_018249.5]; Chr9(GRCh37):g.123280713dup) predicting a null allele. The father was heterozygous carrier (no sample available from the mother).

None of the mutations found in patients 1 to 3 are present in public databases (gnomAD, dbSNP, 1000 Genomes, the NHLBI Exome Variant Server (EVS) and the Exome Aggregation Consortium (ExAC) browser). The variant identified in patients 4 and 5 has been reported only once, in the Latino population of the gnomAD database, and has never been reported in the homozygous state.

Patients 6 and 7 were found to be compound heterozygous for truncating variants by trio whole-exome sequencing as recently described [7]. Each patient carried one unreported variant and one with a very low minor allele frequency, both heterozygous, in the ExAC/gnomAD databases. Patient 6 harbored NM\_018249.5(*CDK5RAP2*):c.[558\_559delGA]+[c.4441C>T], p.[(Glu186Aspfs\*32)]+[(Arg1481\*)], Chr9(GRCh37):g.[123298753\_123298754del];[123171568C>T] with the mother heterozygously carrying c.[558\_559delGA]+[=], p.[(Glu186Aspfs\*32)]+[(=)] (unreported) and the father heterozygously carrying c.[c.4441C>T]+[=], p.[(Arg1481\*)]+[(=)] (rs587783390; ExAC: A=0.00005660 /4, no homozygotes). Patient 7 harbored NM\_018249.5(*CDK5RAP2*):c.[3928G>T];[4546G>T] p.[(Glu1310\*)];[(Glu1516\*)] Chr9(GRCh37):g.[123199600C>A];[123171463C>A] with the mother heterozygously carrying c.[4546G>T];[=] p.[(Glu1516\*)];[(=)] (rs374351172; ExAC: A=0.00002472/3, no homozygotes, gnomAD: A=0.00002844/7, no homozygotes) and the father heterozygously carrying c.[3928G>T];[=] p.[(Glu1310\*)];[(=)] (unreported).

## 2. Clinical characteristics

Clinical details are summarized in Table 1. Microcephaly was confirmed at birth in all cases and was even detectable during the 2<sup>nd</sup> or 3<sup>rd</sup> trimester of pregnancy in 5/7 patients before reaching a mean OFC of -6 +/- 1.7 SD during infancy. Nevertheless, an unusual improvement of the OFC after age 2 for patients 1, 2 and 4 was noted (Supplementary Figure 1). None of the patients had short stature beyond age 5. Areas of hypo- or hyperpigmentation of the skin were systematically found in all patients. Motor development was also normal. Suspected around age 3 because of speech delays, the main functional consequence of brain volume reduction and simplified gyri, both obvious on brain MRI (Supplementary Figure 2), was ID, ranging from mild to moderate (Full Scale IQ [FSIQ] from 40 [patient 5] to 67 [patient 1], see Supplementary Figure 3). We cannot exclude the possibility that patient 5, with moderate ID may have a second hit related to the context of consanguinity. However,

in all patients, a relative preservation of verbal comprehension (VCI 78 and 75 for patients 1 and 2) and visuospatial abilities (71 and 75 for patients 2, 3 and 6) as well as a preservation of mental manipulation was detected (78 and 73 for patients 1 and 4). None of the patients exhibited epilepsy.

### 3. Developmental hypothalamic defect

Patients 1, 2, 4, 5 and 7 displayed interhypothalamic adhesion (IHA), a grey matter band that connects the dorsomedial and ventromedial hypothalamic nuclei and/or arcuate, paraventricular or preoptic nuclei on both sides of the third ventricle [36, 37] (Figure 2). IHA differs from hamartoma as it doesn't extend into the third ventricle. For patient 6, brain MRI was performed with another protocol including a series of 5mm slices without T1-weighted 3D sequence, which did not allow to formally identify IHA. IHA was already noticed on the brain MRI performed at 1 year of age in patient 1, and was not enhanced after gadolinium injection, as classically described for glioma, another differential diagnosis of IHA. Taken together, these observations suggest that IHA, which is present at least in 5/7 patients, may be caused by a failure of the separation of hypothalamic nuclei at early developmental stages.

### 4. Neurosensory impairment (Tables 1-2, Figures 3-4)

#### ***Retinal pigmentation defects:***

Ophthalmological assessment showed normal bio-microscopy and no oculomotor disorder. The axial length was below the normal range in all patients (average: 20.9, from 20.3 to 22.3 mm; normal age for age > 3 years old: 23.5 mm).

In all patients, examination of the ocular fundus revealed round or oval regions of focal hyper- or hypopigmentation scattered in the periphery and all along the vascular arcade. Hyperpigmented lesions consisted of either extended dark grey lesions or small dark lesions scattered in the macular zone and in the retinal periphery (Figure 3A-B). Small round spots of hypopigmentation were either located in the macula (Figure 3B) or scattered in the periphery (Figure 3A-B). All hyperpigmented lesions were hypo-autofluorescent (Figure 3C-D), while hypopigmented spots were hyper-autofluorescent (Figure 3C-D). OCT was performed and found normal in patients 1 and 4.

All patients being hyperopic, best-corrected visual acuity was 20/20 except in the youngest patient (patient 3), aged 4 and non-cooperative, whose visual acuity was superior to 20/40 in each eye. Scotopic and photopic flash ERGs as well as the EOG and pERG were similar to age-matched controls (data not shown).

#### ***Progressive hearing loss associated with congenital inner ear malformation:***

Patients 1, 3, 5 and 6 developed progressive uni- or bilateral hearing loss, respectively diagnosed at age 9, 6, 7 and 10 (Table 1). Audiometric evaluations at diagnosis showed severe hearing loss from 70 to 80 Hz in at least one ear for all four patients. Patient 1 had a unilateral scotoma of the left ear at 6000 Hz at 60dB, and a 77.5 dB threshold in the right ear (Figure 4 B-C). Close to normal hearing levels were restored with conventional hearing aids. However, patient 5 did not receive early intervention/rehabilitation. This delayed medical support is likely to have increased the receptive and expressive speech impairment caused by PM itself and worsened ID. Surprisingly, although they had been stable for at least 4 years, patient 1 has recently undergone an improvement of his auditory thresholds, which is now at 42dB in the right ear and 26dB in the left ear on average, with a concordant vocal threshold.



As sensorineural hearing loss (SNHL) is very unusual in patients with PM, a temporal bone CT scan was performed for all patients except patient 6 (parental rejection), and revealed the same cochlear malformation in each case: small cochlea compared to normal values on CT [32] and an absence of the interscalar septa at the apical turn (Figure 4A and Table 2), i.e. compatible with the diagnosis of incomplete partition type II of the Sennaroglu classification [33]. In addition, patient 2 displayed an enlarged vestibular aqueduct associated with this cochlear dysplasia. In all patients, the diameter of the bony cochlear nerve canal was reduced ( $<1.7$  mm), in favor of a cochlear nerve hypoplasia [32].

Table 2

| Neuroradiological findings  | Patient 1<br>10 y                           | Patient 2<br>4.5 y  | Patient 3<br>4.5 y                          | Patient 4<br>11.5 y                         | Patient 5<br>10 y                           | Patient 6<br>5 y                         | Patient 7<br>13.75 y                        |
|-----------------------------|---|---|---|---|---|--|---|
| Corpus callosum             | Raw / 3rd percentile                        | Raw / 3rd percentile  | Raw / 3rd percentile                        | Raw / 3rd percentile                        | Raw / 3rd percentile                        | Raw / 3rd percentile                     | Raw / 3rd percentile                        |
| APD (mm)                    | <b>45.7</b> (59.1)                          | <b>47.91</b> (55.5)   | <b>42.43</b> (55.5)                         | <b>55.86</b> (59.7)                         | <b>49.06</b> (59.1)                         | <b>30.09</b> (56.2)                      | <b>29.39</b> (61.7)                         |
| FOD (mm)                    | <b>121.6</b> (148.6)                        | <b>113.2</b> (140.5)  | <b>113</b> (140.5)                          | <b>130.1</b> (145)                          | <b>114.5</b> (148.6)                        | <b>113</b> (142.3)                       | <b>125</b> (147.5)                          |
| APD/FOD                     | <b>0.37</b> (0.37)                          | 0.42 (0.36)   | 0.37 (0.36)                                 | 0.42 (0.37)                                 | 0.42 (0.37)                                 | <b>0.27</b> (0.36)                       | <b>0.23</b> (0.38)                          |
| GT (mm)                     | 9.48 (7.3)                                  | 7.25 (6.7)  | 7.36 (6.7)                                  | 8.72 (7.4)                                  | 7.96 (7.3)                                  | <b>6.2</b> (6.9)                         | <b>7.22</b> (7.6)                           |
| BT (mm)                     | 7.42 (4)                                    | 5.39 (3.5)  | 6.93 (3.5)                                  | 5.46 (4)                                    | 6.42 (4)                                    | 7.8 (3.7)                                | 5.23 (3.9)                                  |
| IT (mm)                     | 7.3 (2.4)                                   | 4.82 (1.9)  | 6.02 (1.9)                                  | 6.14 (2.4)                                  | 6.32 (2.4)                                  | 6 (2)                                    | 4.6 (2.5)                                   |
| ST (mm)                     | 9.09 (7.7)                                  | 10.02 (6.7)   | 8.11 (6.7)                                  | 11.17 (7.7)                                 | 10.13 (7.7)                                 | <b>3.9</b> (6.9)                         | <b>0</b> (7.1)                              |
| Gyrations                   | simplified                                  | simplified  | simplified                                  | simplified                                  | simplified                                  | simplified                               | simplified                                  |
| Interhypothalamic adhesions | yes   | yes   | no  | yes   | yes   | ?<br>Midline sagittal sections<br>> 5 mm | yes   |
| Internal ear                | type II incomplete partition of the cochlea | type II incomplete partition of the cochlea<br>Enlarged right vestibular aqueduct | type II incomplete partition of the cochlea | type II incomplete partition of the cochlea | type II incomplete partition of the cochlea | Not performed                            | type II incomplete partition of the cochlea |

APD: anteroposterior diameter of the corpus callosum, FOD \_ fronto-occipital diameter, GT \_ genu thickness, BT: body thickness, IT: isthmus thickness, ST: splenium thickness (according to Garel et al. AJNR, 2011).

### ***CDK5RAP2 detection in the fetal cochlear epithelium***

The CDK5RAP2 protein is known to be expressed in the VZ of both mouse and human telencephalon throughout neurogenesis [16]. However, its presence in the inner ear has not been documented so far. Using immunohistochemistry for CDK5RAP2 on human fetal tissues, we found that it is expressed in the developing cochlea in addition to the fetal brain. Co-labeling of CDK5RAP2 with  $\gamma$ -TUBULIN on human fetal brain sections at early (WG8) and mild (WG12) stages confirmed that CDK5RAP2 is highly expressed at the centrosome of the apical radial glial cells (aRGCs) in the VZ (Supplementary Figure 4). Immunolabeling on human fetal cochlea at WG12, showed that CDK5RAP2 is expressed in MYOSIN VI (a protein expressed in the cytoplasm of outer hair cells of the organ of Corti) positive cells (Figure 4 F). At the apical pole of these outer hair cells, CDK5RAP2 was also co-expressed with  $\gamma$ -TUBULIN (Figure 4 G) at the centrosome, concordant with its expression within the VZ. Thus CDK5RAP2 is expressed early at the centrosome in the sensory epithelium of the organ of Corti as in the neuroepithelium.

## **DISCUSSION**

In this study, we identify neurosensory impairments associated with the CDK5RAP2 microcephaly, including retinal pigmentation defects and a specific cochlear malformation responsible for progressive sensorineural hearing loss. All patients examined with our protocol displayed IHA, suggesting a failure in hypothalamic nuclei separation. CDK5RAP2 is thus crucial for neurosensory development in addition to brain development.

### **CDK5RAP2 and brain development**

In agreement with the congenital defects observed, CDK5RAP2 is highly expressed during neurocorticogenesis. Although the protein has been detected from WG18 onward [16], we find that CDK5RAP2 is present as early as WG8 in the VZ of the human fetal cortex, at the centrosome of aRGCs. This highlights CDK5RAP2 requirement for early progenitors' division and may explain why microcephaly is often detected antenatally. Similarly, aRGCs switch from symmetric (proliferative) to asymmetric (neurogenic) division in *Cdk5rap2-deficient* mice, which results premature cell cycle exit, increased cell death, and a reduced number of neurons [15].

Interestingly, OFC suddenly increased after age 2 in 3 patients (Supplementary Figure 1), a phenomenon not reported previously in PM. The partial compensation observed could result from postnatal white matter development, myelination and/or synaptogenesis [34, 35]. Brain MRI early follow-up, including T2 weighted images and tractography should therefore be envisioned to assess myelination and synaptogenesis in CDK5RAP2 patients. Despite the reduction in neuron number, it is possible that neurons that have reached maturity manage developing a fairly efficient network, a situation that would be consistent with the mild ID observed, unlike ASPM patients who have more pronounced ID [30]. In agreement with this, patients whose OFC increases after age 2 also showed a higher IQ. Larger cohorts would greatly help validating this hypothesis.

IHA was detected in 5 patients and may be similar to a forme fruste of holoprosencephaly (HPE), which is restricted to the ventral part of the diencephalon and characterized by the non-separation of hypothalamic nuclei along the midline without fusion of the thalami. IHA is often associated with

other CNS midline defects including corpus callosum dysgenesis, as seen in patients 6 and 7, or other defects not observed in our patients such as an absence of the septum pellucidum, optic pathway hypoplasia, hippocampal dysgenesis [36, 37]. However, IHA may also occur as an isolated asymptomatic feature [36]. At their last examination, the children of our series did not exhibit any obvious endocrine disorder attributable to the hypothalamic defect: growth was within the normal range for weight and height; no diabetes insipidus or high blood pressure was noted. Although no micropenis was detected, tracking puberty onset would be relevant. Interestingly, patients 1 and 4 showed excessive weight gain (after age 8 and 9, respectively). During the 2<sup>nd</sup> decade of life, we propose to assess energy metabolism in these patients.

PM and HPE have already been associated in patients carrying variants in the MCPH7 gene *STIL* [41]. These patients exhibit a mild lobar form of HPE and PM with gyral simplification and interhemispheric cysts. This suggests that, as for *STIL*, *CDK5RAP2* can be added to the list of genes causing a forme fruste of HPE in humans.

### **CDK5RAP2 and ear development**

We show here that *CDK5RAP2* loss of function also affects the development of the spiral organ of Corti. Malformations of the inner ear are found in 30-40% of children with sensorineural hearing loss (SNHL) on imaging (CT, MRI) [43–46]. Incomplete partition of the cochlea type II [33] refers to a cochlea that has a normal basal turn but a cystic apex with hypoplasia of the modiolus and absence of the interscalar septum. This malformation was obvious in all examined patients. Patient 2 also showed a dilated vestibule and vestibular aqueduct enlargement (Mondini dysplasia). Incomplete partition of the cochlea type II is a congenital cause of sensorineural hearing loss. This cochlear malformation typically occurs during WG7 [47]. Hearing loss may be mild to profound and must be evaluated with systematic audiometry. As *CDK5RAP2* variants described in the present study have not been reported previously, one cannot rule out the possibility that the associated neurosensory impairments are variant-specific. However, hearing loss have been occasionally mentioned in previous *CDK5RAP2* patients carrying other variants [19, 26] and our systematic study suggests that the neurosensory defects are constitutive of the *CDK5RAP2* phenotype and may have been overlooked previously.

The most common etiology of Mondini dysplasia is a variation in the *SLC26A4* gene involved in Pendred syndrome [48] and in a non-syndromic hearing loss [49], but variants in more than 100 genes cause severe congenital or progressive hearing loss [50]. Genes involved in genetic SNHL encode proteins playing a role in gene regulation, fluid homeostasis, synaptic transmission and hair cell and neuronal maturation, but also in stereocilia function [50]. To date, no interaction between such proteins and *CDK5RAP2* has been reported. In humans, the cochlea develops between WG6-7 and 10-12, and the vestibule between WG9 and 13 [51], i.e. at the beginning of neurogenesis. It has been shown that the brain volume reduction related to *Cdk5rap2*-deficient mice is due to reduced neural progenitor proliferation, cell death and early neuronal differentiation during neurogenesis [15]. It is thus possible that the observed simplification of the cochlear spiral also reflects defective proliferation or cell death leading to a depletion of the pool of progenitors and the early differentiation of the cochlear epithelium. Given that hearing loss was not congenital and was identified when the youngest patient was 6 years old, we recommend that audiograms be performed systematically at the time of diagnosis, and possibly, repeated on a yearly basis. Neurosensory

management of these patients is crucial as it considerably impacts communication, learning and integration abilities.

### **CDK5RAP2 and eye development**

PM is not commonly associated with microphthalmia except in specific syndromes such as Warburg-Micro syndrome (#600118, *RAB3GAP1*, #614225, *RAB3GAP2*, #614222, *RAB18*, #615623, *TBC1D20*), microcephaly and chorioretinopathy type 3 (#616335, *TUBGCP4*, [52]) or type 6 (#251270, *TUBGCP6*, [53]) or microcephaly with or without chorioretinopathy, lymphedema and mental retardation (#152950, *KIF11*, [54]). In the present study, we have shown that microphthalmia is a cardinal feature of the CDK5RAP2-associated phenotype. More surprising were the retinal pigmentation changes found in all the patients. Retinal hyperpigmentation could be related to skin hyperpigmentation spots and is probably developmental due to migration defects in melanocytes. Hypopigmented spots that are hyper-autofluorescent are reminiscent of lipofuscin deposits/accumulation in the Retinal Pigmentary Epithelium (RPE). No functional consequences on visual acuity or ERG were noticed. OCT was normal for both patients tested, suggesting that lipofuscin accumulation does not alter the RPE-photoreceptor layer. Our patients still being children, their ophthalmological status obviously needs to be reassessed to check whether lipofuscin deposits are stable or increase with time and/or impact visual function. These retinal pigmentation changes do not resemble the well-characterized chorioretinopathies associated with microcephaly such as those caused by *PLK4*, *TUBGCP6* (#251270, [53]), *TUBGC4* (#616335, [52]), or *KIF11* (#152950, [54]) variants. We propose that this new entity, which resembles that described by Abdel-Saam et al. prior to gene identification [55], may be related to CDK5RAP2 variants, because it is present in all patients of the present series.

### **CONCLUSION**

Overall, our findings extend the phenotype associated with CDK5RAP2 microcephaly and indicate that MCPH3 is distinct from other MCPH by its association with neurosensory impairments. These results shed new light on the role of CDK5RAP2 in brain, ear and eye development and maturation.

### **CONTRIBUTORSHIP STATEMENT**

HN analysed and interpreted all clinical data. LV and NT analysed the ocular and ENT phenotypes respectively for all patients with the help of MM. ME analysed brain MRI and temporal bone CT-scan for all patients. HN, LV, NT and ME helped writing the manuscript. AE performed the neuropsychological assessment for all patients. KS, AA, MLM, DH, YA and MZ were involved in patient recruitment and clinical management. SGC and FK conducted the research protocol. PL and FG performed the immunostaining in human tissues. VEG, AV and PG revised and edited the manuscript. SD and AR analysed and interpreted NGS data and performed segregation analysis. SP conceived the study, interpreted the results and wrote the manuscript.

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#### COMPETING INTERESTS

The authors declare no competing interests.

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#### DATA AVAILABILITY STATEMENT

Data used in the manuscript are available by request to the authors.

#### LICENCE STATEMENT

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## LEGENDS of FIGURES

Figure 1: Novel and previously described *CDK5RAP2* variants.

A: Pedigree of seven individuals from European (families 2, 4 and 5) or North of Africa's families (families 1 and 3)

B: Scheme representing novel variants in our series and the known variants in the human *CDK5RAP2* gene and corresponding domain of the protein. The variants reported previously are shown above the horizontal line; novel variants from the present study are indicated below this line and just above the exons. Various symbols represent missense, nonsense, splice, and frameshift variants as indicated. Abbreviations:  $\gamma$ Turc (gamma-tubulin ring complex); CM1 (centrosomin (cnn) motif 1); SMC (structural maintenance of chromosomes protein).

Figure 2: Developmental hypothalamic defects in patients with *CDK5RAP2* variants.

Coronal (A, C) and Sagittal (B, D) T1-weight images showing in patients 1, 2, 4, 5 and 7 a structure iso-intense to grey matter consistent with an inter-hypothalamic adhesion (IHA, white arrows), and absent in the healthy control. This grey matter band on coronal view and nodular on sagittal view is located at the level of the upper midbrain, closed to the anterior/inferior third ventricle. IHA is known to connect both dorso-medial and ventromedial hypothalamic nuclei and/or arcuate, paraventricular or pre-optic nuclei on both sides of the third ventricle.

Figure 3: Retinal pigmentary changes in patients with *CDK5RAP2* variants.

A-B: Ocular fundus photography of patients 1 and 4 showing focal regions of hyper- and hypopigmentation in the retinal pigment epithelium (RPE). Note that hyper-pigmented lesions appeared as small dark spots (patient 1, white arrows) or dark grey extended lesions (patient 4, black arrow) and were located in the macular zone and/or in the retinal periphery. Small round spots of hypopigmentation were scattered in the posterior pole (yellow arrows in patients 1 and 4).

C-D: Ocular fundus autofluorescence showing that hyper-pigmented lesions appear as well-defined patches of hypo-auto-fluorescence (Figure 3C-D, white and black arrows), whereas hypo-pigmented spots appear as areas of hyper-fluorescence, reminiscent of lipofuscin deposits/accumulation in the RPE in both patients (yellow arrows).

Figure 4: Congenital inner ear malformation responsible for progressive deafness in patients with *CDK5RAP2* variants.

A: Temporal bone coronal CT scan images in patient 1 and 5 showing small cochlea with no visibility of the interscalar septa at the apical turn of the cochlea and a confluence of middle and apical turns of the cochlea, evocative of an incomplete partition type II according to Sennaroglu classification.

B-C: Tonal and vocal audiograms of patients 1 (B) and 5 (C). Circles and X symbolize joint air and bone-conducted hearing thresholds respectively for right and left ears.

B: On the right ear, the ascending curve corresponds to a severe hearing loss whereas the left ear demonstrates a normal hearing threshold for conversational frequencies associated with a scotoma at 6000 Hz.

C: The mean hearing threshold of 80dB on the right ear and 70dB on the left ear indicate a bilateral hearing loss.

D-E: Horizontal section of temporal bone (D) of a control fetus at WG12 stained by Hematoxylin and Eosin and high magnification on the cochlea (E) highlighting the structure of a normal fetal cochlea. RM = Reissler's membrane, OC = Organ of Corti, SV = Stria vascularis. Scale bars: 25 mm (D), 500µm (E).

F-G: Immuno-histochemistry in the adjacent section of the cochlea of the control fetus at WG12 shown in D; RM = Reissler's membrane, OC = Organ of Corti, SV = Stria vascularis. White squares indicate regions of the cochlea that are shown in higher magnification in the right part of the panel. F: Co-labeling of CDK5RAP2 (red) and MYOSIN VI (green, that stains the outer hair cells of the cochlea) and DAPI (blue) showing that CDK5RAP2 is expressed in MYOSIN VI positive cells at the apical pole of outer hair cells. G: Co-labeling of CDK5RAP2 (red) and GAMMA TUBULIN (green, that stains the pericentriolar material at the centrosome) demonstrating that CDK5RAP2 is localized at the centrosome of the outer hair cells of the cochlea. Scale bars: 90µm (first left panel), 15µm (second left panel), 15µm (right panel)

Supplementary Figure 1: Clinical characteristics of patients with *CDK5RAP2* variants.

A. Morphological features of patients with *CDK5RAP2* variants. No dysmorphic feature has been noticed except a retrognathism in all patients and a sloping forehead in patients 4 and 5.

B. OFC curve of patients with *CDK5RAP2* variants. Microcephaly was obvious at birth for all patients. Head circumference at last examination ranged from -3 SD to -7.2 SD (mean -6 SD). OFC curve slowed down with age with an increase of the z-score for all patients prior age 2, whereas a noteworthy and unusual ascent of OFC after age 2 was noticed in patients 1, 2 and 4.

Supplementary Figure 2: Neuroimaging characteristics of patients with *CDK5RAP2* variants.

Axial (A), Sagittal (B) and coronal (C) T1-weight images showing the typical MRI features of patients with *CDK5RAP2* variants, with a reduced fronto-occipital diameter (FOD) largely under the 3<sup>rd</sup> percentile for age (see also Table 2). Gyration appeared simplified as the number of second and/or third gyri were reduced and the sulci were shallow. Note that Patient 6 and 7 had a posterior agenesis of the CC. The CC was short for the other patients if only referring to the measurement of the antero-posterior diameter of the CC (APD). Nevertheless, when it is reported to the FOD, the APD/FOD ratio was within the normal range (see Table 2), suggesting that the CC is not short taking the FOD into account. No abnormal signal within the periventricular or the subcortical white matter on T2-weight images was detected (D).

Supplementary Figure 3: Cognitive abilities of patients with *CDK5RAP2* variants.

Cognitive profile of patients carrying *CDK5RAP2* variants according to Wechsler scales.

Six patients out of seven were able to perform all the subtests of Wechsler scales (WISC IV, n = 6: patients 1, 4, 5, 6 and 7, WPPSI III, n = 2: patients 2 and 3). Patients 3 and 6 couldn't be assessed totally, especially regarding Verbal Comprehension Index (VCI) and (POI) because she did not speak our language.



According to the 10th Revision of the Internal Classification of Disease (ICD-10) and to the fourth revision of the American Psychiatric Association's Diagnostic and Statistical Manual (DSM-IV) classification, five patients obtained a full scale intellectual quotient (FSIQ) within the mild intellectual disability (ID) zone ( $50 < \text{FSIQ} < 70$ , light blue rectangle) and one patient had a FSIQ in the moderate ID zone ( $\text{FSIQ} = 40$ , dark blue rectangle; see also Table 1). For the latter, we cannot exclude a second hit responsible for lower scores. Scores were homogeneous whatever the domain under evaluation without any significant differences between scales (one way ANOVA,  $n = 7$ , ns). Except for patient 5, all patients obtained a score in the lower range or in the mild ID zone for VCI ( $57 < \text{VCI} < 78$ ,  $n=4$ ), POI ( $52 < \text{POI} < 79$ ,  $n= 7$ ) and PSI ( $50 < \text{PSI} < 78$ ,  $n= 5$ ). WMI scale was the most dropped index among school-aged patients ( $50 < \text{WMI} < 60$ ,  $n = 4$ ), without significant differences with other scales (one way ANOVA,  $n = 7$ , ns).

Supplementary Figure 4: The CDK5RAP2 protein is expressed and located at the centrosome of the apical radial glial cells in the VZ at early and mild neurogenesis

WG8 (A-C) and WG12 (D-F) sections from control cortices stained for GAMMA TUBULIN (green) and CDK5RAP2 (red) and DAPI (blue); inserts show high magnification of A in the lower panels (B-C and E-F) and showed that CDK5RAP2 co-localized with GAMMA TUBULIN at the centrosome of both mitotic apical radial glial cells (aRGc) and non-dividing (aRGc) in the VZ at early (WG8) and mild (WG12) neurogenesis. CDK5RAP2 was also expressed within the nucleus of (aRGc), although to a lesser extent. VZ: ventricular zone, SVZ: sub ventricular zone. Scale bars: 90  $\mu\text{m}$  (A, D), 5 $\mu\text{m}$  (B, C, F), 10 $\mu\text{m}$  (E).

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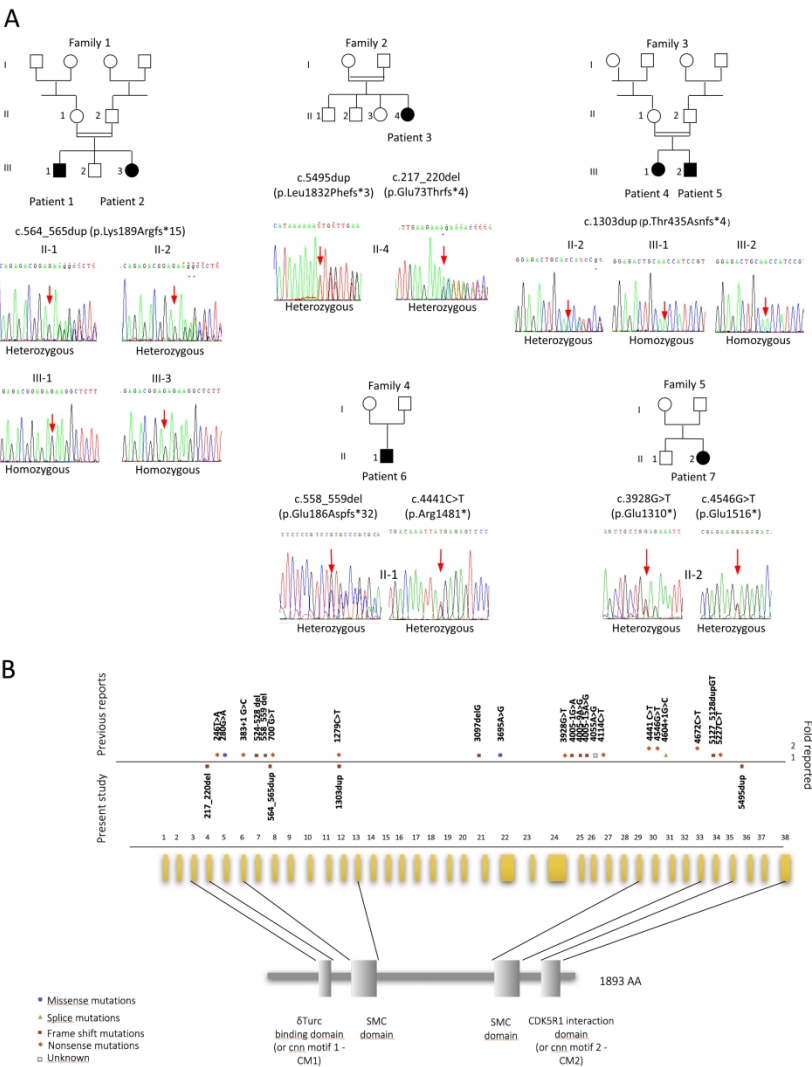


Figure 1

Figure 1

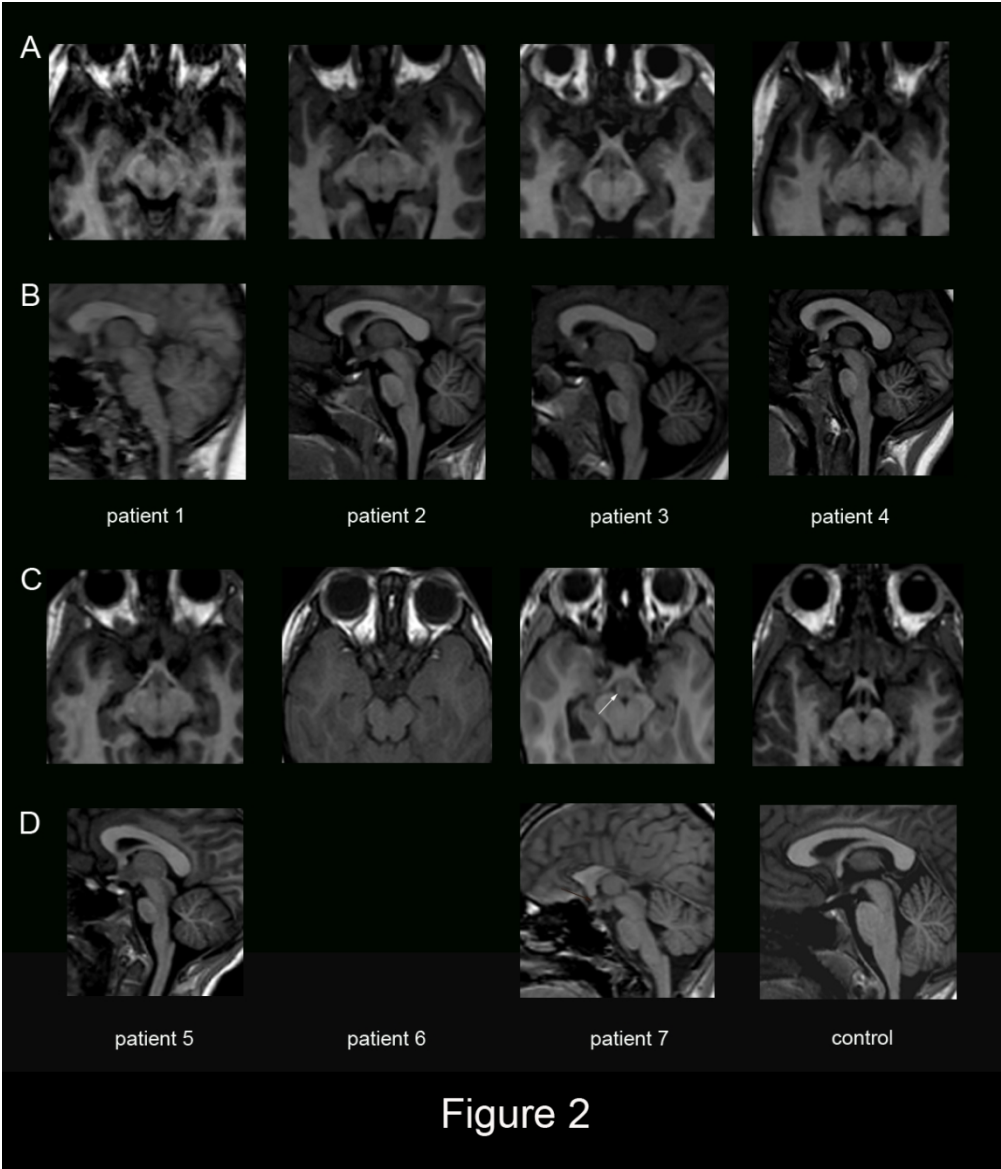


Figure 2

Figure 2  
102x119mm (300 x 300 DPI)



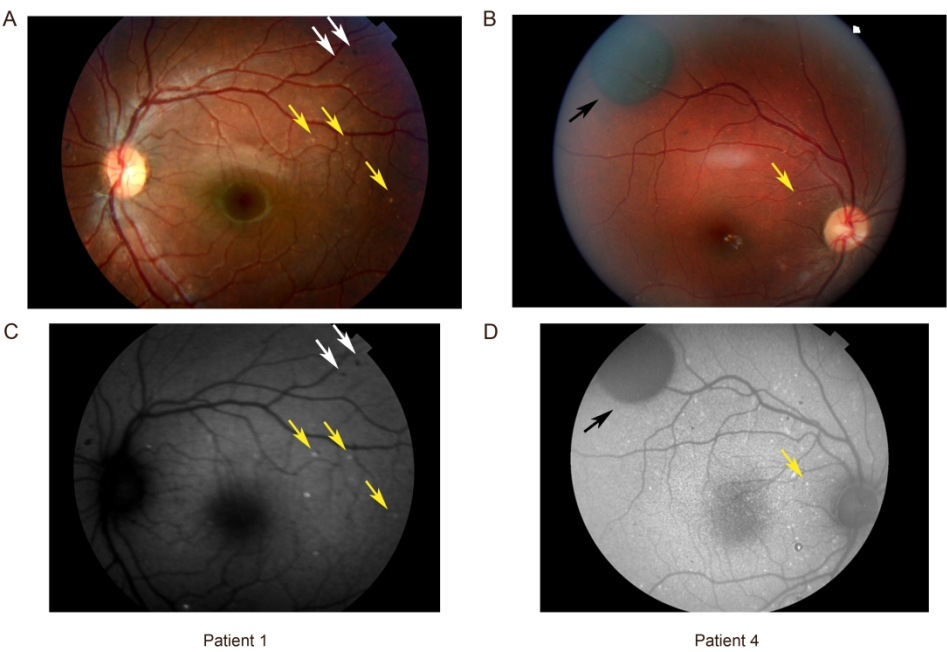


Figure 3

Figure 3

299x237mm (300 x 300 DPI)

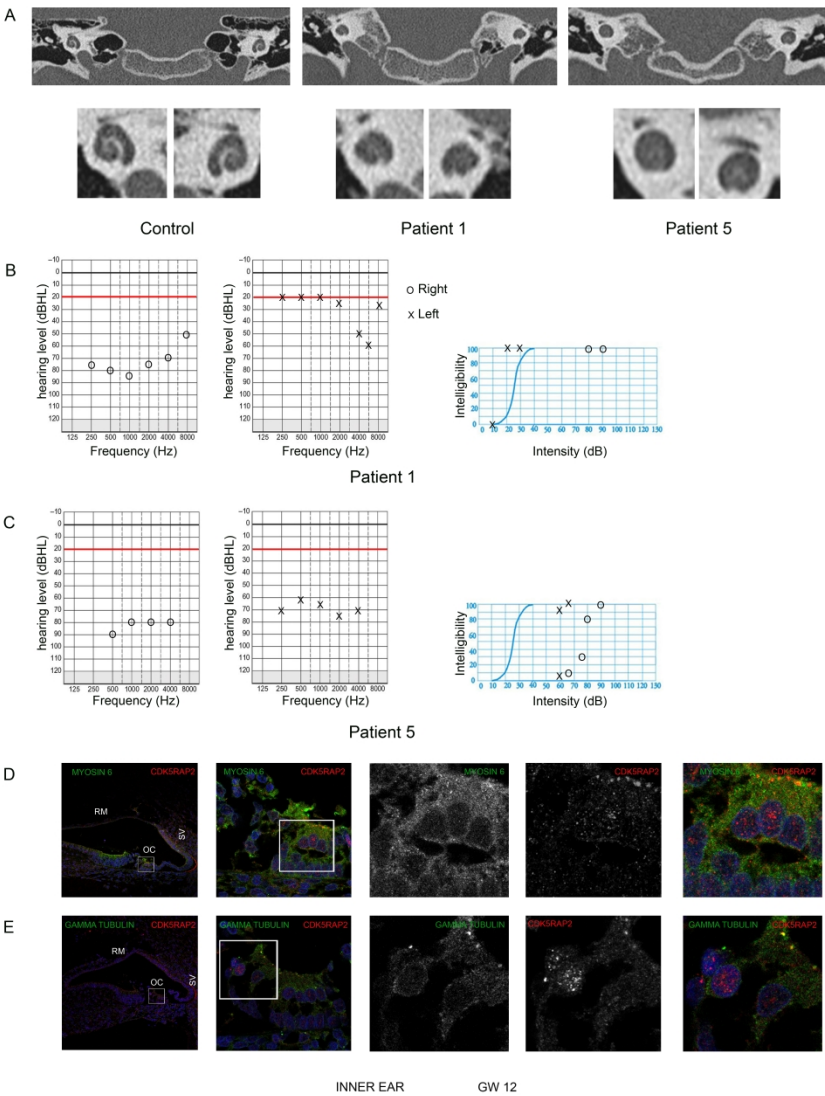
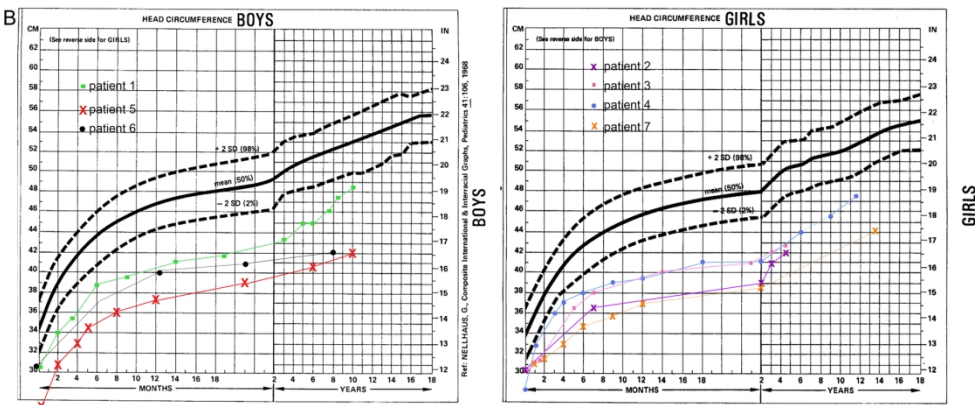


Figure 4

Figure 4

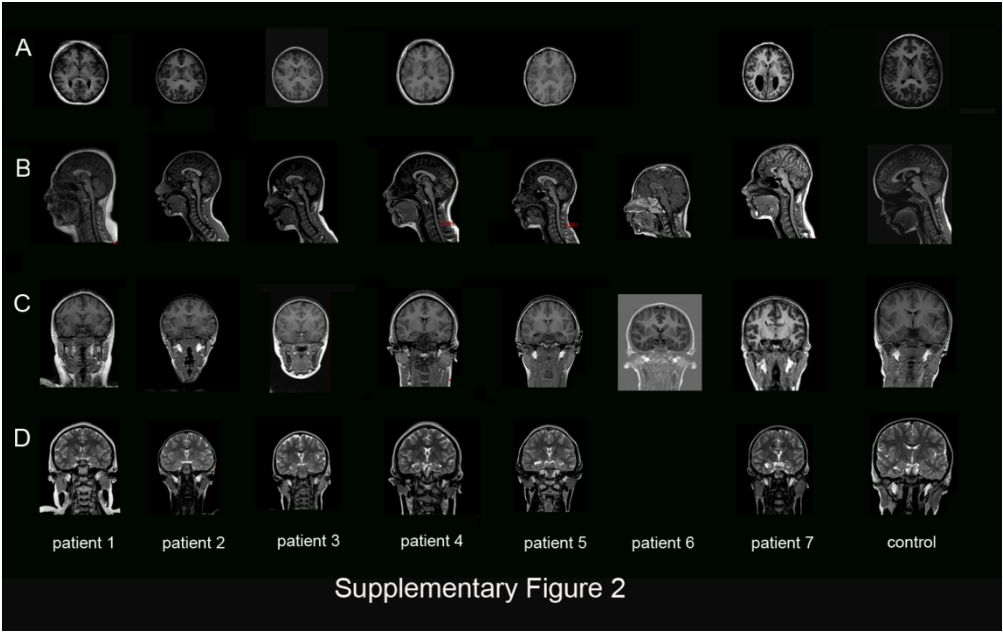
439x603mm (300 x 300 DPI)

| PATIENTS    | REFERENCE                               | FAMILY | ORIGIN       | CONSAGUINITY | AGE AT DIAGNOSIS (years) | Mutation 1                             | Mutation 2                             | PRENATAL FEATURES                | SEX | OFC AT BIRTH (SD) | WEIGHT AT BIRTH (SD) | HEIGHT AT BIRTH (SD) | AGE AT TIME OF EXAMINATION (years) | OFC AT TIME OF EXAMINATION (DS) | WEIGHT AT TIME OF EXAMINATION (DS) | HEIGHT AT TIME OF EXAMINATION (DS) | BRAIN IMAGING   | NEURODEVELOPMENTAL OUTCOME                   | EPILEPSY   | OTHER SYMPTOMS  |
|-------------|---|--------|--------------|--------------|--------------------------|--|--|----------------------------------|-----|-------------------|----------------------|----------------------|------------------------------------|---------------------------------|------------------------------------|------------------------------------|---|--|--|---|
| Patient #1  | Moynihan, 2000/ Bond, 2005              | #1     | Pakistan     | Yes          | Not Provided             | c.246T>A (p.Ser82*)                    | c.246T>A (p.Ser82*)                    | Not Provided                     | M   | Not Provided      | Not Provided         | Not Provided         | Not Provided                       | -6                              | Not Provided                       | Not Provided                       | Not performed   | Mild intellectual disability                 | No   | No  |
| Patient #2  | Moynihan, 2000/ Bond, 2005              | #1     | Pakistan     | Yes          | Not Provided             | c.246T>A (p.Ser82*)                    | c.246T>A (p.Ser82*)                    | Not Provided                     | F   | Not Provided      | Not Provided         | Not Provided         | Not Provided                       | -8                              | 0,0                                | 0,0                                | Not performed   | Moderate intellectual disability             | Yes - rare tonic/clonic seizures                   | Profound congenital neurosensory deafness   |
| Patient #3  | Moynihan, 2000/ Bond, 2005              | #1     | Pakistan     | Yes          | Not Provided             | c.246T>A (p.Ser82*)                    | c.246T>A (p.Ser82*)                    | Not Provided                     | F   | Not Provided      | Not Provided         | Not Provided         | Not Provided                       | Not Provided                    | Not Provided                       | Not Provided                       | Not performed   | IQ 86  | No   | No  |
| Patient #4  | Heneý, 1992/ Moynihan, 2000/ Bond, 2005 | #1     | Pakistan     | Yes          | Not Provided             | c.246T>A (p.Ser82*)                    | c.246T>A (p.Ser82*)                    | Not Provided                     | F   | Not Provided      | Not Provided         | Not Provided         | Not Provided                       | Not Provided                    | Not Provided                       | Not Provided                       | Not performed   | IQ 89  | No   | Acute Lymphoblastic Leukemia causing death - skin areas of hypopigmentation                                 |
| Patient #5  | Bond, 2005                              | #2     | Pakistan     | Yes          | Not Provided             | c.4005-15A>G (p.E386fs*4)              | c.4005-15A>G (p.E386fs*4)              | Not Provided                     | F   | Not Provided      | Not Provided         | Not Provided         | Not Provided                       | -5                              | Not Provided                       | Not Provided                       | Not Provided  | Moderate intellectual disability             | Not Provided                                       | Not Provided  |
| Patient #6  | Bond, 2005                              | #2     | Pakistan     | Yes          | Not Provided             | c.4005-15A>G (p.E386fs*4)              | c.4005-15A>G (p.E386fs*4)              | Not Provided                     | F   | Not Provided      | Not Provided         | Not Provided         | Not Provided                       | -7                              | Not Provided                       | Not Provided                       | Not Provided  | Moderate intellectual disability             | Not Provided                                       | Not Provided  |
| Patient #7  | Hassan, 2007                            | #3     | Pakistan     | Yes          | 18-30                    | c.246T>A (p.Ser82*)                    | c.246T>A (p.Ser82*)                    | Not Provided                     | F   | Not Provided      | Not Provided         | Not Provided         | Not Provided                       | -4                              | Not Provided                       | Not Provided                       | Not Provided  | IQ 51  | No   | No  |
| Patient #8  | Hassan, 2007                            | #3     | Pakistan     | Yes          |                          | c.246T>A (p.Ser82*)                    | c.246T>A (p.Ser82*)                    | Not Provided                     | F   | Not Provided      | Not Provided         | Not Provided         | Not Provided                       | -7                              | Not Provided                       | Not Provided                       | Not Provided  | IQ 65  | No   | No  |
| Patient #9  | Hassan, 2007                            | #3     | Pakistan     | Yes          |                          | c.246T>A (p.Ser82*)                    | c.246T>A (p.Ser82*)                    | Not Provided                     | M   | Not Provided      | Not Provided         | Not Provided         | Not Provided                       | Not Provided                    | Not Provided                       | Not Provided                       | Not Provided  | No   | No   | No  |
| Patient #10 | Hassan, 2007                            | #3     | Pakistan     | Yes          |                          | c.246T>A (p.Ser82*)                    | c.246T>A (p.Ser82*)                    | Not Provided                     | M   | Not Provided      | Not Provided         | Not Provided         | Not Provided                       | Not Provided                    | Not Provided                       | Not Provided                       | Not Provided  | No   | No   | No  |
| Patient #11 | Pagnamenta, 2012                        | #4     | Somalia      | Yes          | 6                        | c.700G>T (p.Glu234*)                   | c.700G>T (p.Glu234*)                   | Not Provided                     | F   | -3,7              | -2,4                 | -3,7                 | 6                                  | -8,9                            | -1,1                               | -1,6                               | No abnormalities  | Mild intellectual disability                 | No   | severe gastric reflux - Moderate to severe bilateral neurosensory deafness - Slight hyperlaxity - Hypotonia |
| Patient #12 | Issa, 2013                              | #5     | Italy        | Yes          | Not Provided             | c.4441C>T (p.Arg1481*)                 | c.4441C>T (p.Arg1481*)                 | Not Provided                     | M   | Not Provided      | 0,5                  | Not Provided         | 5                                  | -6                              | Not Provided                       | -4,1                               | Simplified gyral pattern (mostly frontal) - corpus callosum agenesis                  | IQ 69  | No   | Abdominal hernia - Skin areas of hyperpigmentation - Behavioral disorder - Tic disorder                     |
| Patient #13 | Issa, 2013                              | #5     | Italy        | Yes          | Not Provided             | c.4441C>T (p.Arg1481*)                 | c.4441C>T (p.Arg1481*)                 | Not Provided                     | M   | -3,5              | -0,5                 | -0,5                 | 2                                  | -5                              | Not Provided                       |                                    | Simplified gyral pattern (mostly frontal) - corpus callosum and cerebellar hypoplasia | IQ 56  | No   | Skin areas of hyperpigmentation - Behavioral disorders  |
| Patient #14 | Lancaster, 2013                         | #6     | Not Provided | No           | Not Provided             | c.4546G>T (p.Glu1516*)                 | c.4672C>T (p.Arg1558*)                 | Microcephaly                     |     | -6,5              | -3,9                 | -5                   | 3                                  | -13,2                           | Not Provided                       | -6,7                               | Simplified gyral pattern  | Not Provided                                 | No   | Neurosensory deafness   |
| Patient #15 | Tan, 2014                               | #7     | Caucasian    | No           | 5                        | c.524_528del(p.Gln175Argfs*42)         | c.4005-1G>A (fs*)                      | Microcephaly                     | F   | Not Provided      | Not Provided         | Not Provided         | 6                                  | -8,9                            | -1,5                               | -3                                 | No abnormalities  | IQ 75  | No   | Asthma - Allergies - Sleep apnea  |
| Patient #16 | Li, 2015                                | #8     | Hispanic     | No           | 0,5                      | c.4441C>T (p.Arg1481*)                 | c.5227C>T (p.Gln1743*)                 | No                               | M   | -3,1              | -0,3                 | -1,5                 | Not Provided                       | Not Provided                    | Not Provided                       | Not Provided                       | Short corpus callosum   | Not Provided                                 | No   | Failure to thrive (enteral nutrition)   |
| Patient #17 | Yigit, 2015                             | #9     | Turkey       | Yes          | Not Provided             | c.4005-9A>G (p.Arg1335Serfs*3)         | c.4005-9A>G (p.Arg1335Serfs*3)         | No                               | M   | Not Provided      | -2                   | Not Provided         | 9                                  | -4                              | Not Provided                       | -3,2                               | Not Provided  | Not Provided                                 | No   | Bone age delay -5th finger clinodactyly - 11 pairs of ribs  |
| Patient #18 | Yigit, 2015                             | #9     | Turkey       | Yes          | Not Provided             | c.4005-9A>G (p.Arg1335Serfs*3)         | c.4005-9A>G (p.Arg1335Serfs*3)         | Not Provided                     | F   | Not Provided      | Not Provided         | Not Provided         | 10                                 | -4                              | Not Provided                       | -3,4                               | No abnormalities  | Mild intellectual disability                 | No   | Congenital hip dislocation  |
| Patient #19 | Yigit, 2015                             | #10    | Pakistan     | Yes          | Not Provided             | c.383+1G>C(p.Lys129*)                  | c.383+1G>C(p.Lys129*)                  | Not Provided                     | F   | Not Provided      | -2                   | Not Provided         | 2                                  | -6,2                            | Not Provided                       | -5                                 | No abnormalities  | Not Provided                                 | No   | Not Provided  |
| Patient #20 | Jouan, 2016                             | #11    | Canada       | No           | 53                       | c.280G>A (p.Gly94Arg)                  | c.3695A>G (p.Asn1232Ser)               | No                               | F   | Not Provided      | Not Provided         | Not Provided         | 53                                 | 1,0                             | Not Provided                       | 0,0                                | Corpus callosum agenesis  | IQ 84  | No   | Learning disabilities   |
| Patient #21 | Jouan, 2016                             | #11    | Canada       | No           | 52                       | c.280G>A (p.Gly94Arg)                  | c.3695A>G (p.Asn1232Ser)               | No                               | F   | Not Provided      | Not Provided         | Not Provided         | 52                                 | -1,3                            | Not Provided                       | -0,5                               | Corpus callosum agenesis  | IQ 78  | No   | Mutism - Intermittent ataxia  |
| Patient #22 | Jouan, 2016                             | #11    | Canada       | No           | 44                       | c.280G>A (p.Gly94Arg)                  | c.3695A>G (p.Asn1232Ser)               | Not Provided                     | M   | Not Provided      | Not Provided         | Not Provided         | 44                                 | -1,3                            | Not Provided                       | 0,5                                | Corpus callosum agenesis  | IQ 77  |  | Resolutive obsessive-compulsive disorder  |
| Patient #23 | Pagnamenta, 2016                        | #12    | Not Provided | No           | 11                       | c.4604+1G>C                            | c.3097delG (p.V1033 fs*41)             | Not Provided                     | M   | Not Provided      | Not Provided         | Not Provided         | 11                                 | -5,5                            | Not Provided                       | Not Provided                       | No abnormalities  | developmental quotient: 45                   | No   | Café au-lait spots  |
| Patient #24 | Abdullah, 2017                          | #13    | Pakistan     | Yes          | 30                       | c.5127_5128dupGT (p.Ser1710CysfsTer22) | c.5127_5128dupGT (p.Ser1710CysfsTer22) | Not Provided                     | F   | Not Provided      | Not Provided         | Not Provided         | 30                                 | Not Provided                    | Not Provided                       | Not Provided                       | Severe intellectual disability - walking  | No   |  |   |
| Patient #25 | Abdullah, 2017                          | #13    | Pakistan     | Yes          | 32                       | c.5127_5128dupGT (p.Ser1710CysfsTer22) | c.5127_5128dupGT (p.Ser1710CysfsTer22) | Not Provided                     | M   | Not Provided      | Not Provided         | Not Provided         | 32                                 | Not Provided                    | Not Provided                       | Not Provided                       | Severe intellectual disability - walking  | No   |  | Moderate hearing impairment   |
| Patient #26 | Abdullah, 2017                          | #13    | Pakistan     | Yes          | 12                       | c.5127_5128dupGT (p.Ser1710CysfsTer22) | c.5127_5128dupGT (p.Ser1710CysfsTer22) | Not Provided                     | M   | Not Provided      | Not Provided         | Not Provided         | 12                                 | Not Provided                    | Not Provided                       | Not Provided                       | Moderate intellectual disability  | No   |  |   |
| Patient #27 | Abdullah, 2017                          | #13    | Pakistan     | Yes          | 8                        | c.5127_5128dupGT (p.Ser1710CysfsTer22) | c.5127_5128dupGT (p.Ser1710CysfsTer22) | Not Provided                     | F   | Not Provided      | Not Provided         | Not Provided         | 8                                  | Not Provided                    | Not Provided                       | Not Provided                       | Moderate intellectual disability  | No   |  |   |
| Patient #28 | Ahmad, 2017                             | #14    | Pakistan     | Yes          | Not Provided             | c.1279C>T (p.Arg427*)                  | c.1279C>T (p.Arg427*)                  | Not Provided                     | NP  | Not Provided      | Not Provided         | Not Provided         | Not Provided                       | Not Provided                    | Not Provided                       | Not Provided                       | Not Provided  | Not Provided                                 | Not Provided                                       | Not Provided  |
| Patient #29 | Sukumaran, 2017                         | #15    | Pakistan     | Yes          | 16                       | c.4114C > T (p.Arg1372*)               | c.4114C > T (p.Arg1372*)               | Not Provided                     | F   | Not Provided      | Not Provided         | Not Provided         | 16                                 | -5                              | Not Provided                       | Not Provided                       | Mild intellectual disability  |  |  | No  |
| Patient #30 | Sukumaran, 2017                         | #15    | Pakistan     | Yes          | 12                       | c.4114C > T (p.Arg1372*)               | c.4114C > T (p.Arg1372*)               | Not Provided                     | M   | Not Provided      | Not Provided         | Not Provided         | 12                                 | -9                              | Not Provided                       | Not Provided                       | Mild intellectual disability  |  |  | No  |
| Patient #31 | Alfares, 2018                           | #16    | Saudi Arabia | Yes          | 16                       | c.4055A>G (p.Glu1352Gly)               | c.4055A>G (p.Glu1352Gly)               | Not Provided                     | M   | -2                | 1100                 | Not Provided         | Not Provided                       | Not Provided                    | Not Provided                       | Not Provided                       | White matter abnormalities  | Severe intellectual disability - non walking | Yes - epileptic encephalopathy with hypsarrhythmia | Bilateral cataract - White matter abnormalities - Tetrapyramidal syndrome                                   |
| Patient #32 | Alfares, 2018                           | #16    | Saudi Arabia | Yes          | 10                       | c.4055A>G (p.Glu1352Gly)               | c.4055A>G (p.Glu1352Gly)               | Not Provided                     | M   | Not Provided      | Not Provided         | Not Provided         | 10                                 | -2                              | -4                                 | -4                                 | moderate ventriculomegaly   | Severe intellectual disability - non walking | Yes  | Bilateral cataract - Tetrapyramidal syndrome - Dystonia   |
| Patient #33 | Alfares, 2018                           | #16    | Saudi Arabia | Yes          | 8                        | c.4055A>G (p.Glu1352Gly)               | c.4055A>G (p.Glu1352Gly)               | Not Provided                     | F   | Not Provided      | Not Provided         | Not Provided         | 8                                  | -4                              | -4,5                               | -5                                 | Thin corpus callosum  | Severe intellectual disability - non walking | Yes - epileptic encephalopathy with hypsarrhythmia | Bilateral cataract - Tetrapyramidal syndrome  |
| Patient #34 | Alfares, 2018                           | #16    | Saudi Arabia | Yes          | 6                        | c.4055A>G (p.Glu1352Gly)               | c.4055A>G (p.Glu1352Gly)               | Intra uterine growth retardation | M   | -2                | 1700                 | Not Provided         | 0,75                               | -4                              | Not Provided                       | Not Provided                       | Thin corpus callosum  | Not Provided                                 | Yes  | Bilateral cataract - Hypospadias - Bilateral cryptorchidism - Cardiac congenital defect                     |
| Patient #35 | Alfares, 2018                           | #17    | Saudi Arabia | Not Provided | 3                        | c.4672C>T p.(Arg1558*)                 | c.4672C>T p.(Arg1558*)                 | Not Provided                     | M   | -3,5              | Not Provided         | Not Provided         | Not Provided                       | Not Provided                    | Not Provided                       | Not Provided                       | Decreased cerebral volume   | Intellectual disability                      | Yes  | Cataract  |

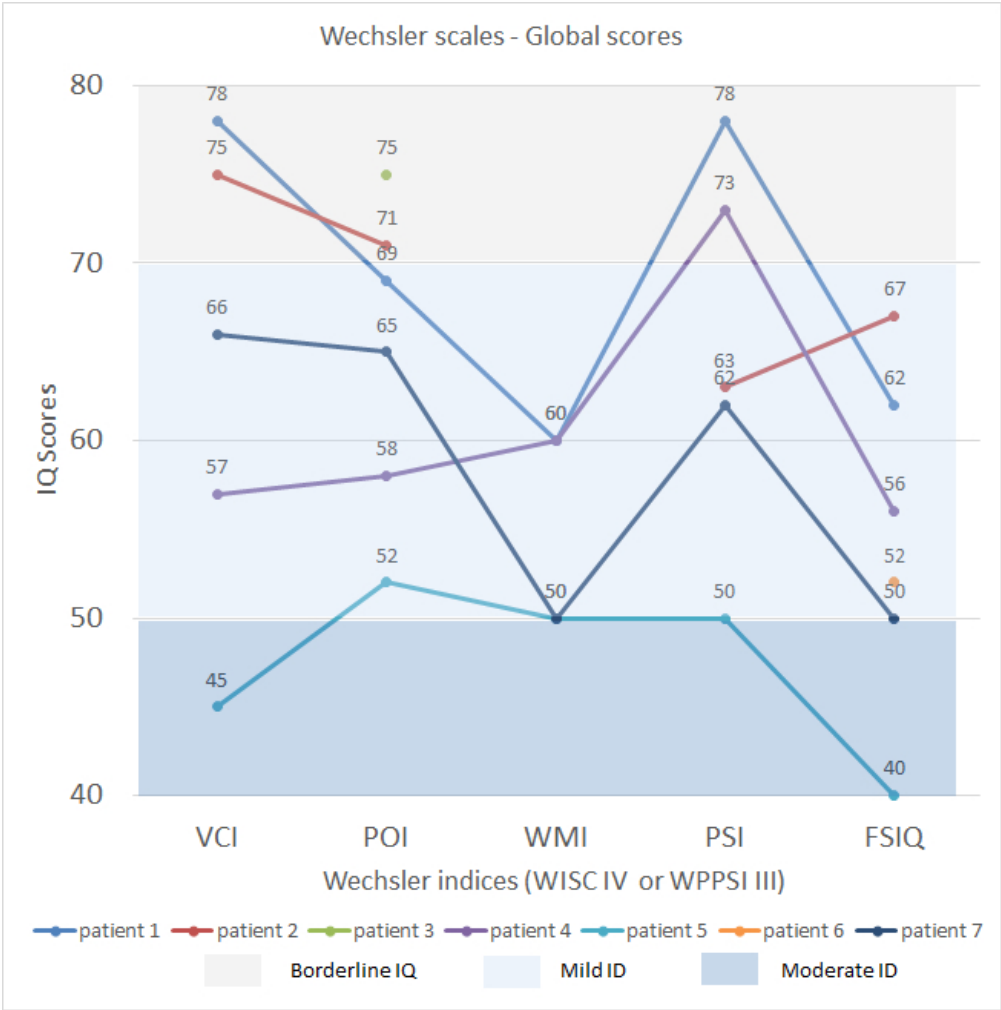


Supplementary Figure 1

172x90mm (300 x 300 DPI)

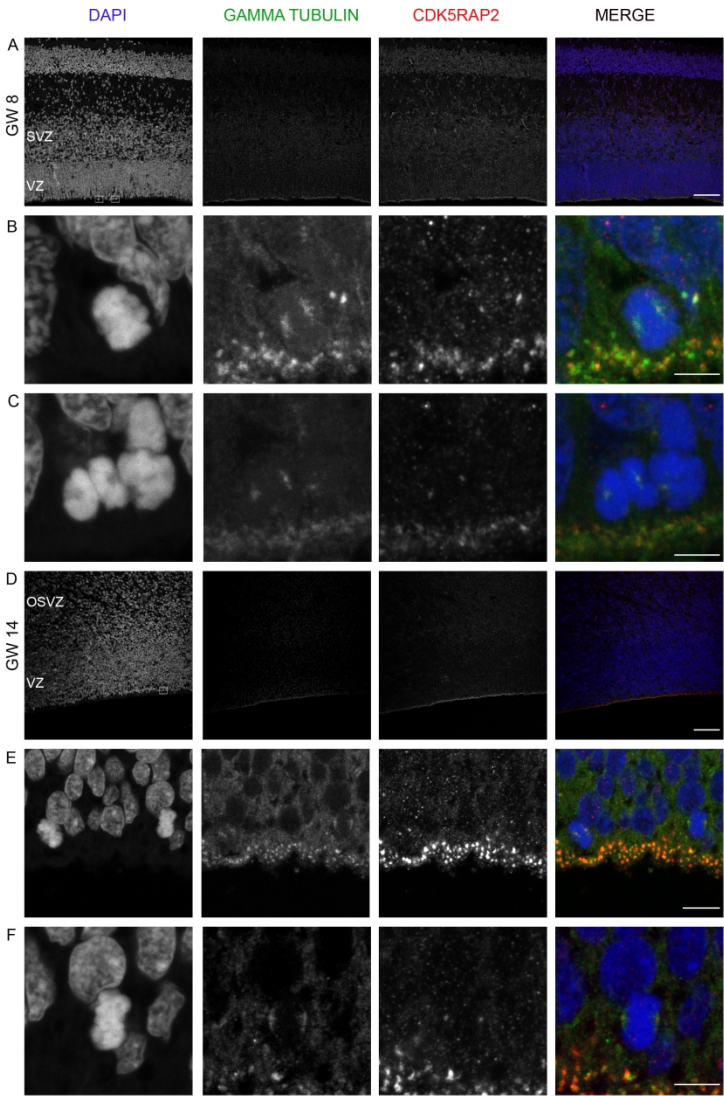


128x81mm (300 x 300 DPI)



Supplementary Figure 3

55x60mm (300 x 300 DPI)



Supplementary Figure 4

199x308mm (300 x 300 DPI)