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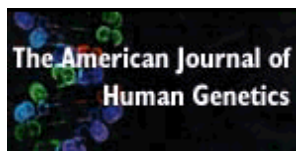
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CGG-Repeat Expansion in the *DIP2B* Gene Is Associated with the Fragile Site FRA12A on Chromosome 12q13.1

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Abstract

A high level of cytogenetic expression of the rare folate-sensitive fragile site FRA12A is significantly associated with mental retardation. Here, we identify an elongated polymorphic CGG repeat as the molecular basis of FRA12A. This repeat is in the 5' untranslated region of the gene *DIP2B*, which encodes a protein with a DMAP1-binding domain, which suggests a role in DNA methylation machinery. *DIP2B* mRNA levels were halved in two subjects with FRA12A with mental retardation in whom the repeat expansion was methylated. In two individuals without mental retardation but with an expanded and methylated repeat, *DIP2B* expression was reduced to approximately two-thirds of the values observed in controls. Interestingly, a carrier of an unmethylated CGG-repeat expansion showed increased levels of *DIP2B* mRNA, which suggests that the repeat elongation increases gene expression, as previously described for the fragile X-associated tremor/ataxia syndrome. These data suggest that deficiency of *DIP2B*, a brain-expressed gene, may mediate the neurocognitive problems associated with FRA12A.

The eukaryotic genome contains tissue-specific patterns of cytosines covalently modified by the addition of a methyl group.¹ Of all CpG dinucleotides of the mammalian genome, 60%–90% are subject to this kind of epigenetic modification. Most of the unmethylated CpG sites are located in the promoter region of genes, where changes in their methylation status are linked to long-term changes in expression level of the underlying genes.² Methylated cytosines may be targeted by methyl-binding domains of transcriptional repressors—which, on binding, recruit members of chromatin-remodeling complexes—resulting in the deacetylation of core histone proteins and transcriptional silencing of the underlying gene.³

Methylation abnormalities have been linked to a broad range of diseases. In tumor cells, the promoter regions with a CpG island are often hypermethylated. This epigenetic change is found in every type of tumor and is associated with a loss-of-gene transcription.³ Alterations of genomic imprinting or loss of imprinting has been shown to play a pivotal role in malignant tumorigenesis; for example, see the works of Robertson² and Muller et al.⁴ Methylation defects of the DNA-methylation machinery itself can also give rise to different human diseases. For instance, mutations in the *DNMT3B* gene are known to cause human immunodeficiency syndrome (ICF) (immunodeficiency, centromeric heterochromatin, facial anomalies, mental retardation, prolonged respiratory infections, and infections of the skin and digestive system) because of hypomethylation of the pericentromeric satellite DNA, which causes centromeric decondensation and chromosomal rearrangements.⁵ In addition, faithful maintenance of the DNA-methylation pattern and the enzymes that bind methylated CpG residues are essential for proper functioning and survival of the CNS.^{6–8}

Excessive DNA methylation may also be responsible for gene silencing at rare folate-sensitive fragile sites. Fragile sites are breakage-prone spots in the DNA that express as gaps and breaks in chromosomes when cells are grown in vitro under specific cell-culture conditions. Rare fragile sites are further subdivided according to specific culture conditions by which they are induced. Six folate-sensitive, rare fragile sites have been characterized at the molecular level—namely, FRAXA,⁹ FRAXE,¹⁰ FRAXF,¹¹ FRA11B,¹² FRA16A,¹³ and, more recently, FRA10A.¹⁴ All six were shown to be associated with an expanded CGG repeat. After repeat expansion, the CGG repeat and the surrounding CpG island located in the promoter become hypermethylated. Several of the rare folate-sensitive fragile sites have been associated with cognitive dysfunction; for example, fragile X syndrome, caused by expression of the fragile site FRAXA, is characterized by mental retardation and specific dysmorphic and behavioral features. Expansion of the FRAXE repeat is associated with a mild form of mental retardation. In vivo chromosome breakage at or near the *FRA11B* locus has been implicated in Jacobsen syndrome, characterized by mental retardation and specific associated abnormalities.

In the present study, we have cloned the fragile site FRA12A, another rare folate-sensitive fragile site that has been linked to mental retardation (for an overview, see the work of Berg et al.¹⁵), and we have identified the associated gene. The protein product of the FRA12A-associated gene *DIP2B* contains a DMAP1-binding domain and might thus be involved in DNA-methylation processes.

Subjects and Methods

Clinical Diagnosis and Chromosome Analysis

Family A The proband (III.1; [fig. 1A](#)) was a 5-year-old girl who had global delay in her neurodevelopment during infancy, significant learning disability, subtle craniofacial dysmorphisms, and recurrent, inexplicable chest infections.¹⁵ She had bullous ichthyosiform erythroderma and areas of hyperkeratosis, particularly around the knees, elbows, wrists, and neck. Both the mother (II.2; [fig. 1A](#)) and the grandmother (I.2; [fig. 1A](#)) of the proband were phenotypically unaffected. Examination of metaphase chromosome spreads from the proband revealed a fragile site at 12q13 in 8 of the 20 metaphases examined. An identical fragile site was observed in metaphase chromosomes of the mother (4 of 20) and the grandmother (4 of 40). After receipt of informed consent, a peripheral-blood sample from the grandmother was obtained. Epstein-Barr virus (EBV)–transformed lymphoblastoid cell lines of the proband and her mother were obtained from the European Collection of Cell Cultures.

Family B The proband (subject II.2; [fig. 1B](#)) was a 15-year-old mentally retarded boy who had epileptic seizures and serious behavioral problems.¹⁶ A fragile site at 12q13 was seen in 20 of 50 of his

metaphase chromosomes. His mother (subject I.2; [fig. 1B](#)) and sister (subject II.1; [fig. 1B](#)) showed the same fragility in 13 of 50 and 11 of 50 of their metaphase chromosomes, respectively. Yet they did not show any phenotypical abnormality. Peripheral-blood samples from the proband and his mother were obtained, with informed consent. From these peripheral-blood samples, EBV-transformed lymphoblastoid cell lines were established at our laboratory.

Family C Subject II.1 ([fig. 1C](#)) of this family was a 35-year-old phenotypically unaffected man. The fragile site FRA12A was detected in peripheral-blood cells with a frequency of 2 of 63 metaphases in standard low-folate medium.¹⁷ When we added methotrexate and *BrdU* to the standard medium, frequency was increased to 11 of 50 metaphases. The addition of *FudR* also induced the FRA12A site in fibroblast cells, with a frequency of 8 of 77 metaphases. Peripheral-blood cells of his mother also displayed the site in standard medium (4 of 63 metaphases). Peripheral-blood samples from subject II.1 were obtained after informed consent, and we established an EBV-transformed lymphoblastoid cell line. None of the children of subject II.1 inherited the FRA12A fragile site.

FISH Mapping

Slides for FISH analysis were made from fixed cell suspensions of subject I.1, by standard methods. BAC clone RP11-831J22 (Roswell Park Cancer Institute library) was obtained from the BACPAC Resource Center. The probe was labeled with digoxigenin-11-2-deoxyuridine 5-triphosphate (dUTP) or biotin-16-dUTP (Roche) by nick translation. DNA hybridization and antibody detection were performed in accordance with standard protocols. At least five metaphases were analyzed for each hybridization, with a Zeiss Axioplan 2 fluorescence microscope equipped with a triple band-pass filter (#83000 for 4,6-diamidino-2-phenylindole, fluorescein isothiocyanate, and Texas Red [Chroma Technology]). Images were collected using a cooled CCD camera (Princeton Instruments Pentamax [Roper Scientific]) and were analyzed using IPLab software (Scanalytics).

PCR Amplification and Hybridization of the FRA12A-Associated CGG Repeat

PCR amplification of the normal allele of the FRA12A repeat-containing region was performed with the aid of 2.5×PCR× Enhancer solution (Invitrogen) with use of primers derived from the sequences flanking the repeat (DIP2B-CGG-F primer, 5'-GTCTTCAGCCTGACTGGGCTGG-3', and DIP2B-CGG-R, primer 5'-CCGGCGACGGCTCCAGGCCTCG-3'). To amplify the expanded allele, a PCR with use of deaza-dGTP (2-deoxyguanosine 5-triphosphate) instead of common dGTP was performed as described by Sarafidou et al.¹⁴ The expanded allele could not be detected on an agarose gel. Therefore, the PCR products were electrophoresed on a 1.5% agarose gel and, after denaturation and neutralization, were transferred to Hybond N⁺ membranes. Hybridization was performed at 65°C with use of a 255-bp PCR CGG probe, produced using primers DIP2B-CGG-F and DIP2B-CGG-R. Triplet-repeat primed PCR was performed according to the principles described by Warner et al.,¹⁸ to detect CAG-repeat expansion. We adapted their protocol by using 2.5×PCR× Enhancer solution (Invitrogen), to be able to amplify the CGG repeat. Three different primers were added to the PCR mixture: a single forward fluorescently labeled primer (DIP2B-CGG-F) and a combination of two reverse primers (P4, 5'-TACGCATCCCAGTTTGAGACGGCCGCGCCGCGCCGCGC-3', and P3, 5'-TACGCATCCCAGTTTGAGACG-3') in a 1:10 ratio. The reverse primer P4 anneals at different sites of the CGG repeat, which produces PCR products of different lengths that differ from each other by a multiple of three residues. After depletion of the P4 primer, the P3 primer takes over and amplifies the PCR products of different lengths. PCR products were size fractionated on a Prism ABI 3100 DNA sequencer (Applied Biosystems). The methylation status of the CGG repeat and the surrounding region was analyzed by bisulphite sequencing. Genomic DNA was bisulphite treated using the EZ DNA methylation kit

(Baseclear). Bisulphite treatment converts all nonmethylated cytosines into thymines, whereas methylated cytosines remain unchanged. Primers specific for the methylated bisulphite-converted DNA were developed (*DIP2B*-FM, 5'-GATAGATATATATTGTTAAGTTTAG-3', and *DIP2B*-RM, 5'-CCGCATAAAACAAAATCACGTAAACG-3'). After PCR amplification, the CGG surrounding area was sequenced using primers *DIP2B*-FM and *DIP2B*-RM on a Prism ABI 3100 DNA sequencer (Applied Biosystems).

Gene-Expression Analysis

We used a multiple-tissue northern blot (FirstChoice Northern Human Blot I [Ambion]) to determine the size of the *DIP2B* transcript. The specific *DIP2B* probe was an 855-bp cDNA PCR product (forward primer, 5'-CCGCAGACACAAGAACTGA-3', located at the exon 2-exon 3 boundary; reverse primer, 5'-AGCAGGAGGCCAGTTACAGA-3', located in exon 8) and was radiolabeled by the addition of [α -³²P]dATP and [α -³²P]dCTP (MP). Hybridization was performed according to the manufacturer's instructions. Detailed information on the expression pattern of the *DIP2B* gene was obtained from a multiple tissue expression (MTE) array (BD Biosciences) that was hybridized to the radiolabeled 855-bp *DIP2B* cDNA probe. Hybridization was performed according to the manufacturer's instructions.

The European Bioinformatics Institute ([EBI](#)) database was searched for coding SNPs (cSNPs) within the coding sequence of the *DIP2B* gene. The presence of cSNPs in the FRA12A carriers and patients was tested by sequencing at the genomic level. RNA was isolated for EBV-transformed lymphoblastoid cells of the FRA12A carriers through use of Trizol (Invitrogen) and was converted to cDNA through use of Superscript III reverse transcriptase (Invitrogen). cDNA was tested for the presence of cSNPs in FRA12A carriers and patients by PCR followed by sequencing. The following primer sets were used: for exon 16, 5'-GATGCACACAATCAGCGTAC-3' (forward) and 5'-TCAGGCTTCAGTCCATGACT-3' (reverse); for exon 20, 5'-AATCTGTGTTAGCTCCAGAACT-3' (forward) and 5'-TCTACAGCCAATCCAGTAGC-3' (reverse); for exon 24, 5'-CTCCACTAGGAGGAATCCAT-3' (forward) and 5'-ACTGTAGGATCTCTGCCAGA-3' (reverse); and, for exon 37, 5'-AGCGTCATGATGCATTGTATG-3' (forward) and 5'-CCAACGATGAGGTAATGCTC-3' (reverse). Big Dye Terminator sequencing was performed, and sequencing products were analyzed using an ABI 3100 (Applied Biosystems) automated sequencer.

Real-time quantitative PCR was performed using gene-specific Assays-on-Demand containing two PCR primers (forward and reverse) and a TaqMan MGB probe (FAM dye labeled), obtained from Applied Biosystems. Seven independent control individuals were included in the first experiment, and five control individuals in the second experiment. PCR products were synthesized from RNA samples first converted to cDNA, through use of the qPCR Mastermix Plus without UNG (Eurogentec). The reactions were run on an ABI Prism 7000 sequence-detection system. The thermal cycler conditions were 2 min at 50°C and 10 min at 95°C, followed by 40 cycles at 95°C for 15 s and 60°C for 1 min. The raw fluorescence data were analyzed using the Sequence Detection Software (SDS [Applied Biosystems]). The SDS results were subsequently exported as comma-separated value files and were imported into the relative quantification software [qBase](#) for further analysis. For normalization of the quantitative expression values, the geometric mean of three housekeeping genes (*GAPD*, *UBC*, and *YWHAZ*)¹⁹ were used. The final results were checked by the nonparametrical Mann-Whitney *U* test.

Sequencing the *DIP2B* Gene

All 38 exons of the *DIP2B* gene were PCR amplified at the genomic level. All primers used for mutation analysis of the *DIP2B* gene can be obtained from the authors on request. Big-Dye Sequencing products

were analyzed on an ABI 3100 (Applied Biosystems) automated sequencer.

Results

Cytogenetic Expression of FRA12A and Likelihood of Mental Retardation

The percentage of cells expressing FRA12A on cytogenetic analysis was available for 41 individuals from 16 published families ([table 1](#)). In seven of the individuals with significant neurocognitive problems, the average level of expression was 43.7% (95% CI 33.6%–54.8%). In two of these individuals, the FRA12A expression was de novo; for three individuals, information about the parents was not available; and the remaining two individuals with mental retardation inherited the fragile site maternally, and approximately half of the cells of the unaffected mother expressed FRA12A. The average level of FRA12A expression in the individuals with FRA12A but without mental retardation was 16.6% (95% CI 14.3%–19.0%). Thus, the percentage of cytogenetic expression is significantly associated with the clinical phenotype of the patients.

FISH Mapping of FRA12A

Through use of FISH experiments on metaphase chromosome spreads obtained from lymphoblastoid cells of proband III.1 of family A ([fig. 1A](#)), a single BAC clone RP11-831J22 (49080K–49260K) (National Center for Biotechnology Information [[NCBI](#)]) spanning the fragile site FRA12A was identified ([fig. 2](#)). Since all hitherto cloned folate-sensitive rare fragile sites are caused by a CGG-repeat expansion, the identified BAC clone was searched for CGG repeats. A single (CGG)₇ repeat was identified, located in the CpG island at the 5' end of the Disco-interacting protein 2 homologue *DIP2B* gene (alternate names *KIAA1463*, *FLJ34278*, and *MGC104005*). The *DIP2B* gene maps to chromosome 12q13.12.

Analysis of the *DIP2B* CGG Candidate Repeat

PCR-based amplification of the *DIP2B* candidate CGG repeat in 70 controls—a total of 140 chromosomes—revealed 13 different alleles, with a range of 6–23 CGG-repeat units; the most common allele contained 6 repeats ([fig. 3](#)). We analyzed six individuals with cytogenetic expression of the fragile site FRA12A (subjects I.2, II.2, and III.1 of family A; subjects I.2 and II.2 of family B; and subject II.1 of family C). A single CGG allele in the normal-size range was identified in all FRA12A carriers through use of primers flanking the repeat and standard PCR techniques. Triplet-repeat primed-PCR analysis¹⁸ did show a characteristic ladder on the fluorescence trace, with a periodicity of 3 bp, for all FRA12A-expressing individuals but not for controls, which indicates repeat expansion (not shown). Subsequent PCR amplification incorporating deaza-dGTP, followed by Southern blotting and hybridization with use of a CGG-repeat probe, confirmed the presence of an expanded allele in all patients and carriers with FRA12A ([fig. 4](#)). This experiment was repeated several times, with comparable outcomes. All individuals expressing FRA12A showed much larger PCR fragments, indicating CGG-repeat expansion. The length of the expanded allele in the unaffected FRA12A carriers (subjects I.2 and II.2 of family A and subject I.2 of family B) exceeded that in control individuals (250 bp) by at least 400–600 bp. In the two patients with FRA12A (subject III.1 of family A and subject II.2 of family B), the length of the expanded allele exceeded the normal length by 800–900 bp.

To assess the methylation status of the expanded CGG allele and the surrounding CpG island, a 304-bp region of the promoter CpG island was subjected to bisulphite sequencing. The Gene2Promotor program ([Genomatix](#)) identified a 601-bp promoter region starting at –656 bp before the start codon of the *DIP2B* gene. The FRA12A-associated CGG repeat and the surrounding CpG island lie within the

predicted promotor. The bisulphite sequencing method allowed a detailed analysis of methylation in a particular region by converting all nonmethylated cytosines into thymines; methylated cytosines remained unchanged. In control individuals, the promotor region was unmethylated. In family A, this promotor region was fully methylated in FRA12A-affected patient III.1 but was unmethylated in the patient's unaffected FRA12A-expressing mother (subject II.2) and grandmother (subject I.2). In family B, methylation of the 5' promotor region of the *DIP2B* gene was detected in FRA12A-affected patient II.2 and his FRA12A-carrier mother (subject I.2). Also in the unaffected FRA12A carrier (subject II.1) of family C, the promotor region was methylated ([table 2](#)). All cytosines at CpG dinucleotides in the *DIP2B* promotor in the patients and carriers were methylated as indicated in [figure 5](#).

DIP2B Protein

The *DIP2B* gene contains 38 exons, which encode a putative 1,576-aa protein. The *DIP2B* gene is a member of the Disco-interacting 2 family ([Ensembl](#) family ENSF00000000904), which contains two additional members: *DIP2A* on chromosome 21q22.3 and *DIP2C* on chromosome 10p15.3. Percentage similarities, as calculated by the program Align ([EBI](#)), are listed in [table 3](#). The DIP2B protein contains three putative functional domains: one DMAP1-binding domain (location 14–131) and two AMP-binding domains (locations 370–822 and 1025–1495) ([fig. 6](#)). The conserved domain database ([NCBI](#)) points out that the DMAP1-binding domain is highly conserved throughout the eukaryotic kingdom ([fig. 7](#)), an indication of its important function.

DIP2B Gene Expression

For northern-blot analysis, we used a specific *DIP2B* probe that contained exons 3–7. A single transcript of 9 kb, which corresponds well to the predicted cDNA size, was detected in several human tissues ([fig. 8](#)). *DIP2B* expression was found in brain, placenta, skeletal muscle, heart, kidney, pancreas, lung, spleen, and colon. Hybridization of an MTE array (data not shown) indicated that the *DIP2B* gene is expressed in several brain regions: the cerebral cortex; the frontal, parietal, occipital, and temporal lobes; the paracentral gyrus; the pons; the corpus callosum; and the hippocampus. Highest expression levels in the brain were found in the cerebral cortex and the frontal and parietal lobes.

To detect possible differences in the expression levels of *DIP2B* among FRA12A-affected patients, FRA12A carriers, and control individuals, real-time PCR was performed. To have a representative sampling, cDNA of each FRA12A carrier and patient was prepared three times independently, and each preparation was analyzed in duplicate ([fig. 9](#)). This procedure was repeated, with comparable results ([table 4](#)). As expected, for both patients with FRA12A, *DIP2B* expression was reduced to about half the amount of that in controls (55% [$P=.002$] and 45% [$P=.001$] in the first and second experiment, respectively). For subject II.2 of family A, a carrier of an unmethylated FRA12A expansion, real-time PCR results revealed enhanced expression values (200% [$P=.009$] and 142% [$P=.013$]). Subjects I.2 of family B and II.1 of family C, both carriers of a methylated FRA12A expansion, showed a reduction of *DIP2B* expression, approximately two-thirds of that of controls (67% [$P=.056$] and 63% [$P=.001$]).

To verify whether the reduced gene expression in patients with FRA12A is due to the silencing of the expanded allele, cSNP analysis was performed by sequencing. Four cSNPs were found in the *DIP2B* cDNA sequence ([GenBank](#) accession number [NM_173602](#)): one at position 2073 in exon 16 (c.2073A/T), one at position 2530 in exon 20 (c.2530A/G), one at position 3102 in exon 24 (c.3102T/C), and one at position 4587 in exon 37 (c.4587C/T). All FRA12A carriers and patients were tested for these cSNPs. The cSNP in exon 16 appeared informative in DNA of carrier I.2 of family B (genotype g.2073A/T). By sequencing the cDNA of subject I.2, only the c.2073A allele could be detected, indicating the

transcriptional silencing of the T allele. Determination of the inheritance in family B demonstrated that it is the FRA12A allele that is silenced: descendant II.2 of carrier I.2 has the genotype g.2073T/T. The fact that he expresses FRA12A indicates that the c.2073T allele is linked to the CGG-repeat expansion.

Finally, all exons of the *DIP2B* gene were sequenced at the DNA level, and intron/exon boundaries were checked for both FRA12A-affected patients. No sequence alterations could be detected in the *DIP2B* coding sequences.

Discussion

Our study provides evidence that the molecular basis of the rare folate-sensitive fragile site FRA12A is a CGG-repeat expansion at the 5' end of the *DIP2B* gene. The *DIP2B* CGG repeat was shown to be expanded in individuals expressing the FRA12A site. An expanded CGG repeat has also been found in previously cloned folate-sensitive rare fragile sites. The FRA12A CGG repeat, located in the CpG island in the promotor region of *DIP2B*, is polymorphic in the general population and is expanded on transmission to the next generation in the FRA12A-affected families we analyzed.

The putative *DIP2B* protein contains a highly conserved DMAP1-binding domain and two AMP-binding domains. The AMP-binding domains are rich in serine, threonine, and glycine and possess a catalytic activity. The DNMT1-associated protein (DMAP1) was discovered recently and has a transcription-repressor activity.³¹ The chief enzyme that maintains DNA methylation of the mammalian genome during S phase is DNA (cytosine-5)-methyltransferase 1 (DNMT1).^{32,33} The carboxy-terminal catalytic domain of DNMT1 has a strong affinity for hemimethylated DNA and transfers methyl groups from S-adenosylmethionine to cytosines in CpG regions, whereas the N-terminal portion directs the enzyme to the replication foci. More recently, it has been shown that the noncatalytic N-terminal region of DNMT1 also forms a repressive transcription complex consisting of DNMT1, the histone deacetylase HDAC2, and the DNMT1-associated protein DMAP1. The DMAP1-*DIP2B* interaction may modify the DNMT1 enzymatic activity to perform an effective repression by methylation. If this is the case, the methylation of the *DIP2B* promotor is able to directly influence the DNMT1 methylation machinery itself.

The *DIP2B* protein is a member of the *DIP2* protein family. We demonstrated expression of *DIP2B* in the brain. In situ hybridization of the mouse *dip2* homologue suggested that *mdip2* may participate in defining the segmental borders in the CNS and may have a role in providing positional cues for axon pathfinding and patterning.³⁴ A *Drosophila* homologue of *DIP2B*, discovered by the yeast-2-hybrid interaction-trap technique, is a protein that interacts with the *Drosophila disconnected (disco)* gene shown to be required for proper neuronal connections in both the larval and adult visual system of *Drosophila*.^{35,36}

For FRAXA, the most extensively studied folate-sensitive rare fragile site, a clear-cut relationship exists in males between fragile-site expression and disease. Patients with fragile X express the FRAXA fragile site in vitro because of large CGG-repeat expansions, which cause hypermethylation of the promotor region of the underlying *FMR1* gene. These so-called full mutations originate from smaller expansions of the *FMR1* CGG repeat (premutations), which are unmethylated and do not cause fragile X syndrome. A comparable situation is described for family A, where a repeat grows stepwise in three generations to a methylated CGG-repeat expansion in the youngest generation. It is tempting to speculate that the de novo gene silencing of the *DIP2B* gene is causative of the mental retardation in this patient. A relationship between fragile-site expression and mental retardation was predicted, since patients with cytogenetic FRA12A expression and mental retardation expressed the fragile site in a significantly higher percentage of cells than did unaffected FRA12A carriers. However, in family B, the unaffected mother of the patient (subject I.2) has a methylated repeat expansion as well, which could imply that

the coexistence of *DIP2B* methylation and the mental retardation in patient II.2 is purely coincidental. However, it can also be speculated that disease penetrance is not 100%. This phenomenon is also seen in FRAXE mental retardation, where expansion and methylation of the CGG repeat in front of the *FMR2* gene is not always associated with mental retardation.³⁷ Some evidence of nonpenetrance is provided by investigation of the *DIP2B* expression in our FRA12A-affected families; it seems to suggest that gene expression in unaffected carriers is higher than in patients.

Other proteins involved in methylation metabolism have been implicated in disease as well. Mutations in the methyl-CpG binding protein 2 gene (*MECP2*) cause Rett syndrome, a form of severe mental retardation in females. *MECP2* is a transcriptional repressor that binds to methylated CpG residues and forms chromatin remodeling complexes with various transcriptional repressors.^{38–40} Absence of the *MECP2* protein in mice results in neurological abnormalities and premature death.^{41,42} Additional evidence of the importance of the DNA methylation machinery for the brain comes from studies on *Dnmt1*-knockout mice. Normal mice contain surprisingly high levels of *Dnmt1* in neurons,^{43–46} whereas absence of this enzyme in the brain results in death shortly after birth.⁴⁷ Moreover, in brains containing both normal and *Dnmt1*-negative cells, the *Dnmt1*-negative cells are rapidly lost and mutant neurons are quickly eliminated.⁴⁷ Mutations in one of the methyltransferases, the *DNMT3B* gene, causes ICF, in which neurological abnormalities are also present.⁴⁸ Finally, additional suggestions for a link between DNA methylation defects and brain disorders come from the ATR-X syndrome (α -thalassemia/mental retardation syndrome, X linked), characterized by severe psychomotoric retardation, characteristic facial features, genital abnormalities, and α -thalassemia. It was shown that the syndrome is caused by mutations in the *ATR-X* gene, resulting in changes in the methylation pattern of several highly repeated sequences, including the rDNA arrays, a Y-specific satellite, and subtelomeric repeats.⁴⁹ More recently, the *ATR-X* gene has also been shown to be involved in other X-linked mental retardation syndromes.^{50,51}

A second interesting finding, although based on a single case, is the observation that the elongated, unmethylated CGG repeat of carrier II.2 for family A has a 1.4–2-fold overexpression of the *DIP2B* gene. This implies two-to-threefold overexpression of the expanded allele, with the assumption of unaffected expression of the unexpanded allele. A similar situation is found in fragile X premutation carriers, who have an enhanced expression of the *FMR1* mRNA of unmethylated elongated repeats. Our data thus suggest that CGG-repeat expansion in general may act as a transcriptional enhancer. Males with premutation fragile X alleles are at risk for a neurodegenerative disorder called “FXTAS” (fragile X-associated tremor/ataxia syndrome).⁵² It would therefore be of interest to determine the phenotype of aged FRA12A carriers.

In conclusion, we report the identification of the molecular cause of the fragile site FRA12A. The associated *DIP2B* gene possesses a DMAP1-binding domain and might therefore be involved in the methylation machinery. Our finding warrants further investigations of the possible role of the *DIP2B* gene in mental retardation.

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Web Resources

Accession numbers and URLs for data presented herein are as follows:

EBI, <http://www.ebi.ac.uk/> (for the Align program)

Ensembl, <http://www.ensembl.org/>

GenBank, <http://www.ncbi.nlm.nih.gov/Genbank/> (for *DIP2B* cDNA sequence [accession number NM_173602], *R. norvegicus* protein similar to DIP2B [accession number XP_235656], *M. musculus* LOC239667 [accession number NP_766407], *B. taurus* protein similar to DIP2B, partial [accession number XP_580943.1], *M. fascicularis* protein similar to DIP2B, partial [accession number BAC41762], *H. sapiens* DIP2C [accession number NP_055789], and *H. sapiens* DIP2A [accession number NP_055966])

Genomatix, <http://www.genomatix.de/> (for the Gene2Promotor program)

NCBI, <http://www.ncbi.nlm.nih.gov/>

qBase, <http://medgen.ugent.be/qbase/>

UniProtKB/Swiss-Prot, <http://au.expasy.org/uniprot/> (for Q8BWT5)

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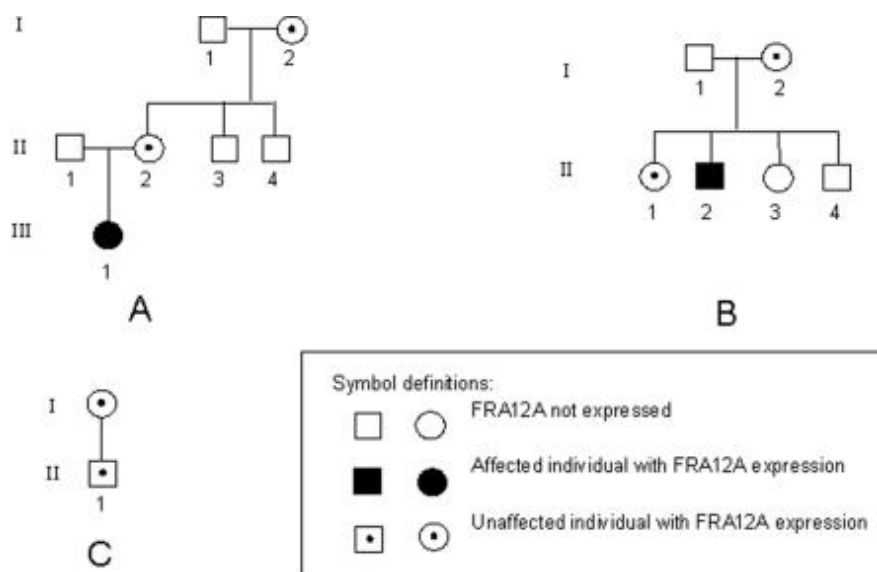
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Figures and Tables

Figure 1.



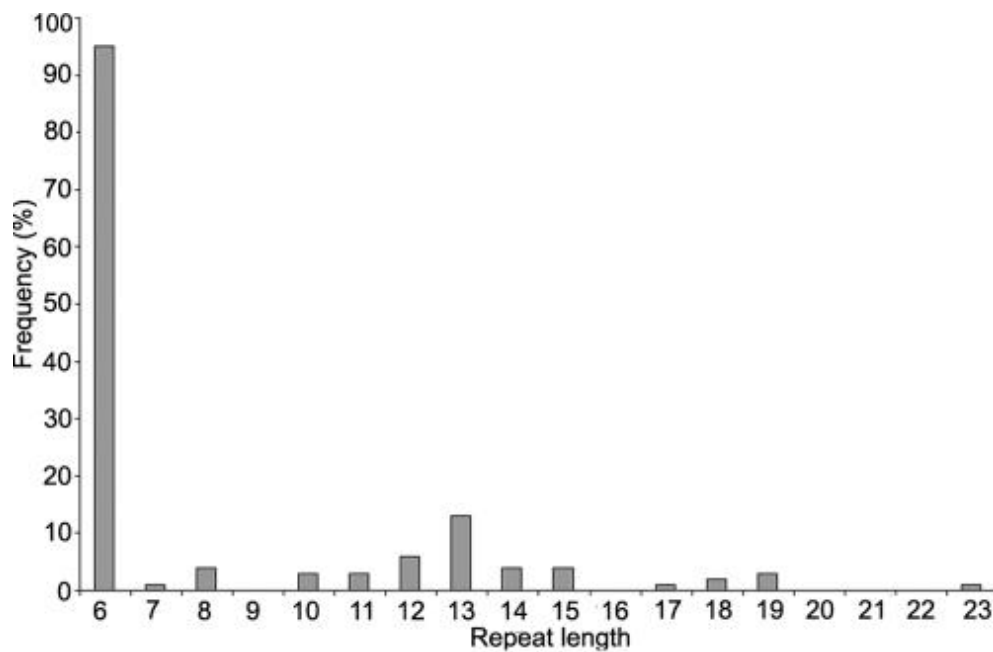
Family pedigree of FRA12A-expressing families A–C

Figure 2.



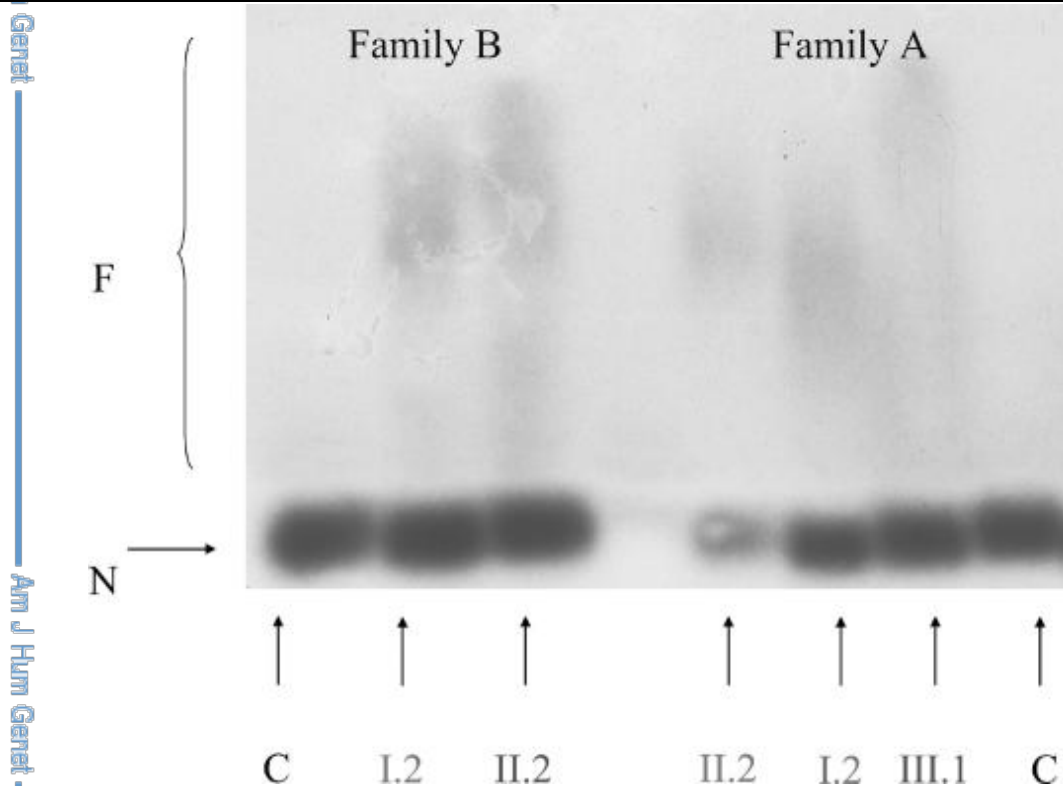
FISH mapping of BAC clone RP11-831J22, spanning the fragile site FRA12A in patient I.1. The fragile site FRA12A is indicated by an arrow.

Figure 3.



Allele frequencies of the FRA12A-associated 5' CGG repeat in a population of 70 control individuals.

Figure 4.



Analysis of the *DIP2B* CGG repeat in controls (“C”) and individuals expressing the FRA12A fragile site (in family B, subjects I.2 and II.2; in family A, subjects I.2, II.2, and III.1). In addition to a normal allele (“N”; 250 bp), individuals who cytogenetically express the FRA12A site show additional PCR fragments of larger size (“F”), indicating repeat expansion. The length of the expanded allele in the unaffected FRA12A carriers (subjects I.2 and II.2 of family A and subject I.2 of family B) is 650–850 bp. In the two patients with FRA12A (subject III.1 of family A and subject II.2 of family B), the length of the expanded allele is >1,050–1,150 bp.

Figure 5.

A

TAAAG**CGG**ACAGTCCATACCATGAG**CCC**ATTT**CGCG**CAGGGAAA
 TT**CGAG**TGGTGGGCTTGGG**CCCGT**CGTTCTGGTAGCTT**CCC**AGTA
 GAGGG**CGCCG**TAAGCATATG**CGT**CTTCAG**CC**TGACTGGGCTGGA
 GAGG**CGGTGCGT**CCTCCTGG**CCG**GGGGG**CGGGGGCCGT**

B

TAAAG**TCGAT**AGT**TT**ATA**TT**ATGAG**TTT**ATTT**CGCGT**AGGGAAAT
 TCGAGTGGTGGG**TTT**GGG**TT**CGT**CGT**TTTGGTAG**TTTT**AGTAG
 AGGG**CGT**CGTAAGTATATG**CGT**TTTTAG**TT**TGAT**TT**GGG**TT**GGAGA
 GG**CGGTGCGT**TTTTTTGGT**CGGGGGGCGGGGGT**CGT

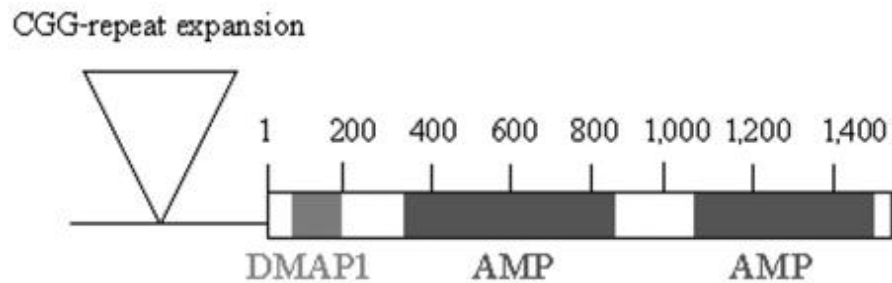
C

TAAAG**TTGAT**AGT**TT**ATA**TT**ATGAG**TTT**ATTT**TGT**AGGGAAAT
 TTGAGTGGTGGG**TTT**GGG**TTT**GT**T**TTGGTAG**TTTT**AGTAGA
 GGG**TGT**TGTAAGTATATG**TGT**TTTTAG**TT**TGAT**TT**GGG**TT**GGAGAG
GTGGTGTGTTTTTTTGG**TTGGGGGGTGGGGGTTGT**

A, Sequence of the *DIP2B* promoter region. All non-CpG cytosines are indicated in dark blue; CpG cytosines are indicated in green. **B**, Sequence of the *DIP2B* promoter after bisulphite treatment in FRA12A-expressing individuals with a methylated CGG-repeat expansion. All nonmethylated cytosines are converted into thymines (*blue*), whereas

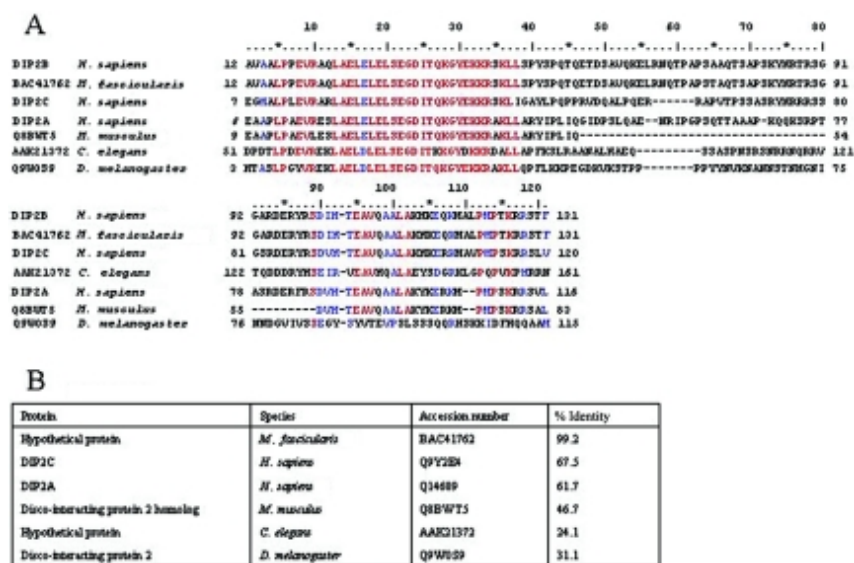
methylated cytosines remain unchanged (*red*). *C*, Sequence of the *DIP2B* promoter after bisulphite treatment in FRA12A-expressing individuals with an unmethylated repeat expansion. All cytosines are converted into thymines (*blue*).

Figure 6.



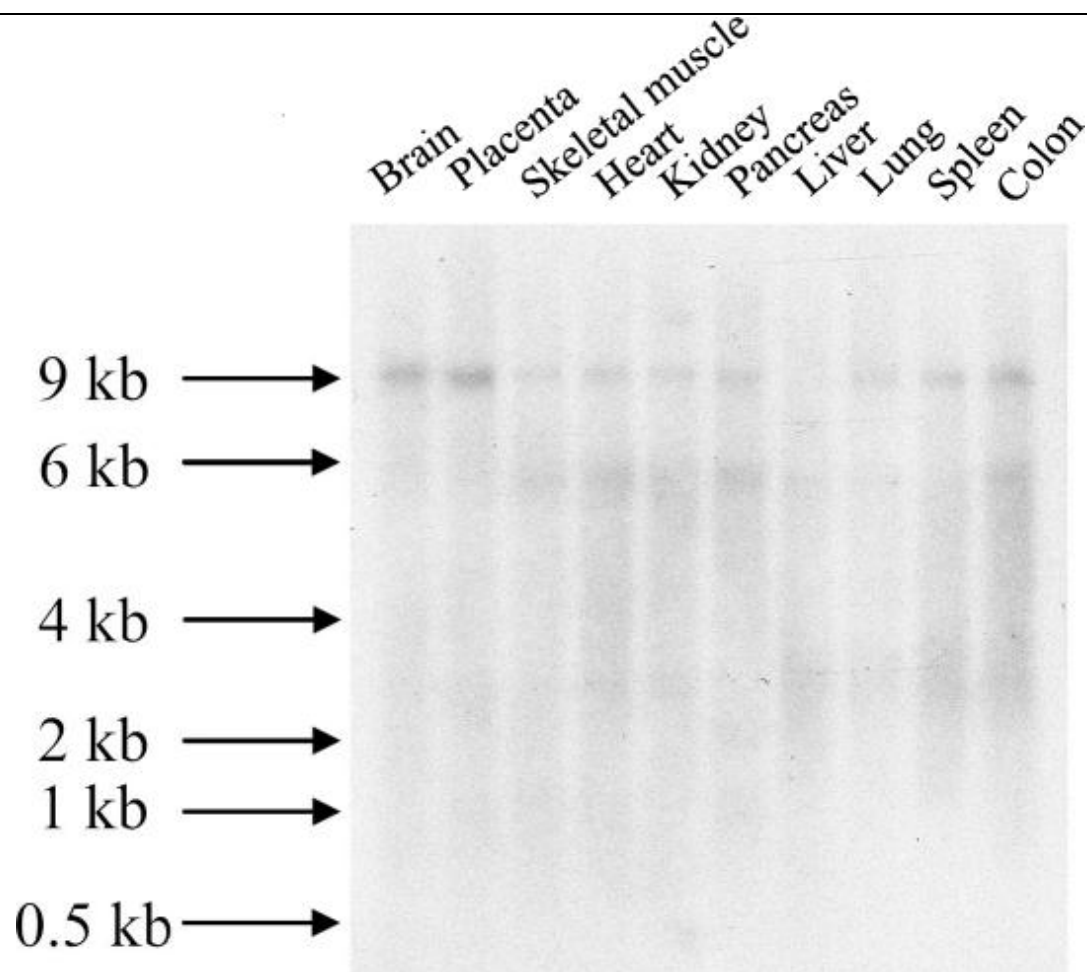
Position of the three conserved domains in the DIP2B protein.

Figure 7.



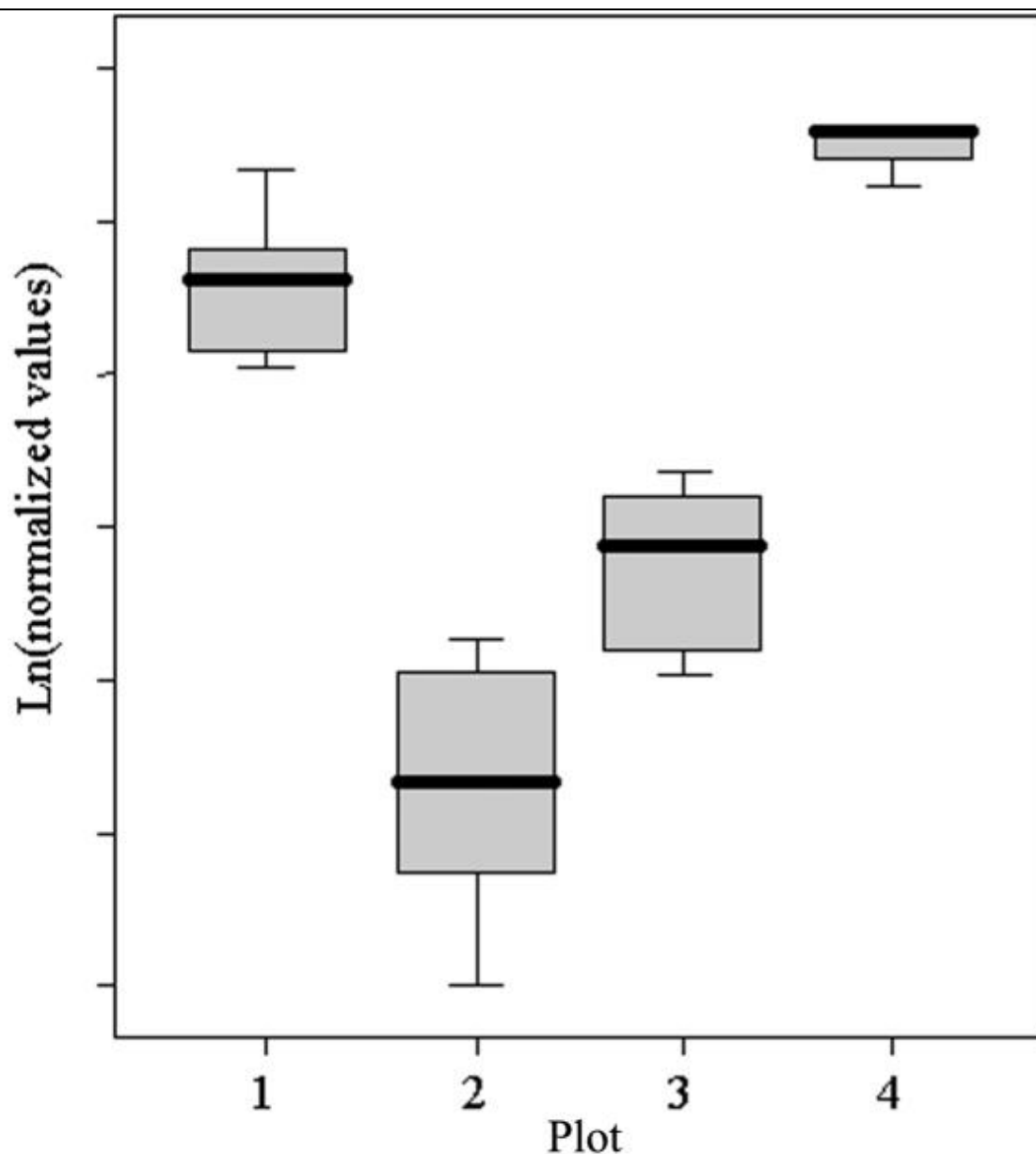
A, Alignment of the six most similar homologues of the DMAP1-binding domain of the DIP2B protein. Amino acid–sequence positions are indicated at the beginning and end of each line. Identical amino acids are indicated in red, and the similar ones in blue. The source species and the accession number of the sequence are indicated at the left of each line. *B*, Percentage similarity among the DIP2B DMAP1-binding domain and its homologues.

Figure 8.



Northern-blot analysis of the *DIP2B* gene. A specific cDNA probe was hybridized to a human multiple-tissue northern blot. A single transcript of 9 kb was detected in various human tissues.

Figure 9.



Box plot showing the significant difference in the normalized amount of RNA for *DIP2B* in function of the methylation status and the disease status. Plot 1 represents control individuals, plot 2 represents patients with a methylated repeat expansion, plot 3 represents healthy individuals with a methylated repeat expansion, and plot 4 represents a healthy individual with an unmethylated repeat expansion. The significance was tested using one-way ANOVA (analysis of variance).

Table 1.

Overview of FRA12A Expression of 41 Individuals from 16 Published Families

Family and Subject Sex	Percentage of Cells with FRA12A Expression	Phenotype and Remarks	Reference (Year)
1:			Giraud et al. ²⁰ (1976)

1	F	N		Mother of baby with lethal arthrogryposis, baby had no evidence of FRA12A (subject 9)	
2:					Giraud et al. ²⁰ (1976)
2	M	Y		Male with MR and multiple congenital abnormalities (not detailed) (subject 10)	
3:					Stetten et al. ²¹ (1988)
3	F	N		Woman, aged 38 years, with two miscarriages	
4:					Smeets et al. ¹⁶ (1985)
4	M	Y	40	Male with MR (40% of cells), unaffected sister (22% of cells), and unaffected mother (26% cells)	
5	F	N	22	Unaffected sister of subject 4	
6	F	N	26	Unaffected mother of subject 4	
5:					Moric-Petrovic and Laca ²² (1984)
7	M	Y		Male with MR, hypotonia, cryptorchidism, and micropenis; neither parent had fragile site	
6:					Donti et al. ²³ (1979)
8	F	Y	30	Female with MR, hypotonia, facial dysmorphisms, arachnodactyly, intracranial calcification; neither parent had fragile site	
7:					Mules et al. ¹⁷ (1983)
9	M	N		Incidental finding in the father of child with ring chromosome 13	
10	F	N	8	Mother of subject 9; low-folate culture failed, 4 of 63 FRA12A seen on standard-culture cultured fibroblasts 2 of 63 in subject 9	
8:					Sutherland and Hecht ²⁴ (1985)
11	M	N	13	Phenotypically unaffected male neonate	
12	F	N	14	Phenotypically unaffected mother of subject 11	
13	F	N	24	Phenotypically unaffected maternal grandmother of subject 11	
14	M	N	12	Phenotypically unaffected maternal great-grandfather of subject 11	
9:					Sutherland and Hinton ²⁵ (1981)
15	M	N	16	Found in family with autosomal dominant epidermyolysis bullosa simplex (EBS), unaffected	
16	M	N	16	Brother of subject 15; unaffected with EBS	
17	F	N	16	Sister of subject 15; affected with EBS	
18	F	N	28	Mother of subject 15; affected with EBS	

19	F	N	12	Maternal aunt of subject 15	
20	F	N	18	Maternal first cousin of subject 15	
21	M	N	30	Maternal first cousin of subject 15	
22	F	N	14	Maternal aunt of subject 15; unaffected with EBS	
23	F	N	11	Maternal first cousin of subject 15	
24	F	N	12	Maternal grandmother of subject 15; affected with EBS	
25	M	N	8	Maternal great-uncle of subject 15	
10:					Kumar et al. ²⁶ (1986)
26	F	N	30	Probably incidental finding in a woman with craniofrontonasal dysplasia	
27	F	N	27	Unaffected sister of subject 26	
28	F	N	20	Mother of subject 26; also affected with craniofrontonasal dysplasia	
11:					Webb et al. ²⁷ (1987)
29	F	Y		Female with mild nondysmorphic MR; parents not available	
12:					Guichaoua et al. ²⁸ (1982)
30	F	N		Mother of an infant with multiple malformations	
13:					Guichaoua et al. ²⁸ (1982)
31	M	Y		Male, aged 7 years, with dysmorphic features	
14:					Giardino ²⁹ (1990)
32	M	N	9	Male whose wife had multiple miscarriages	
33	F	N	14	Unaffected daughter of subject 32	
34	F	N	16	Unaffected daughter of subject 32	
15:					Kahkonen ³⁰ (1989)
35	F	N	25	Unaffected female; coincidental finding on investigation of familial t(X;5)	
36	F	N	12	Unaffected mother of subject 35	
37	F	N	18	Unaffected maternal aunt of subject 35	
38	M	N	10	Unaffected maternal uncle of subject 35	
16:					Berg et al. ¹⁵ (2000)
39	F	Y	40	Girl, aged 11 years, with MR, pulmonary stenosis, and bullous ichthyosiform erythroderma	
40	F	N	20	Mother of subject 39; unaffected	
41	F	N	2	Maternal grandmother of subject 39; unaffected	

^aMental retardation (MR) or developmental delay.

Table 2.

Overview of Methylation and Disease Status of FRA12A-Expressing Individuals

Family and Subject Methylation	Status
A:	
II.2	No Unaffected
III.1	Yes Affected
B:	
I.2	Yes Unaffected
II.2	Yes Affected
C:	
II.1	Yes Unaffected

Table 3.

Percentage Identity among the Human DIP2B Protein and Other Homologous Protein Sequences

Protein and Species	Accession Number	Percentage Identity
Protein similar to DIP2B:		
<i>Rattus norvegicus</i>	XP_235656 (GenBank)	97.5
LOC239667:		
<i>Mus musculus</i>	NP_766407 (GenBank)	96.1
Protein similar to DIP2B, partial:		
<i>Bos taurus</i>	XP_580943.1 (GenBank)	93.3
<i>Macaca fascicularis</i>	BAC41762 (GenBank)	83.7
DIP2C:		
<i>H. sapiens</i>	NP_055789 (GenBank)	72.7
Disco-interacting protein 2 (DIP2) homologue:		
<i>M. musculus</i>	Q8BWT5 (UniProtKB/Swiss-Prot)	70.7
DIP2A:		
<i>H. sapiens</i>	NP_055966 (GenBank)	71.2

Table 4.Relative *DIP2B* Expression for Families A, B, and C in Comparison with That for Control Individuals for Two Independent Real-Time PCR Analyses

Experiment and Subject(s)	Relative Expression	
	(%)	<i>P</i>
1:		
Healthy individual with unmethylated repeat expansion	200	.01
Affected individuals with methylated repeat expansion	55	.01
Healthy individuals with methylated repeat expansion	67	.05
2:		

Healthy individual with unmethylated repeat expansion	142	$\leq .01$
Affected individuals with methylated repeat expansion	45	.01
Healthy individuals with methylated repeat expansion	63	.01

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