

Mutations in *PHF8* are associated with X linked mental retardation and cleft lip/cleft palate

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Truncating mutations were found in the *PHF8* gene (encoding the PHD finger protein 8) in two unrelated families with X linked mental retardation (XLMR) associated with cleft lip/palate (MIM 300263). Expression studies showed that this gene is ubiquitously transcribed, with strong expression of the mouse orthologue *Phf8* in embryonic and adult brain structures. The coded *PHF8* protein harbours two functional domains, a PHD finger and a JmjC (Jumonji-like C terminus) domain, implicating it in transcriptional regulation and chromatin remodelling. The association of XLMR and cleft lip/palate in these patients with mutations in *PHF8* suggests an important function of *PHF8* in midline formation and in the development of cognitive abilities, and links this gene to XLMR associated with cleft lip/palate. Further studies will explore the specific mechanisms whereby *PHF8* alterations lead to mental retardation and midline defects.

X linked mental retardation (XLMR) is an inherited condition that causes failure to develop cognitive abilities because of mutations in several genes on the X chromosome. XLMR is a highly heterogeneous condition affecting around 1.6/1000 males, with a carrier frequency of 2.4/1000 females.^{1,2} More than 200 XLMR conditions have been described,³ which can be divided as follows:

- non-syndromic forms (MRX), in which mental retardation is the only clinical manifestation; 81 MRX families have been reported so far;
- syndromic forms (MRXS), in which mental retardation is associated with biochemical abnormalities, neurological features, or recognisable physical signs such as skeletal abnormalities or facial dysmorphism.^{3,4}

Both MRX and MRXS genes have been located at various regions on the X chromosome. To date, more than 40 genes responsible for MRXS and 24 genes responsible for MRX have been cloned.⁵ Furthermore, it has been shown that mutations in a single gene—such as *ARX*,^{6,7} *MECP2*,^{8,9} or *RSK2*¹⁰—could lead to a wide spectrum of clinical features that encompass both MRX and MRXS conditions. Based on the distribution of linkage intervals in 125 unrelated MRX families, Ropers and others previously showed that 30% of all mutations cluster on proximal Xp.¹¹ We report here the identification of a new gene, *PHF8* (PHD finger protein 8), located in Xp11 and mutated in families with XLMR associated with cleft lip/cleft palate.

METHODS

Patients and families

The family pedigrees are shown in figs 2 and 3. DNA and RNA were extracted from blood samples and immortalised

lymphoblastoid cell lines (family N42) that were established from peripheral lymphocytes using standard protocols.

Fluorescence in situ hybridisation (FISH)

BACs on chromosome Xp11.21 were selected from Ensembl database (<http://www.ensembl.org>) and the UCSC genome browser (<http://genome.ucsc.edu>) and obtained from the CHORI Institute (<http://bacpac.chori.org>). Hybridisation experiments were carried out as previously described.¹²

Mutation analysis of *PHF8*

Polymerase chain reaction (PCR) was carried out in 50 µl reaction volumes containing 100 ng of genomic DNA from the patients, 10 pM of each primer, 1.25 mM of dNTPs, 0.5 U *Taq* DNA polymerase (Promega, Madison, Wisconsin, USA), and 1.5 mM MgCl₂. An initial denaturation of five minutes at 94°C was followed by 30 cycles of one minute at 94°C, one minute of annealing, a one minute extension at 72°C, and a final extension step of seven minutes at 72°C. The products were then checked on a 1.5% agarose gel to verify amplification before analysis by denaturing high performance liquid chromatography (DHPLC) using the WAVE 3500 HT system (Transgenomic, Santa Clara, California, USA). For each amplified exon, melting profiles and temperatures were predicted by the Transgenomic Navigator software version 1.5.1 (DHPLC conditions available upon request). Pairs of amplified fragments from patient and control PCR products were pooled, denatured at 95°C for three minutes, and cooled to 40°C in decreasing increments of 0.05°C per second. The products were then injected and eluted with an acetonitrile gradient at a flow rate of 1.5 ml/min, with a mobile phase composed of two buffers (buffer A, 0.1 M triethylammonium acetate or TEAA; buffer B, 0.1 M TEAA with 25% acetonitrile). For each sample pair that showed an abnormal elution profile, the PCR products were purified and sequenced on an ABI377 DNA sequencer (Perkin Elmer, Norwalk, Connecticut, USA).

RT-PCR and expression studies

For reverse transcriptase polymerase chain reaction (RT-PCR) experiments on mouse material, total RNA samples were prepared from embryonic, newborn, and postnatal (P60) mouse brains. Cells were derived from brains of randomly bred Swiss mice. Glial cells were from newborn murine cerebral hemispheres, and 95% of the cells were identified as type 1 astrocytes. Cultures of neuronal cells were set up from single cell suspension of fetal brains at 15 days of gestation. Cultures consisted predominantly of neurones (>95%).

Abbreviations: DHPLC, denaturing high performance liquid chromatography; FISH, fluorescence in situ hybridisation; MRX, non-syndromic forms of X linked mental retardation; MRXS, syndromic forms of X linked mental retardation; XLMR, X linked mental retardation

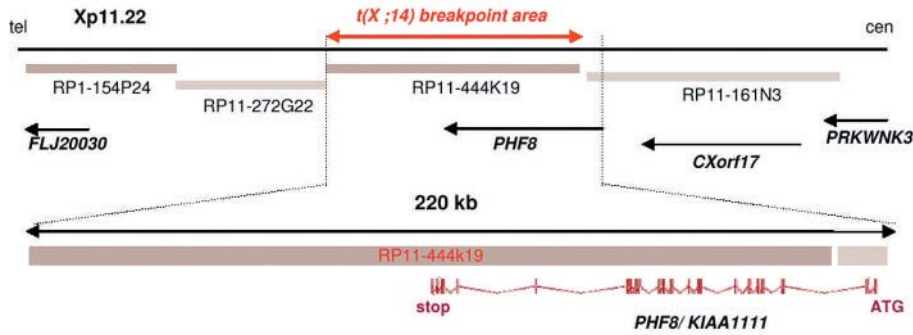


Figure 1 Characterisation of the *PHF8* gene. Transcriptional map of the Xp11 area including the X chromosomal breakpoint of the t(X;14) translocation. The minimum critical region is estimated at 220 kb and includes the *PHF8* gene that is transcribed from centromere to telomere.

Amplification by RT-PCR was carried out according to standard procedures.

Non-radioactive in situ hybridisation was undertaken as described elsewhere.¹⁹ Mouse embryonic (16 days postconception) and adult brain sections were hybridised with the sense and the antisense RNA generated from the 3' untranslated part of *Phf8*.

RESULTS

Characterisation of the *PHF8* gene and identification of mutations in families with XLMR and cleft lip/cleft palate

We previously investigated a balanced translocation (X;14)(p11.2;p10) found in two half sisters and their mother who all had non-syndromic mental retardation and epilepsy. To establish the physical location of the chromosomal aberration, we carried out FISH experiments with BAC clones obtained from the CHORI institute. The DNA clones were prepared by standard techniques, were labelled by nick translation, and were used as probes in FISH, as previously described.¹² The breakpoint of the chromosome 14 was located on the short arm. As rearrangements of the short arms of acrocentric chromosomes, particularly robertsonian

translocations, do not lead to any abnormal phenotypes, we suggested that the Xp chromosomal breakpoint would probably be involved in the phenotype of the patients. We localised the Xp breakpoint within the BAC clone RP13-444K19 (GenBank Accession number AL732374) (fig 1). BLAST analysis of the genomic region revealed the presence of only one cDNA called *KIAA1111* (GenBank accession number AB029034). It is expressed in human adult brain and represents a novel gene named *PHF8* (PHD finger protein 8) (GenBank accession number NM_015107) (fig 1).

Based on these findings, the *PHF8* gene appeared to be a strong candidate for XLMR. We screened the *PHF8* gene for mutations in 40 families with XLMR and with a linkage interval covering the candidate region in Xp11. The families were collected by the EuroMRX consortium (24 families) and the Greenwood Genetic Center/University of Miami XLMR study (16 families).¹³ We analysed the coding sequence of *PHF8* by PCR experiments using genomic DNA and primers that amplified both the exonic sequences and the exon-intron junctions (table 1).

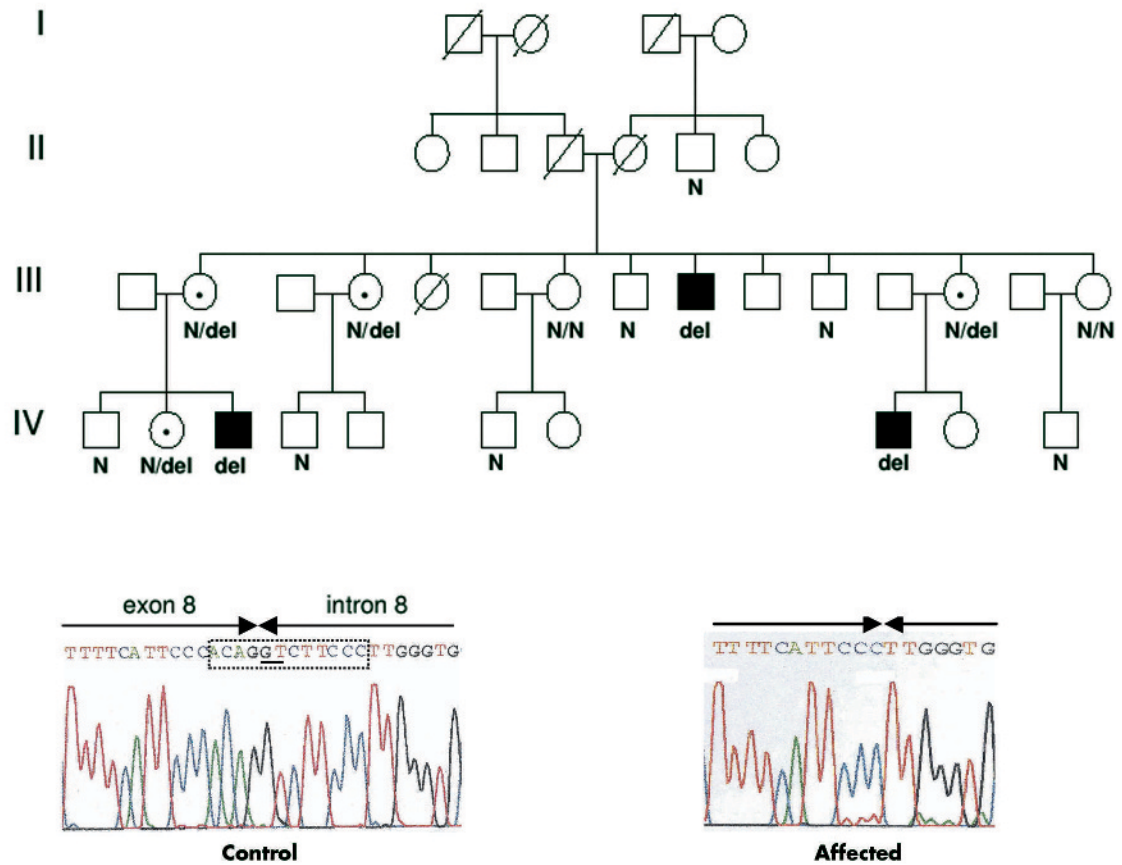
Initially, we identified a mutation in *PHF8* in family N42, previously described by Siderius *et al.*¹⁴ Affected males of this family have a syndromic form of XLMR associated with cleft

Table 1 Primers for mutation detection and polymerase chain reaction amplification conditions

Exon	Forward primer (5'→3')	Reverse primer (5'→3')	Annealing temperature (°C)
1	tcaagtcactggctctcga	atgagaggagggtgaactcggf	62
2	actgagggtggfgcaagg	gagagcaagtgcaacacactg	63
3	acagcactgtctagacctca	cttctctgtctctctct	56
4	gctcaggcdttgtcttggt	gggaaagctgggaagaggf	63
5	gtggcagcctccaataag	aaacagattggaggggaagg	59
6	ggactgatgaggggacaagaga	agagaacaacgaaatatacactag	59
7	gctgtggggatatttactcaggc	gattcaaatgtttctgtgctgc	59
8	gtttggagcatgggagaaa	ftaatgtctgtggggccaa	60
9	ftgagatgagtaggtagtgagaga	cttctacattatactctcact	58
10	catccagaacaaaactgattc	tatcatgtctgactcgtttac	59
11	gttgttaacaagaagtcacca	ctctcaaaaacatctaccca	60
12	tctcattgaattgctctgg	atgctaactgcagaggcctaa	58
13	aggaaacagacctctgcttta	ggatagcctgttttgaac	59
14	ccagtgaacacagagggaact	ctgtctcagtgccatattacc	59
15	ctgtctagcgaactcaatac	tccgtctcaaacacaacaaaca	62
16	Gtatttgctgaaatgctaact	ccaactagaatgacaactgtctc	59
17	tcaatgcccctgactctgg	ctgattggctgacctggcac	64
18	gatgaccgatttactctgacag	gatggagactgggactgagg	64
19	tcatttactctaccctttg	catctctgtatttctct	59
20	agagcttggaggtagggat	tctaatgcaaacctgattagt	59
21	gtctagttggtctctctgg	taagatcctcggttacaacc	59
22	ttgggcccagatttgatgtg	gaaggcaggcaggatgctcta	63

Note: the coding sequence of *PHF8* begins at exon 2 and ends at exon 22.

A



B

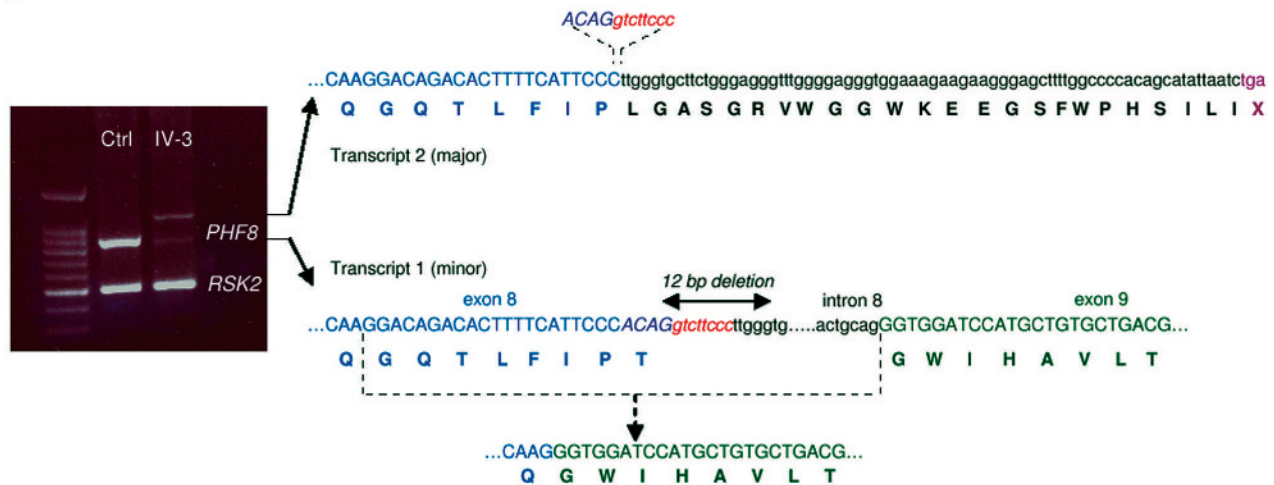


Figure 2 Mutation of *PHF8* in family N42 associated with X linked mental retardation and cleft lip/palate. (A) Pedigree of family N42 showing the affected male patients (black squares) who carry the 12 bp deletion (del), the unaffected male and female patients (white squares, N), and the heterozygous female carriers (N/del). The sequencing electropherograms show that the deletion causes the elimination of the splice donor site of intron 8. (B) Reverse transcriptase polymerase chain reaction (RT-PCR) and sequencing results of both *PHF8* transcripts expressed in lymphoblastoid cell lines of individual IV-3. One microgram of RNA from affected individual IV-3 and control was reverse transcribed followed by polymerase chain reaction (PCR) with one forward primer (5'-TAACCTTGTTGGAGACACCGAA-3') located in exon 7, one reverse primer (5'-AAGAGCAGATGATCGCAACT-3') located in intron 8, and a second reverse primer (5'-CTAGTGCTAATGCACATCAG-3'). In the same reaction, a *RSK2* transcript fragment (486 bp) was amplified as an internal control,¹⁰ using the following primers (forward 5'-GGACAGCAAATTATGGATGA-3' and reverse 5'-CTAGTGCTAATGCACATCAG-3'). The PCR products were migrated in a 1.2% agarose gel and showed two *PHF8* transcripts in the affected individual, one weakly expressed transcript (transcript 1 fragment, 822 bp) with the 8 bp deletion compared to the control, and a higher expressed transcript (transcript 2 fragment, 1068 bp) composed of coding sequence of exon 7 extended into intron 8, and absent in the control lane.

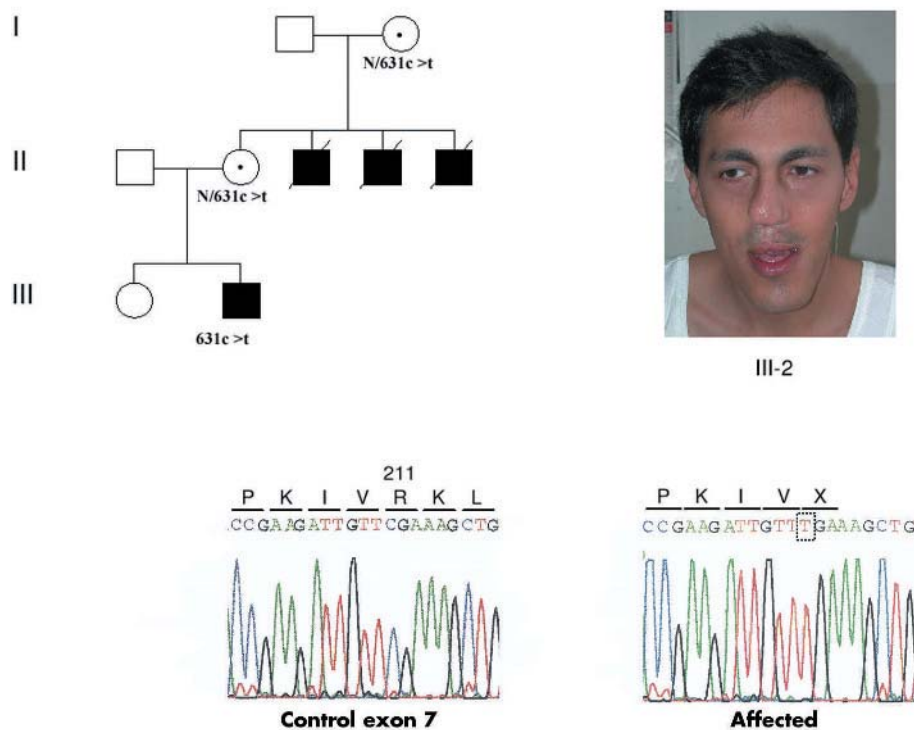


Figure 3 631C→T (R211X) mutation in family 2. Representation of the pedigree of the Italian family and facial features of patient III-2 (written consent from the patient and his legal guardian was obtained for publication of the images in print and online). The chromatogram of the mutant sequence is shown, along with a control sequence. A C→T substitution located in exon 7 was identified in blood DNA from patient III-2. The carrier females (I-2 and II-2) are heterozygous for this mutation. (Photograph reproduced with patient's consent.)

lip/cleft palate. The family consists of three affected males in two generations (III-9, IV-3, and IV-8; fig 2A), who showed, at the time of clinical examination, mild to borderline mental retardation, minor facial anomalies such as a long face and broad nasal tip, cleft lip/cleft palate in two (bilateral for individual III-9 and unilateral for IV-3), and large hands (75th to 97th centile for IV-3; >97th centile for IV-8 and III-9). Previous linkage analysis assigned the putative XLMR gene to Xp11.3–q21.3, between the markers *DXS337* and *DXS990*, defining a linkage interval of 25 cM.¹⁴ The mutation analysis for *PHF8* in the three affected males of N42 family revealed a 12 base pair (bp) deletion at the exon 8/intron 8 junction (fig 2A), that was predicted to suppress the splice donor site of intron 8. To test the presence of an abnormal splicing event, RT-PCR was carried out on RNA extracted from lymphoblasts of both the affected individuals and controls. The RT-PCR analysis showed the presence of two transcripts in the affected individuals. One was a very weakly expressed transcript composed of a portion of exon 8, joined to the normal exon 9. A higher expressed second band was a transcript in which the coding sequence extended into intron 8 up to a stop codon (fig 2B).

Based on this finding, additional screening of cases with mental retardation plus cleft lip/cleft palate was undertaken. As a result, another mutation was found in a second family with males who had mental retardation and clinical features similar to the N42 family (fig 3). The proband (III-2) was the second child of two unrelated parents. Two maternal uncles died in the neonatal period and the mother stated they had bilateral cleft lip and palate. A third maternal uncle, who died in a car accident, suffered from mild mental retardation without clefting. The proband was born by spontaneous delivery after an uneventful pregnancy. At birth, his weight was 2550 g (5th to 10th centile), length

47 cm (10th to 25th centile), and head circumference 33 cm (5th to 10th centile). At 25 years old, his weight was 60.5 kg (25th to 50th centile), and his height was 178 cm (75th centile). He had mental retardation and minor facial dysmorphism with a scar from an operated cleft lip (fig 3). He also had long hands (20 cm, >97th centile) with long and thin fingers, and flat feet with long and thin toes. Computed tomography and magnetic resonance imaging were normal. Cytogenetic analysis revealed a normal 46, XY karyotype, and molecular analysis of the *FMR1* CCG expansion, subtelomeric rearrangements and 22q11 deletion by FISH were negative.

The analysis of the coding sequence of *PHF8* revealed the presence of a nonsense mutation, p.R211X (c.631C→T), in exon 7 (fig 3), leading to a predicted truncated protein of 211 amino acids compared with the normal protein of 1024 amino acids. We could not test the expression of the altered transcript as only genomic DNA from the proband III-2, his mother, and grandmother were available.

The two mutations described above were present in all tested affected family members. All female carriers in the respective families were heterozygous. The sequence changes were not found in 100 healthy unrelated male and 100 unrelated healthy females (300 X chromosomes), making it unlikely they were rare polymorphisms.

We also analysed the *PHF8* gene in a male patient of a family affected by the Pallister W syndrome (OMIM 311450)¹⁵—which is characterised by median cleft upper lip, central nervous system involvement with strabismus and spasticity, and moderate to severe mental retardation—and in four sporadic male individuals having a similar phenotype to the two mutated families (that is, mental retardation and cleft lip/cleft palate). However, no mutations in the coding sequence of the *PHF8* gene were found.

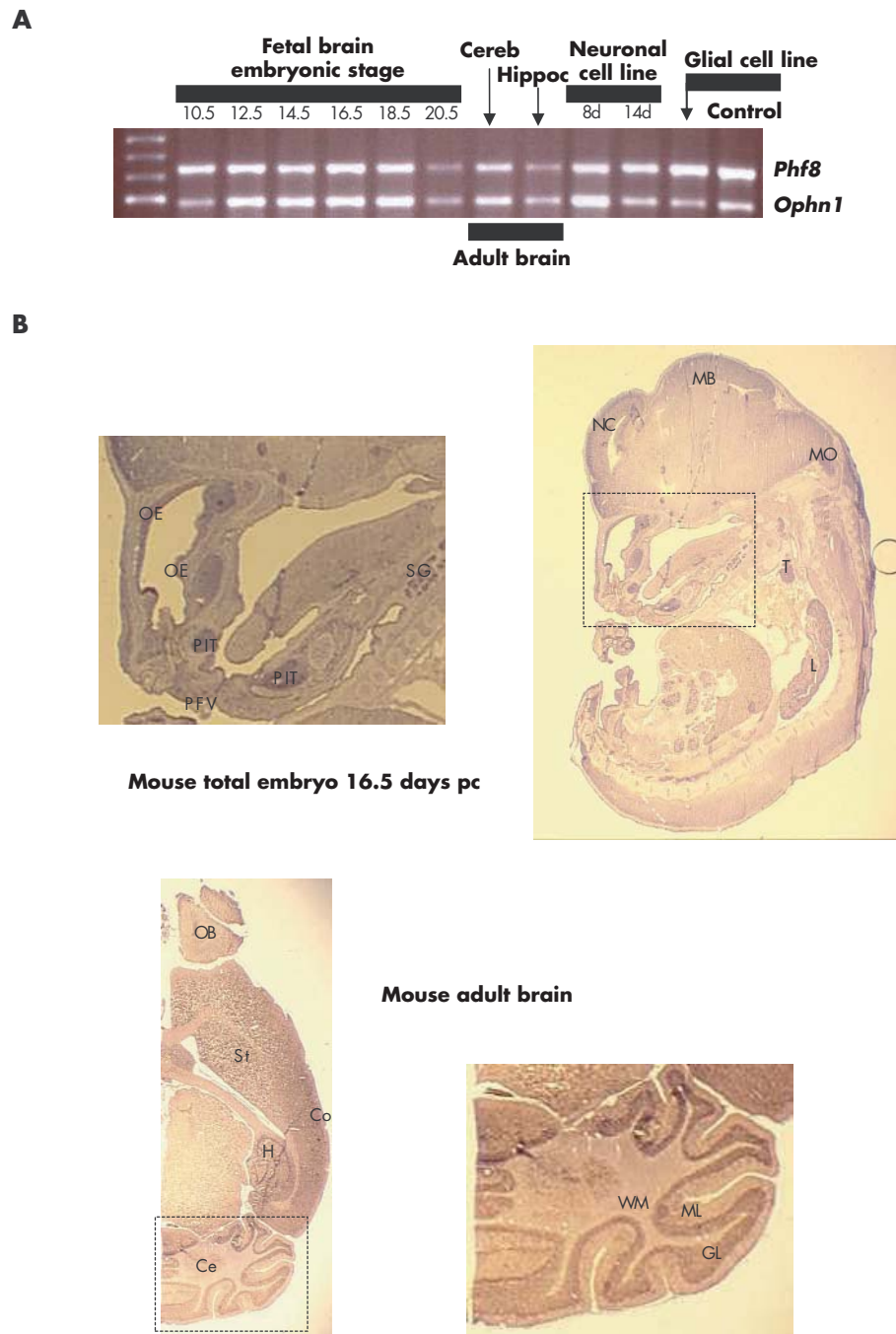


Figure 4 Expression studies of the mouse orthologue *Phf8*. (A) Reverse transcriptase polymerase chain reaction analyses carried out on mouse RNA samples extracted from brains at different embryonic stages, adult brain structures (cerebellum and hippocampus), and neuronal and glial cell lines. *Phf8* was expressed in all tested tissues. (B) In situ hybridisation on mouse embryo (16.5 days postconception (pc) and adult brain section. Non-radioactive in situ hybridisation was carried out as described elsewhere.¹⁹ Mouse embryonic and adult brain sections were hybridised with the sense and the antisense RNA probes generated from the 3' region of the *Phf8* gene (cDNA sequence AK122447; primers forward [5'-AAGGAATGAGGAGGAGCAACA-3'] and reverse [5'-TCACCACACCTCTTCAGAG-3']; amplicon of 620 bp). Mouse total embryo: L, lung; MB, midbrain; MO, medulla oblongata; NC, neopallial cortex; OE, olfactory epithelium; PFV, primordium of follicles of vibrissae; PIT, primordium of incisor tooth; SG, submandibular gland; T, thymus gland. Mouse adult brain: Ce, cerebellum; Co, cortex; GL, granular layer; H, hippocampus; ML, molecular layer; OB, olfactory bulb; St, striatum; WM, white matter.

RT-PCR and expression studies

PHF8 is a novel human zinc finger gene of unknown function. The gene is composed of 22 exons. The transcript has a length of 5776 bp and an open reading frame of 3075 bp. The *PHF8* transcript shows a ubiquitous expression pattern as tested by expressed sequence tag analyses and

northern blot hybridisation (results not shown). *PHF8* domains are also highly conserved among orthologues in other species, including mouse and *X laevis*.

To further investigate *PHF8* expression in adult brain and in primary cultures of mouse neuronal and astroglial cells during development, we derived appropriate primers from

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1   MASVPVYCLC RLPYDVTRFM IECDCMQDWF HGSCVGVVEE KAADIDLYHC PNCEVLHGPS
61  IMKKRRGSSK GHDTHKGKPV KTGSPTFVRE LRSRTFDSSD EVILKPTGNQ LTVEFLEENS
121 FSVPIVLVKK DGLGMLTLPSP SFTVRDVEHY VGSDEIDVI DVTRQADCKM KLGDFVKYYY
181 SGKREKVLNV ISLEFSDTRL SNLVETPKIV ↓ RKLSWVENLW PEECVFERPN VQKYCLMSVR
241 ↓ DSYTDFHIDF GGTSVWYHVL KGEKIFYLIR PTNANLTLFE CWSSSSNQNE MFFGDQVDKC
301 YKCSVKQGQT LFIPTGWIHA VLTPVDCLAF GGNFLHSLNI EMQLKAYEIE KRLSTADLFR
361 FPNFETICWY VGKHILDIFR GLRENRRHPA SYLVHGGKAL NLAFAWTRK EALPDHEDEI
421 PETVRTVQLI KDLAREIRLV EDIFQONVGK TSNIFGLQRI FPAGSIPLTR PAHSTSVMSS
481 RLSLPSKNGS KKKGLKPKEL FKKAERKGE SSALGPAGQL SYNLMDTYSH QALKTGSFQK
541 AKFNITGACL NDSDDDSPDL DLDGNESPLA LLMSNGSTK VKSLSKSRRT KIACKVDKAR
601 LMAEQVMEDE FDLSDDELQ IDERLGKEKA TLIIRPKFPR KLPRAKPCSD PNRVREPGEV
661 EFDIEEDYTT DEDMVEGVEG KLGNGSGAGG ILDLLKASRQ VGGPDYAALT EAPASPSTQE
721 AIQGLCMAN LQSSSSSPAT SSLQAWWTGG QDRSSGSSSS GLGTVSNSPA SQRTPGKRPI
781 KRPAYWRTE EEEEEASLD EQDSLACFK DAEYIYPSLE SDDDDPALKS RPKKKNSDD
841 APWSPKARVT PTLPKQDRPV REGTRVASIE TGLAAAAAKL AQELQKAQK KKYIKKKPLL
901 KEVEQPRPD SNLSLTVAP TVAATPQLVT SSSPLPPPEP KQEALSGSLA DHEYTARPN
961 FGMAQANRST TPHAPGVFLT QRRPSVGSQS NQAGQGRPK KGLATAKQRL GRILKIHRNG
1021 KLLL

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Figure 5 Annotated sequence of the human PHF8 protein. PHF8 protein sequence showing mutations and regions of functional importance. The positions of the mutations are marked by arrows. PHD fingers (amino acid position 7–53) and JmjC (amino acid position 195–294) domains are represented by shaded regions. Putative NLS sequences are underlined and italicised.

the mouse homologous gene and studied its expression by RT-PCR. Figure 4A shows RT-PCR amplification of the mouse *Phf8* mRNA and *Ophn1* mRNA used here as a control.¹⁶ These results showed a relatively high level of expression in the embryonic and early postnatal stages of brain development. The expression spectrum of *Phf8* in sections of mouse embryos is rather ubiquitous, especially in brain structures, with a higher expression in neopallial cortex, midbrain, and the dorsal part of the medulla (fig 4B). The gene is also expressed in the olfactory epithelium, the submandibular gland, and the primordium of follicles of the vibrissae. In addition to the expected expression of *Phf8* in fetal brain at different embryonic stages, a higher expression is observed in cerebellum (granular layer) and hippocampus in adult brain (fig 4B), which are structures potentially involved in the physiological processes underlying memory and learning abilities.

DISCUSSION

We report here the identification of the human *PHF8* gene located in Xp11.21 and encoding a new member of the PHD finger protein family. We showed that truncating mutations of *PHF8* led to XLMR with or without cleft lip/cleft palate in two unrelated families, with a large intrafamilial phenotypic heterogeneity, ranging from non-specific mental retardation (N42 family: individual IV-8; Italian family: individual II-5) to mental retardation and cleft lip/cleft palate (other affected males of both families).

The PHF8 protein is composed of 1024 amino acids and contains a PHD zinc finger domain (amino acid positions 7–53) and a Jumonji C (JmjC) domain (positions 195–294,

fig 5), which are highly conserved among orthologues in other species, including mouse and *X laevis*. The murine *Phf8* encodes a putative 1005 amino acid protein (Genbank accession number BAC65729) and shares 94% of identity at the amino acid level with human PHF8. Furthermore, BLAST analysis identified an orthologue in yeast (Genbank accession number NP_010971), suggesting that the PHF8 protein has been conserved during evolution.

As the large majority of PHD finger-containing proteins are localized in the nucleus, we looked for protein sorting signals using the PSORTII prediction program. We found four putative nuclear localization signals (NLS) with a 4/7 residues pattern, and two bipartite NLS (fig. 5), suggesting that PHF8 is very likely a nuclear protein. Furthermore, the predicted truncated proteins in the two affected families lack five NLS, which probably results in a cytoplasmic localisation of these truncated proteins thereby altering their function.

PHD finger genes are thought to belong to a diverse group of transcriptional regulators affecting eukaryotic gene expression by influencing chromatin structure. This family is composed of more than 15 members and one of them, *PHF6*, has already been implicated in a syndromic form of XLMR known as the Börjeson-Forssmann-Lehman syndrome (MIM 301900).¹⁷

More than 250 proteins share the jmjC domain (Interpro accession number IPR003347). JmjC-domain containing proteins have been implicated in apoptosis and in chromatin remodelling.¹⁸ The secondary structure of the JmjC domain predicts an enzyme activity. Its frequent association with DNA binding motifs, such as PHD finger domains, and its

putative chromatin modification activities suggest a role in regulating the integrity of the chromatin structure.¹⁹ However, the precise enzymatic or biological activity of this domain remains enigmatic.

The two truncating mutations occur nearby or in the MjJC domain of *PHF8* and are associated with a specific form of XLMR. Interestingly, mutations in families with XLMR have been found in the *JARID1C* gene (MIM 314690), which also harbours MjJC and PHD finger domains.²⁰ In the *JARID1C* gene, several disease causing mutations in XLMR families have been described that strengthen the importance of this gene family in the aetiology of mental retardation.

In summary, we have shown that truncating mutations in the *PHF8* gene cause either mild to borderline mental retardation associated with cleft lip/palate (XLMR–cleft lip/cleft palate) or non-specific XLMR. PHD finger proteins are suspected of modifying and regulating the structure of the DNA, and therefore play a critical role in the regulation of transcription. The association of *PHF8* with mental retardation and midline defects highlights the importance of this process, specifically during brain development and midline formation. In further studies we will aim to characterise the specific function of *PHF8* and to understand the consequences of alterations in its function.

GENBANK ACCESSION NUMBERS

Human *PHF8* mRNA accession number NM_015107
 Human *KIAA1111* mRNA accession number AB029034
 Human PHF8 protein accession number NP_055922
 Mouse *Phf8* mRNA accession number AK122447
 Mouse *Phf8* protein accession number BAC65729
 BAC clone RP13-444K19 accession number AL732374

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