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Mutations of *CDKL5* Cause a Severe Neurodevelopmental Disorder with Infantile Spasms and Mental Retardation

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Rett syndrome (RTT) is a severe neurodevelopmental disorder caused, in most classic cases, by mutations in the X-linked methyl-CpG-binding protein 2 gene (*MECP2*). A large degree of phenotypic variation has been observed in patients with RTT, both those with and without *MECP2* mutations. We describe a family consisting of a proband with a phenotype that showed considerable overlap with that of RTT, her identical twin sister with autistic disorder and mild-to-moderate intellectual disability, and a brother with profound intellectual disability and seizures. No pathogenic *MECP2* mutations were found in this family, and the Xq28 region that contains the *MECP2* gene was not shared by the affected siblings. Three other candidate regions were identified by microsatellite mapping, including 10.3 Mb at Xp22.31-pter between Xpter and *DXS1135,* 19.7 Mb at Xp22.12-p22.11 between *DXS1135* and *DXS1214,* and 16.4 Mb at Xq21.33 between *DXS1196* and *DXS1191.* The *ARX* and *CDKL5* genes, both of which are located within the Xp22 region, were sequenced in the affected family members, and a deletion of nucleotide 183 of the coding sequence (c.183delT) was identified in *CDKL5* in the affected family members. In a screen of 44 RTT cases, a single splice-site mutation, IVS13-1G→A, was identified in a girl with a severe phenotype overlapping RTT. In the mouse brain, *Cdkl5* expression overlaps—but is not identical to—that of *Mecp2,* and its expression is unaffected by the loss of *Mecp2.* These findings confirm *CDKL5* as another locus associated with epilepsy and X-linked mental retardation. These results also suggest that mutations in *CDKL5* can lead to a clinical phenotype that overlaps RTT. However, it remains to be determined whether *CDKL5* mutations are more prevalent in specific clinical subgroups of RTT or in other clinical presentations.

Introduction

Rett syndrome (RTT) is a devastating neurodevelopmental disorder that accounts for a significant proportion of severe mental retardation (MR) in females worldwide (Ellaway 2001). It is caused by mutations in the X-linked methyl-CpG-binding protein 2 (*MECP2*) gene in ~75% of cases (Hoffbuhr et al. 2002). Classic RTT is characterized by apparently normal development from birth until ~6 mo of age, followed by regression—with loss of acquired skills and speech—and development of stereotypic hand movements (Hagberg et al. 1985). However, a wide degree of variability has been reported in females with RTT, including severe cases with early-onset seizures and no period of normal development, relatively mild *forme fruste* cases with some preserved skills, and some cases with preserved speech (Hagberg and Rasmussen 1986; Goutiéres and Aicardi 1987; Hagberg and Skjeldal 1994; Zappella 1997).

For a genetic disorder, RTT has a relatively high incidence of ~1/10,000 among females, but familial recurrence is rare (Talvik et al. 1995; Terai et al. 1995; Leonard et al. 1997; Skjeldal et al. 1997; Gill et al. 2003). When there are recurrences within families, they usually follow an X-linked dominant pattern of inheritance, with protective skewing in asymptomatic female carriers and severe disease in affected males (Wan et al. 1999; Villard et al. 2000; Ben Zeev et al. 2002; Geerdink et al. 2002; Hoffbuhr et al. 2002; Gill et al. 2003). Variation observed between females in these families with RTT has generally been ascribed to differences in

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X-inactivation patterns (Miyamoto et al. 1997; Amir et al. 2000; Cheadle et al. 2000; Van den Veyver and Zoghbi 2000; Ishii et al. 2001; Webb and Latif 2001; Hoffbuhr et al. 2002). In addition, an increased prevalence of skewing of X inactivation has been reported in patients with RTT, including those with no identified *MECP2* mutation (Zoghbi et al. 1990*b*; Krepischi et al. 1998; Ishii et al. 2001; Weaving et al. 2003). Skewing has frequently been reported in association with X-linked disorders—and with X-linked MR in particular (Puck and Willard 1998; Plenge et al. 2002)—so its occurrence may be an indication that other X-linked genes cause RTT in a proportion of cases.

Mutations in *MECP2* cause most cases of classic RTT, but a proportion of sporadic cases, and the majority of familial cases, do not appear to have *MECP2* mutations (Cheadle et al. 2000; Huppke et al. 2000; Hoffbuhr et al. 2001; Gill et al. 2003; Weaving et al. 2003), although systematic screenings for large deletions (Laccone et al. 2004) or mutations involving the recently described *MECP2* isoform (Kriaucionis and Bird 2004; Mnatzakanian et al. 2004) have yet to be undertaken. This has raised the possibility that RTT is a genetically heterogeneous disorder, especially among atypical RTT cases, in which the prevalence of *MECP2* mutations may be as low as 44% (Weaving et al. 2003).

Here, we report patients from two families whose conditions show considerable clinical overlap with RTT and report mutations in *CDKL5* in the first family and in one affected individual from the second family. These results suggest that the phenotypic variability seen in RTT is, in part, due to genetic heterogeneity and that a clinical picture that overlaps RTT may be caused by mutations in *CDKL5*.

Material and Methods

MECP2 *Mutation Screening, X-Inactivation Analyses, and X-Chromosome Exclusion and Haplotype Mapping Studies*

MECP2 screening was performed as described elsewhere (Leonard et al. 2001; Weaving et al. 2003). In addition, we performed multiplex-ligation–dependent probe amplification [MLPA] analysis (MRC-Holland), in accordance with the manufacturer's instructions. We also performed PCR and direct sequencing, to screen exon 1 of *MECP2* for potentially pathogenic sequence variations. Primers were designed to cover the whole of exon 1, including 112 bp downstream of the intron 1 splice junction and 490 bp upstream of exon 1 (forward primer: 5′-GCACTCGGTGCATCTGTGGACAGAG-3′; reverse primer: 5′-CATCCGCCAGCCGTGTCGTCCGAC-3′). PCR was performed using 100 ng of genomic DNA as a template in a 20-μl reaction with a concentration of 0.166 μM of both primers, and 0.75mM of dNTPs, 1.5 U of Platinum *Taq* polymerase (Invitrogen), 67 mM Tris-HCl (pH 8.3), 3.7 mM $MgCl_2$, 0.5 M betaine, 16.6 mM $(NH_4)_2SO_4$, and 0.05% dimethyl sulfoxide. PCR amplification conditions were 94°C for 7 min; followed by 35 cycles of denaturation at 95°C for 60 s, annealing at 65°C for 60 s, and extension at 72°C for 60 s; and a final extension step for 5 min at 72°C. PCR products were sequenced in forward and reverse directions and were analyzed as reported elsewhere (Weaving et al. 2003), with the forward primer for sequencing being 5′-CAATTGACGGCATCGCCGCTGAGAC-3′ and the reverse sequencing primer being the one used for PCR amplification of this region.

X-chromosome inactivation status was analyzed using DNA extracted from peripheral white blood cells or buccal samples. Skewing of X inactivation was measured in accordance with the method of Allen et al. (1992). For family 2, which was not informative at the androgen receptor locus, X-chromosome inactivation analysis was performed using an FMR1 CGG-repeat PCR-based assay, essentially as described by Carrel and Willard (1996). Digestion conditions, primer sequences, and PCR conditions are available on request.

Preliminary X-chromosome mapping was performed using the ABI Prism set, version 2, panel 28 (X chromosome), obtained from Applied Biosystems. This set comprised primers for 18 fluorescently labeled microsatellite markers dispersed at intervals of ~10 cM over the entire X chromosome. Further primer sequences for amplifying microsatellites within regions identified as potential candidates were obtained from the Genome Database (see Genome Database Web site). Genetic distances and locations in centimorgans and megabases were obtained from the Cedar Genetics X-chromosome map, the Ensembl Human Genome Browser, and the Integrated X Chromosome Database (see Cedar Genetics Map, Ensembl Human Genome Browser, and Integrated X Chromosome Database Web sites). PCR conditions were optimized for individual primers and are available on request. Exclusion mapping was based on the hypothesis of an X-linked dominant disorder shared by the twins and the brother and inherited from the mother in family 1. Alleles that were not shared by the affected individuals were excluded. Alleles shared with the unaffected sister were also considered as highly unlikely candidate regions and were excluded. In family 2, microsatellite markers flanking the *CDKL5* (*DXS987* and *DXS1683*) and the *ARX* (*DXS1684, DXS1177,* and *DXS1108*) loci were used for haplotype analysis.

Candidate-Gene Analyses

After defining candidate regions through exclusion mapping, the Aristaless-like homeobox gene (*ARX*) and

the serine/threonine kinase 9 gene (*STK9*)—also known as "cyclin-dependent kinase–like 5" (*CDKL5* [MIM 300203])—were chosen as candidates for screening. *ARX* screening was performed by sequencing of the coding region by use of the method of Strømme et al. (2002*b*). *CDKL5* was screened by sequencing of the coding region by use of the method of Kalscheuer et al. (2003).

cDNA Analysis of the IVS13-1G→A Mutation

To amplify across *CDKL5* exons 12–15, two primers were designed: E12F, 5′-AGAGCCAACAGCCTGCAACTC-3′; and E15R, 5′-CATGGATCGAATGCTGGTTGTC-3′ (*Tm* = 60°C; amplicon size 365 bp). Peripheral blood RNA of patients II:1 and II:2 from family 2 was isolated using standard techniques. Random hexamer-primed cDNA was used for PCR with primers E12F and E15R. The 365-bp product was directly sequenced and was cloned into pGEM-T (pGEM-T Vector System I [Promega]), in accordance with the manufacturer's instructions. XL10G cells (Stratagene) were used for transformations.

To screen clones for the 1-bp deletion (c.2047delG), we designed a new mutant cDNA-specific primer, E14Rm (5′-TGTGGGTCATGATACACTCCACCT-3′). The E14Rm primer was used in conjunction with the exon 12 primer (E12F) to screen the clones. Despite the specificity of the E14Rm primer for the c.2047delG allele, the normal *CDKL5* alleles (C:A mismatch) were also amplified. However, as a result of the mismatch, the E14Rm primer introduced a base change, compared with the normal *CDKL5* PCR product, which removes a *Dde*I restriction site. After PCR, the products were digested with *Dde*I, to identify clones carrying the c.2047delG mutation. Sequencing (of normal and PCR/*Dde*I-positive clones) was used to confirm the results of the *Dde*I restriction-enzyme digest.

Ethical approval for this study was obtained from the Children's Hospital at Westmead Ethics Committee, and informed consent was obtained from subjects and from the parents or guardians of patients.

In Situ Hybridization of Cdkl5 *Expression in Mouse Brains*

The expression of *Cdkl5* in the mouse brain at postnatal week 8 was examined by whole-mount in situ hybridization. Mouse total RNA was prepared using Trizol, and cDNA was synthesized using Superscript 2 (Invitrogen) with oligodT primers, in accordance with the manufacturer's protocols. Riboprobes that hybridize to a 467-bp sequence of exons 3–10 of the *Cdkl5* gene were prepared using the cDNA as templates (mouse *Cdkl5* sequence obtained from Mouse Genome Informatics; see Mouse Genome Informatics Web site). The forward primer was 5′-AAATGCAGACACAAGGAAACAC-3′, the reverse primer was 5′-AGTAGCAGTTCTGGGGATCG-3′, and PCR was performed with 1 μl of cDNA as template in a 20-μl reaction volume with a concentration of 0.25 μM of both primers, and 0.24 mM of dNTPs, 1.35 U of AmpliTaq Gold polymerase (Applied Biosystems), and 2.0 mM Mg^{2+}. Touchdown amplification conditions were 94°C for 12 min; followed by touchdown with two cycles for each 2°C reduction in the annealing temperature, with denaturation at 94°C for 30 s, annealing at 65°C–55°C for 30 s, and extension at 72°C for 30 s; then 30 cycles with an annealing temperature of 55°C for 30 s and an extension temperature of 72°C for 30 s; and a final extension step at 72°C for 10 min.

Amplified products were cloned into pGEM-T (Promega) plasmids, and clones containing inserts in both orientations were identified and sequenced. Antisense and sense riboprobes were generated from the *Not*I linearized vector from the T7 RNA polymerase site, by use of the Ampliscribe RNA Polymerase Kit (Epicentre) with Digoxigenin-11-UTP (Roche). Brain samples for in situ hybridization were fixed in 4% paraformaldehyde and were dissected into ~1-mm sagittal slices. Specimens were dehydrated and stored in 100% methanol at −20°C until used for in situ hybridization by established protocols (Davidson et al. 1999) with the following modifications: hybridization and high-stringency washes were performed at 65°C, followed by RNase digestion, and BM Purple AP substrate (Roche) was used for color development. Photography was performed under an MZFLIII (Leica) dissecting microscope by use of a Spot digital camera and Spot software, version 3.5 (Diagnostic Instruments).

Mecp2 *Mutant Mouse*

Mecp2-deficient mice were generated by replacing the coding sequence for the methyl-binding domain with a floxed *Pgk-neo* cassette. A nonfunctional splicing site was introduced at the 5′ end of the coding sequence downstream of the selection cassette, to prevent correct splicing of the transcript sequence coding the transcription repression domain, the C-terminal domain, and the 3′ UTR. A 10.8-kb region containing exon 3 and the first 3 kb of exon 4 was excised with *Bam*HI from the BAC clone B22804 containing the *Mecp2* gene and was cloned into a pBC (Stratagene) vector. An *Hpa*I and *Not*I digest removed 2 kb of sequence containing exon 3 and the first ~170 bp of exon 4 that encode the methyl-binding domain. The floxed *Pgk-neo* cassette was inserted into the *Hpa*I/*Not*I ends of the targeting vector. The correctly targeted embryonic stem cells were used to produce mutant mice by germline transmission on an R1-129 background. Mating of the *Mecp2* mutant mice

with Tnap-Cre mice resulted in the recombinase-mediated deletion of the floxed *Pgk-neo*.

MeCP2 Antibody Generation

An N-terminal peptide consisting of human MeCP2 amino acids 9–27 (REEKSEDQDLQGLKDKPLK) was synthesized (Auspep). A homology search, conducted using the BLAST program (see National Center for Biotechnology Information [NCBI] Web site), revealed significant identity to only the MeCP2 protein. This peptide sequence was used to generate antisera in rabbits containing rabbit polyclonal IgG, subsequently referred to as "LR3" (Institute of Medical and Veterinary Science, Adelaide, Australia).

Western Analysis of MeCP2 Protein Expression in the Mouse

Whole brain, liver, and lung tissues were collected from mice after cervical dislocation. Nuclear extracts were prepared using established protocols (Kuramoto et al. 2003). In brief, tissues were homogenized (Polytron Omni 5000) in a low-salt buffer (10 mM HEPES-NaOH buffer [pH 7.9], 10 mM KCl, 1 mM EDTA, 1 mM EGTA, 5 mM dithiothreitol [DTT], and 0.1% Nonidet P-40) containing protease inhibitors and were centrifuged (800 × g at 4°C for 30 min). The pellet was subsequently mixed with high-salt buffer (500 mM of KCl, 50 mM Tris (pH 8.0), 1.5 mM EDTA, 1 mM DTT, 20% glycerol, and 0.1% Nonidet P-40) containing protease inhibitors and was ultracentrifuged (180,000 × g at 4°C for 30 min), and the nuclear fraction in the supernatant was then used for western analyses.

Twenty micrograms of total protein was applied to each lane of a 10% SDS-polyacrylamide gel and was separated by electrophoresis, then transferred to a nitrocellulose membrane (Schleicher and Schuell). Western blotting was performed using established procedures (Sambrook et al. 1989). In brief, the LR3 antibody was diluted 1:1,000 in PBS-T with 5% skim-milk powder, and the secondary horseradish peroxidase-conjugated goat anti-rabbit antibody (Santa Cruz) was diluted 1:2,000. Antibody binding was detected with enhanced chemiluminescence (Amersham Life Science).

Results

Clinical Description of Family 1

The first family in this study consisted of a proband with a clinical phenotype that overlaps RTT (III:1) (fig. 1), her identical twin sister with autistic disorder (III:2), and an elder brother with profound intellectual disability and seizures who died at the age of 16 years (III:3). Monozygosity of the twins was confirmed by X-chromosome microsatellite mapping and by human leukocyte antigen (HLA) and red-cell antigen typing. The family also included an unaffected older sister and an unaffected older brother. The unaffected brother (III:4) (fig. 1) was born with the remains of a dead twin attached to the placenta. The mother also had two first-trimester miscarriages prior to the birth of III:3. The parents were nonconsanguineous and of white descent.

The proband (III:1) (fig. 1) was a female born by caesarean section at 35 wk of gestation, after a normal pregnancy. Her head circumference at birth was 34.6 cm, and her birth weight was 2,340 g; both measures were between the 50th and 98th percentiles. There were no problems in the neonatal period. At 9 wk of age, she developed infantile spasms. Despite this, her general development was normal until 10 mo of age. She sat unsupported at 6 mo and could be pulled to stand at 12 mo of age. She learned to crawl but never walked without assistance. She developed babble, but this was lost between 10 and 15 mo, during which time she became totally withdrawn, showing little interest in others. She had poor eye contact, preferring to look at bright objects or lights. At that time, she also lost her pincer grasp. She subsequently developed hand-wringing and hand-mouthing stereotypies as well as breathing dysfunction with hyperventilation and breath-holding episodes. When seen at 19 years of age, she had a thoracolumbar scoliosis corrected by surgical fusion and was wheelchair dependent. Her head circumference was between the 2nd and 50th percentiles, whereas her weight and height were well below the 3rd percentile. Her hands and feet were small (below the 3rd percentile). She had severe MR and a mixed seizure disorder with generalized tonic clonic, myoclonic, and absence seizures. She had severe constipation and generalized spasticity, hyperreflexia, and peripheral vasomotor disturbance. On the basis of a recent consensus panel review, this patient would fit the criteria for atypical RTT (Hagberg et al. 2002).

III:2 (fig. 1) was the identical twin sister of III:1. Her birth weight was 1,820 g (below the 10th percentile), and her head circumference 30.5 cm (10th percentile). In contrast to III:1, III:2 was described as a "stroppy," or hard-to-please, baby. She was difficult to feed and did not like to be cuddled. She sat unsupported at the age of 9 mo, started to walk at 17 mo, and spoke her first words at 18 mo. Although she was slow to learn new words, she was able to speak in four-five–word sentences at 4 years. At 5 years of age, her speech was clearly abnormal, with echolalia, lack of spontaneity, and an insistence on sameness in replying to others. She avoided eye gaze and interaction with peers. She has never had seizures and has no other recognizable features of RTT. She received a diagnosis of autism at the age of 5 years. When seen at 19 years of age, she was very active and demanding and had reasonable verbal skills but mild MR (IQ assessed at ~70). Her weight was between the

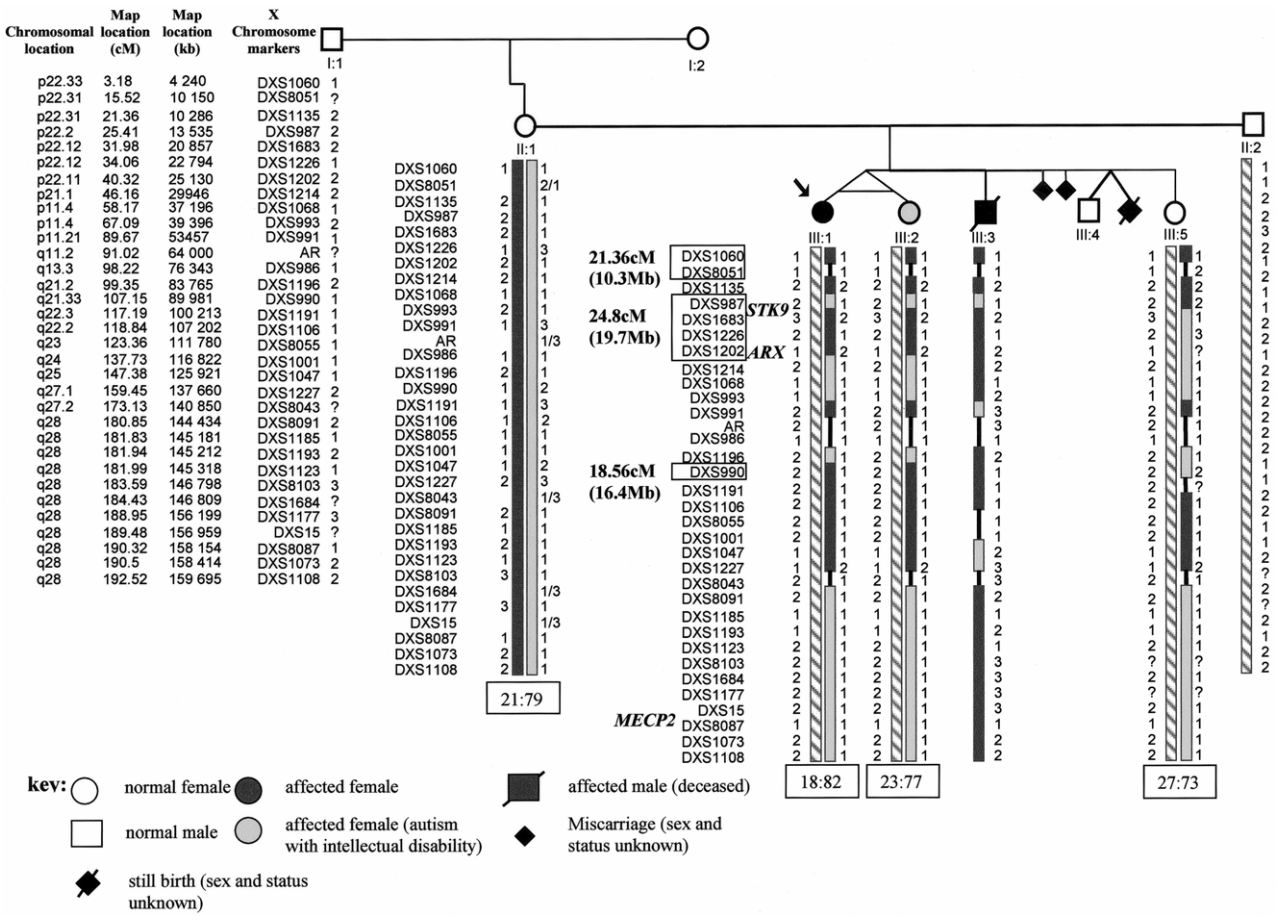

Figure 1 Pedigree of family 1, showing results of microsatellite-exclusion mapping. Haplotypes were derived using Cyrillic v2 software (Cyrillic Software) and are shown as differently shaded bars. The black bars represent grandpaternal alleles, the gray bars represent grandmaternal alleles, and the striped bars represent paternal alleles. Candidate regions are indicated by boxes. X-inactivation patterns are shown as ratios below the haplotypes. Microsatellites used in this study and their chromosomal band and map positions are given at the far left of the pedigree. Map distances were derived from the Cedar Genetics Map of the X Chromosome, the Ensembl Human Genome Browser, and the Integrated X Chromosome Database. The approximate positions of each of the candidate genes screened in this family are also indicated.

10th and 25th percentiles, her height was between the 3rd and 10th percentiles, and her head circumference was between the 50th and 98th percentiles.

III:3 (fig. 1) had a mixed seizure disorder of the "Lennox-Gastaut" type, profound intellectual impairment, severe global developmental delay, spastic quadriparesis, cortical blindness, and a marked kyphoscoliosis. He was born at term after a normal pregnancy, with a birth weight and head circumference in the 50th percentile. He developed infantile spasms in the neonatal period, with a sudden increase in their frequency and severity at 9 wk of age. An electroencephalogram performed at 12 mo of age showed "hypsarrhythmia." His development was reported by his parents to be normal for the first 4 mo of life, but, between 4 and 6 mo, he developed poor head control and adopted a fisting posture of both hands. By 1 year of age, he had severe psychomotor retardation, with no evidence of visual or social responses, although auditory responses were present. At 2 years of age, he showed further developmental regression, with a poor state of consciousness and frequent seizures. His seizures remained very difficult to control, despite numerous anticonvulsants, a trial of adrenocrticotropic hormone, and a ketogenic diet. At 14 years of age, he was unresponsive and had frequent myoclonic jerks, roving eye movements, marked kyphoscoliosis and joint contractures, cortical blindness, and recurrent bouts of pneumonia due to gastroesophageal reflux. He had marked constipation. He was not dysmorphic, his head circumference was in the 2nd percentile, and his weight was below the 3rd percentile. He had no organomegaly and was markedly hypertonic and hyperreflexic. Extensive investigations failed to establish a cause of his problems. These included a cerebral CT scan, ly-

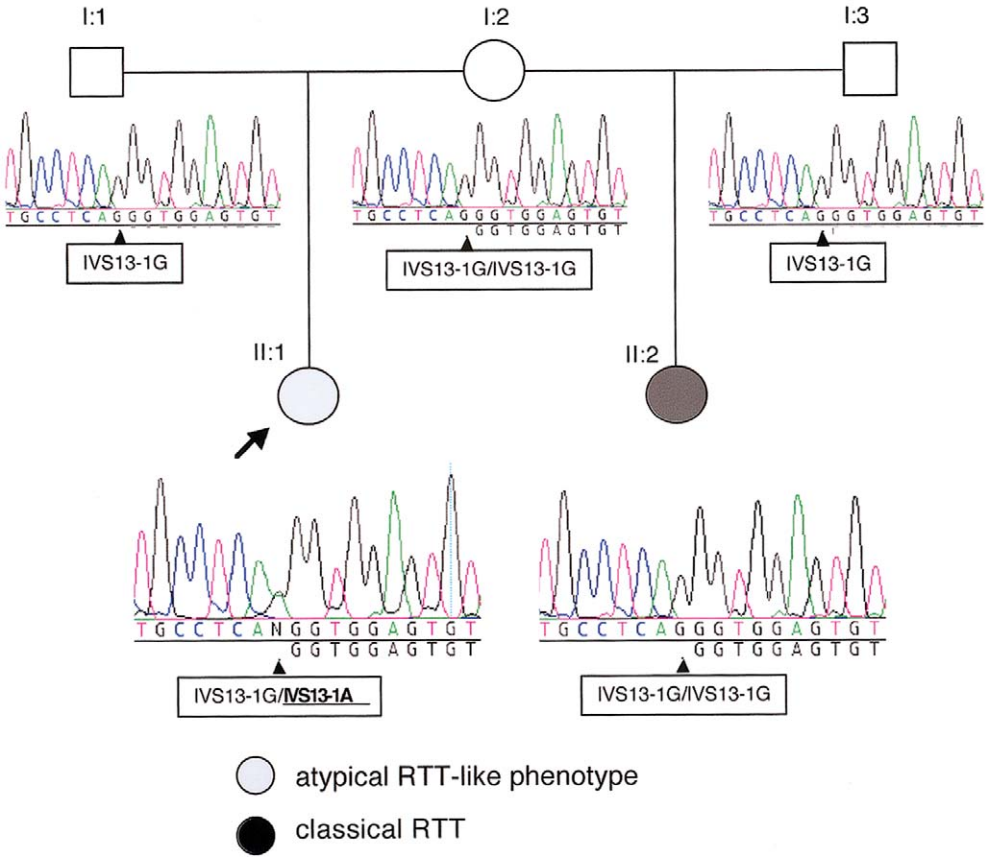

Figure 2 IVS13-1G→A mutation of *CDKL5* in family 2. Chromatograms of the *CDKL5* sequence that is mutated in family 2 are shown. A G-to-A transversion involving the intron 13 splice-acceptor site was identified in blood DNA from the subject with an atypical RTT–like phenotype (II:1) but not in her half sister (II:2) with classical RTT or either of her parents.

sosomal enzymes, karyotype, urinary amino- and organic-acid analyses, and urinary succinylpurines. Plasma electrolytes, copper, acid-base status, urea, calcium, magnesium, uric acid, blood glucose, creatinine, and very-long-chain fatty acids were also normal. His plasma lactate was mildly elevated, but the cerebrospinal fluid (CSF) lactate was normal. CSF neurotransmitters, glucose, and protein and viral studies for herpes simplex, measles, and varicella were normal. He had a normal male karyotype, 46XY. Results of a skin biopsy showed no evidence of a storage disorder or neuroaxonal dystrophy. He did not have the common mtDNA mutations for mitochondrial encephalopathy, lactic acidosis, and stroke-like episodes; myoclonic epilepsy ragged red fibers; Leigh syndrome; or neurogenic ataxia retinitis pigmentosa in a peripheral blood sample. Rectal-biopsy electron microscopy was normal. This boy died of respiratory failure at the age of 16 years, secondary to aspiration pneumonia. In retrospect, his parents report episodes of hyperventilation that were unrelated to feeds and/or aspiration.

Clinical Description of Family 2

This family has been reported elsewhere (family 9 in Gill et al. 2003). The proband (II:1) (fig. 2) was born at term after a normal pregnancy. Her birth weight was in the 50th percentile. She smiled at 6 wk but shortly afterward developed severe infantile spasms. Calcification on her brain CT scan was suggestive of intrauterine cytomegalovirus infection, but viral serology did not support this diagnosis. She developed intractable seizures (generalized tonic clonic, myoclonic, and absence seizures) during childhood, which worsened during adolescence and improved somewhat in adulthood, with daily brief myoclonic seizures. Developmentally, she made very slow progress up to 5 years of age, gaining fine and gross motor skills to a basic level but then becoming clumsy and losing these skills. She was a hypotonic child, who sat unsupported at 17 mo and stood at 4 years of age. She spoke several single words, built a tower of four bricks, spoon fed with help, and turned single pages of a book. She lost these skills between 2

years and 5 years of age. She walked independently at 7 years, with an unsteady gait, and developed a fine pincer grasp. When reviewed at age 28 years, her height, weight, and head circumference were all below the 3rd percentile. She had no verbal communication skills, marked intellectual impairment, and a scoliosis that has not required surgery. Hand stereotypies predominated on the left side, although, in the past, these were midline. She hyperventilated when upset and had peripheral autonomic disturbance. She had a disturbed sleep pattern and laughed spontaneously at night. She was not dysmorphic but was hirsute. She had dystonia with normal reflexes and had small feet (below the 3rd percentile). She was considered to have atypical RTT because of her very poor early progress, early onset of severe epilepsy, late and slight regression, and little, if any, breathing abnormality or stereotypy (A. M. Kerr, personal communication).

II:2 (fig. 2) was the younger half sister of the proband, related through the mother. She was born at term after a normal pregnancy. Her birth weight was between the 50th and 75th percentiles, and her head circumference was in the 9th percentile. There were early maternal concerns regarding placidity, hypotonia, and a slightly vacant look. Her head circumference fell below the 3rd percentile over the first 2 years. She achieved skills to a basic level: babbling at 9 mo, sitting with a rounded back at 19 mo, and standing at 4 years, and she was able to stack four bricks and unscrew lids prior to regression. She regressed between 3 years and 5 years of age, losing her hand skills and stagnating in her motor abilities. She developed typical hand stereotypies, as well as hyperventilation and breath-holding episodes. She made subsequent slow progress, walking independently at 7 years with an unsteady gait and developing a palmar grasp. When seen at 13 years of age, she was a happy child, keen to interact socially. She could babble "mumum" and "dada" and understood several basic commands, but her verbal skills were not considered sufficient for a diagnosis of preserved speech variant. She had a moderate scoliosis and suffered from sleep disturbance, labile mood, peripheral vasomotor disturbance, and mild constipation. She has never had seizures. She had an Angelman-like facial appearance (however, methylation and uniparental disomy studies for Angelman syndrome were negative [results not shown]). Her head circumference and weight were both below the 3rd percentile. She had marked ligamentous laxity and no joint contractures. She had short fourth metatarsals and small feet (below the 3rd percentile). The clinical diagnosis of classical RTT in this patient was independently confirmed by Dr. A. M. Kerr of the Department of Psychological Medicine, University of Glasgow, Scotland (personal communication).

MECP2 *Mutation Screening*

The entire coding region of the *MECP2* gene (including exon 1) was sequenced in the twins from family 1 and their affected brother. A c.426C→T sequence variation was identified in the twins but not in the affected brother. This coded for a silent polymorphism (F142F), but such changes can affect splicing enhancers in some instances (Cartegni et al. 2002). Therefore, the PCR product containing this nucleotide change was sequenced in the other family members. It was not identified in the affected brother or father but was seen in the unaffected sister and mother. It thus seems unlikely that this nucleotide change is pathogenic. No other *MECP2* changes, including large genomic rearrangements screened by MPLA, were identified in this family (results not shown). Similarly, sequencing of the two affected individuals in family 2 revealed no pathogenic sequence variations in any of the four *MECP2* exons, and MLPA analysis did not show any large rearrangements involving this gene.

X-Chromosome Exclusion and Haplotype Mapping

Microsatellite mapping was performed on family 1, to confirm that the twins were identical and to determine whether the Xq28 region containing the *MECP2* gene was shared among the affected siblings. Results of the microsatellite mapping are shown in the family tree in figure 1. The microsatellite mapping was compatible with the twins being identical. The Xq28 region was not shared with their affected brother but was shared between the twins and their unaffected sister. *MECP2* was thus excluded as a disease candidate gene in this family, but the inheritance pattern and phenotypic variation still suggested that this was an X-linked dominant condition.

Three potential candidate regions were identified through exclusion mapping (fig. 1). The first was 10.3 Mb at Xp22.31-pter between Xpter and *DXS1135*, the second was 19.7 Mb at Xp22.12-p22.11 between *DXS1135* and *DXS1214*, and the third was 16.4 Mb at Xq21.33 between *DXS1196* and *DXS1191*. Simonic et al. (1997) observed an increase in the incidence of a breakpoint at Xp22.1 in patients with RTT, which leads us to favor this region. Further support for Xp22 as a candidate region came from the report of a patient with RTT and an (X;3)(p22.11;q13.31) translocation (Zoghbi et al. 1990*a*). The candidate genes *ARX* and *CDKL5* both reside within the Xp22.12-p22.1 region.

In family 2, only microsatellite markers flanking the *CDKL5* and *MECP2* genes were used. These analyses showed that, for both loci, the sisters inherited different maternal and paternal X-chromosome haplotypes (results not shown). These results strongly suggest that their clinical phenotypes of RTT have resulted from different

causes: the *CDKL5* gene mutation in II:1 and an as-yet unknown cause in II:2.

Candidate-Gene Mutation Screening in Family 1

The *ARX* gene was chosen as a candidate for screening because of the pleiotropy of its mutations (Kato et al. 2004; Partington et al. 2004). However, no mutations were detected in the coding region of this gene in family 1.

The *STK9* gene was chosen as a second candidate for screening, because the phenotype of infantile spasms overlapped that described elsewhere in association with *CDKL5* rearrangements (Kalscheuer et al. 2003). A deletion of nucleotide 183 (c.183delT) in exon 5 was identified in the affected siblings but not in any of the unaffected members of family 1, including their mother, for whom the mutation was screened in DNA extracted from blood and buccal cells (fig. 3). This deletion results in a frameshift and a premature truncation of the gene product at amino acid 75.

CDKL5 *Screening of Other* MECP2*-Negative Subjects with RTT*

After the identification of the c.183delT mutation in *CDKL5* in family 1, screening of the *CDKL5* gene was performed in 44 patients with RTT who were *MECP2* mutation negative. These included cases ascertained from Australia and the United Kingdom and one of the probands from each of four familial cases reported by Villard et al. (2000, 2001) who showed highly skewed X inactivation. A splice-site mutation (IVS13-1G→A) was identified in patient II:1 from family 2, who has the early-onset–seizure RTT variant (see "Clinical Description of Family 2" section and fig. 2). This patient had a sister with classic RTT, but neither the sister nor the parents carried the *CDKL5* mutation (fig. 2). This mutation leads to the loss of a *Dde*I site. In unaffected individuals, a 346-bp fragment encompassing this region is cut into 185-bp and 135-bp fragments, whereas the mutant allele is cut into 320-bp and 26-bp fragments (results not shown).

Characterization of the CDKL5 *IVS13-1G→A Mutation*

The *CDKL5* IVS13-1G→A mutation identified in II:1 (family 2) was not found in any of the other 43 *MECP2*-mutation-negative patients with RTT who were investigated or in 236 control chromosomes. To explore the pathogenicity of IVS13-1G→A further, we designed primers that would amplify the cDNA from exons 12–15, inclusive. A fragment of 365 bp was identified in an RT-PCR of blood RNA from patient II:1. An apparently normal *CDKL5* cDNA sequence was obtained from se-

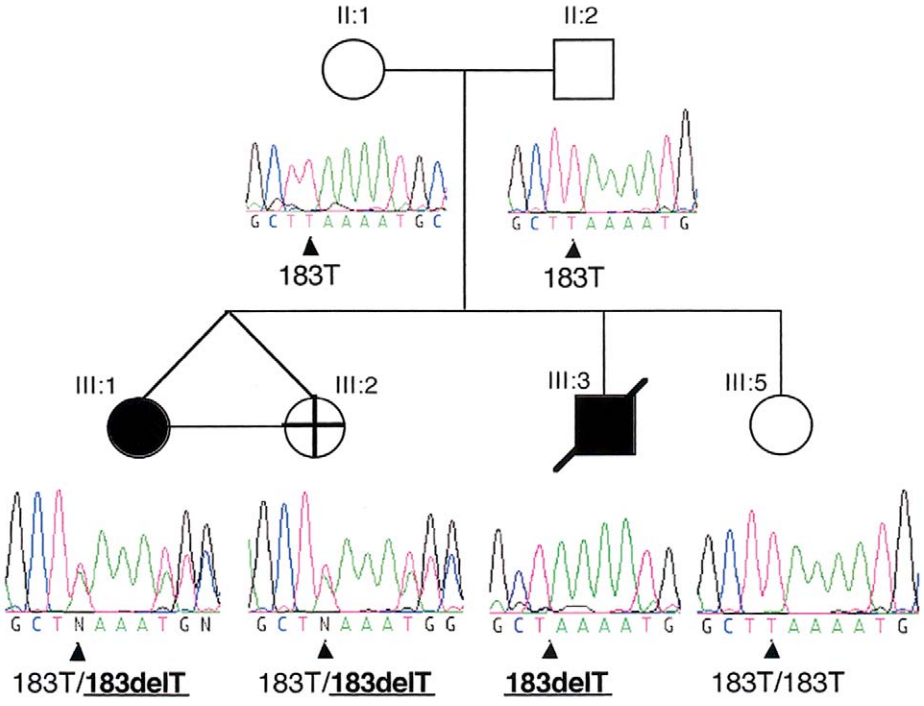

Figure 3 c.183delT mutation of *CDKL5* in family 1. A chromatogram of the *CDKL5* sequence that is mutated in family 1 is shown. Nucleotide numbers begin from the A of the translation start codon in exon 2. Individual ID numbers are derived from figure 1. A deletion of nucleotide 183 was observed in the blood DNA of affected individuals (III:1, III:2, and III:3) but not in unaffected family members.

quencing. However, after a more careful inspection of the sequence chromatograms, a minor second sequence signal could be detected in the background of the normal sequence (mutation shown in fig. 4*A*). Interestingly, the background cDNA sequence appeared to be shifted by only 1 bp. On the basis of this, we hypothesized that the IVS13-1G→A mutation results in the activation of a novel 3′ acceptor splice site only 1 bp away from the original one (fig. 4*B*). To test this hypothesis, peripheral blood RNA of patient II:1 was used to amplify this region (exons 12–15) of the *CDKL5* cDNA by RT-PCR. Using cloning, mismatch PCR, restriction digest, and sequencing (see "Material and Methods" section), we identified 5 clones (of 30 analyzed) that carried the hypothesized 1-bp *CDKL5* cDNA deletion (c.2047delG). The frequency of the c.2047delG clone (16.6%) among the cloned exon 12–15 RT-PCR products is in agreement with the X-inactivation studies, indicating that the chromosome carrying the c.2047delG is active in only 15% of peripheral blood cells (data not shown). Translation of the mutant spliced product would create a premature stop codon (M783X), truncating the protein approximately halfway through exon 16 (fig. 5).

X Inactivation

Results from the *AR* assay suggested that the mother, both of the twins, and their unaffected sister from family 1 had skewed X inactivation (fig. 1). The twins had a high degree of skewing (82% and 77%) in their blood DNA, but this appeared to favor the same X chromosome. In the mother, the grandmaternal chromosome was preferentially active in 79% of her lymphocytes, whereas, in DNA from a buccal sample, it was 96%.

In family 2, X inactivation was studied using an *FMR1* assay. The patient (II:1) had a skewed X-inactivation pattern with a ratio of 85:15. With the use of the available family members, it was not possible to determine which X chromosome (normal or with the *CDKL5* mutation) was preferentially inactivated. However, the anal-

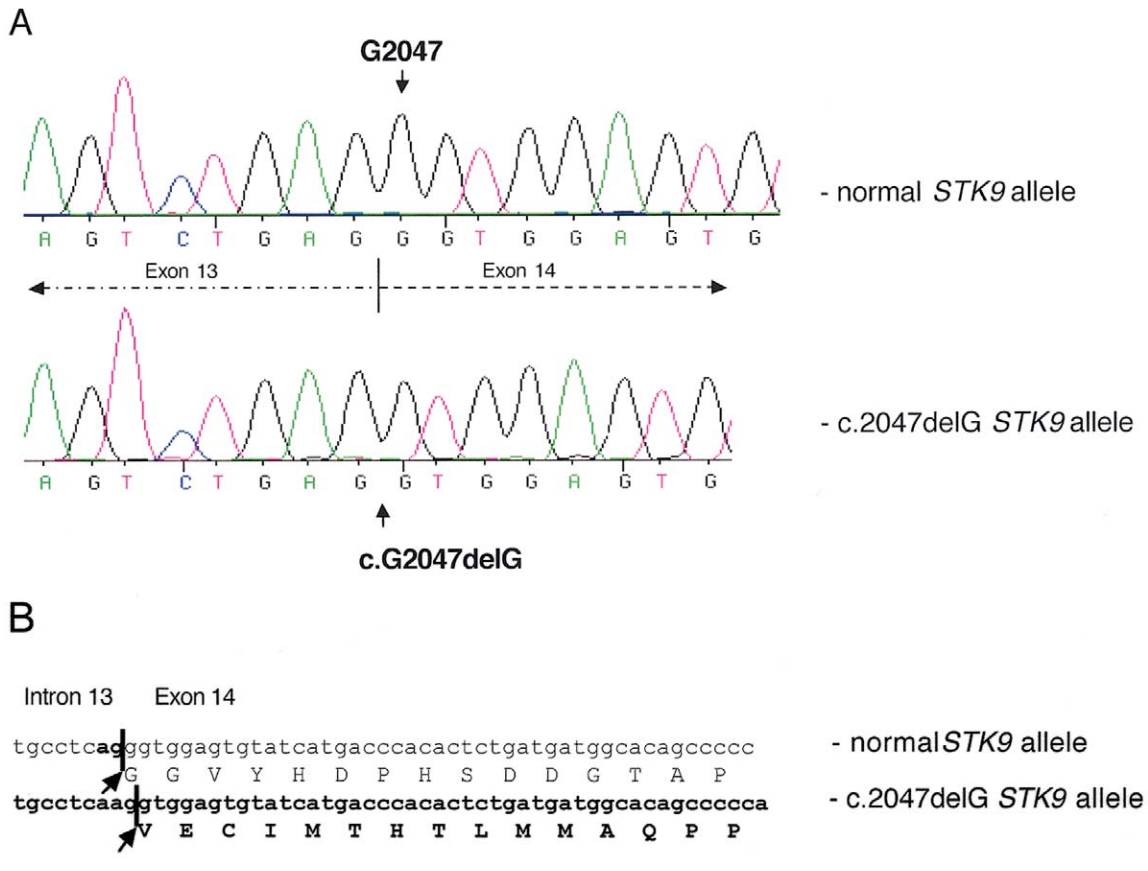

Figure 4 cDNA and translated product of the *CDKL5* IVS-1G→A mutation. *A*, Sequence chromatograms of representative cDNA clones isolated from II:1 (family 2) blood RNA/cDNA. In 5 (16.6%) of 30 clones examined, a c.2047delG *CDKL5* cDNA was observed. *B*, Illustration of the creation of a novel intron 13 acceptor splice site as a result of the IVS13-1G→A mutation in *CDKL5* of individual II:1 from family 2. The protein sequence is shown below the genomic sequence.

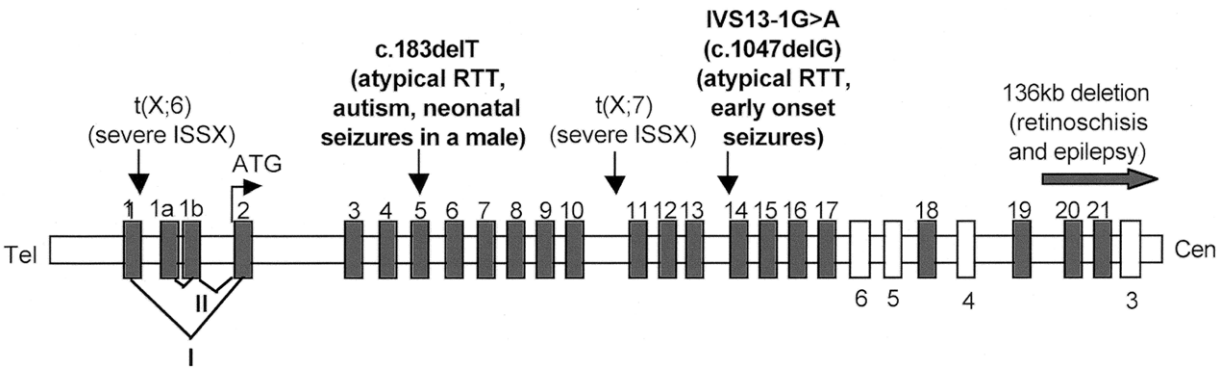

Figure 5 Schematic representation of the *CDKL5* gene. *CDKL5* exons are indicated by black boxes, and the last four exons of *RS1*, a gene overlapping *CDKL5*, are indicated by white boxes. The alternative-splicing patterns are indicated below the diagram for the known isoforms, I and II. Mutations and associated phenotypes that have been reported to date in association with *CDKL5* mutations are given above the diagram, and those described in the present study are shown in bold.

ysis of the *CDKL5* cDNA from this patient suggested that the chromosome carrying the *CDKL5* IVS13-1G→A mutation was preferentially inactivated (active in ~15% of peripheral blood cells).

Expression of Cdkl5 *in Mouse Brains*

In view of the potential link of the activity between *Cdkl5* and *Mecp2*, we examined the activity of *Cdkl5* in the mouse brain, to ascertain whether *Cdkl5* and *Mecp2* are expressed in similar brain regions. Whole-mount in situ hybridization analysis of postnatal week-8 mouse brain revealed regionalized expression of the *Cdkl5* gene in the olfactory bulb, the cerebral cortex, the cerebellum, the hippocampal formation comprising the hippocampus proper and dentate gyrus, the basal ganglia, the thalamus, and the superior and inferior colliculi (fig. 6*A* and 6*B*). A comparison of the expression patterns of *Cdkl5* and *Mecp2* in the brain at the same developmental age showed that there were significant overlaps of the activity of these two genes.

To examine the epistatic relationship of *Cdkl5* and *Mecp2*, we have tested whether *Cdkl5* expression may be influenced by that of *Mecp2*. This was accomplished by studying *Cdkl5* expression in the brain of *Mecp2*-deficient mice by whole-mount in situ hybridization. We have produced, by gene targeting and genetic crosses, *Mecp2*-deficient mice. Real-time RT-PCR assays for the 1.8-kb and 10.2-kb mRNA transcripts of *Mecp2* performed on samples of the cerebellum and substantia nigra of two 2-wk-old *Mecp2*$^{-/Y}$ male mice revealed no real-time RT-PCR products specific to the 10.2-kb or 1.8-kb transcripts in the mutant brains (data not shown). In addition, western analysis showed that there was no detectable Mecp2 protein in the brain tissues (fig. 6*D*). These findings indicate that the targeted mutation has caused a loss of *Mecp2* function. These mice exhibit a phenotype similar to that seen in *Mecp2* null mice published elsewhere (Chen et al. 2001; Guy et al. 2001). In brief, hemizygous males start developing symptoms by 4 wk and died by 6–8 wk, with tremors, stereotypic limb motions, motor impairments, and hypoactivity present. Homozygous females were not generated, and heterozygous females survived well into adulthood. Further details of phenotypic analysis of the mutant mice will be presented in separate publications. Whole-mount in situ hybridization analysis of the brain of week-8 *Mecp2* mutant mice revealed that *Cdkl5* expression was not affected by the loss of *Mecp2* activity (fig. 6*E* and 6*F*). This is an important finding that strongly suggests that *Cdkl5* activity is independent of *Mecp2* activity and that CDKL5 may act either in parallel to or upstream of MeCP2. This is consistent with the working hypothesis that Cdkl5 kinase regulates the function of Mecp2 by phosphorylation of the protein.

Discussion

After exclusion mapping studies in family 1, we searched for genes in the Xp22 region that might be disease candidates. Both the *ARX* and *CDKL5* genes lie within this Xp22 region. *ARX* was chosen as a candidate for screening for several reasons. Mutations in *ARX* have been found in patients with epilepsy, dystonia, spastic paraplegia, autistic behavior, and MR (Bienvenu et al. 2002; Frints et al. 2002; Strømme et al. 2002*a*, 2002*b*; Turner et al. 2002), some characteristics of which are exhibited by the affected siblings in family 1. However, no mutations were identified in *ARX* in this family.

CDKL5 was initially identified as a serine/threonine kinase gene from sequence similarity searches (Montini et al. 1998), but little is known about its function. Chromosome translocations interrupting the gene have been

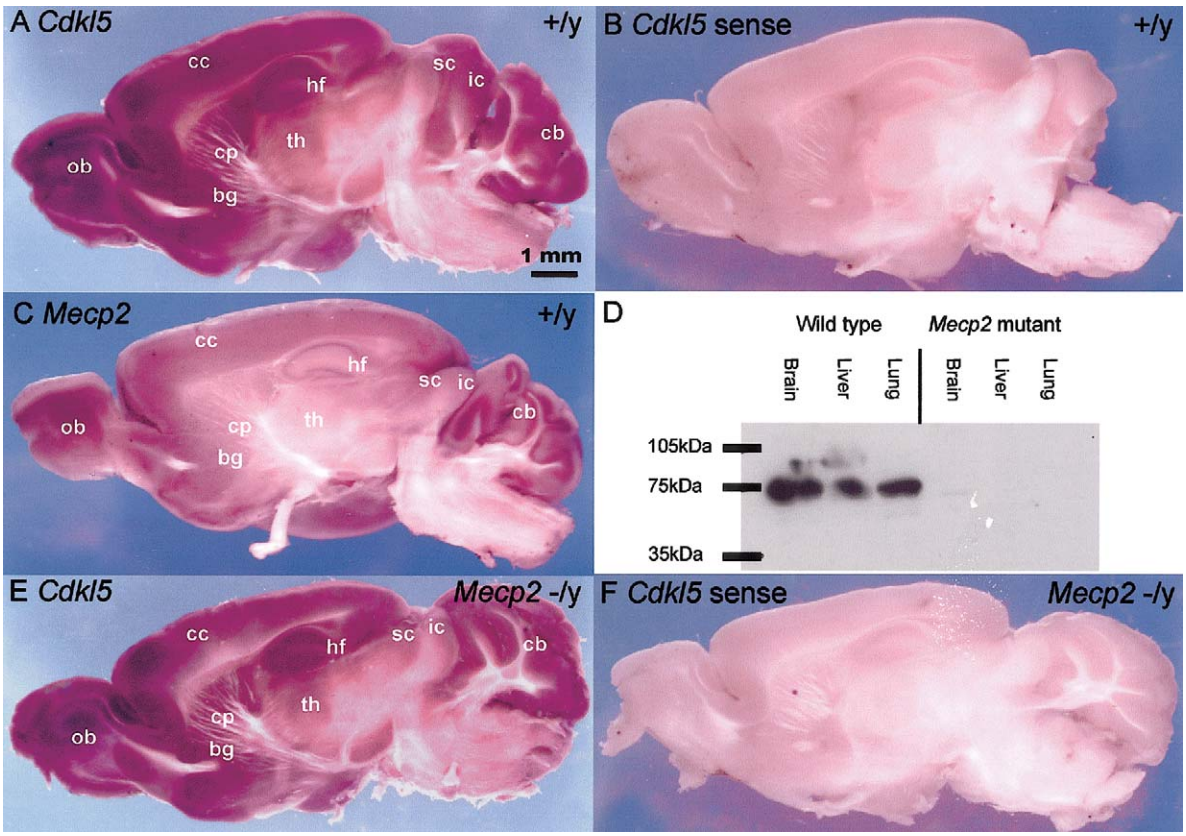

Figure 6 Expression of *CDKL5* in the mouse brain. *A* and *B*, Expression of *Stk9* in the brain of wild-type postnatal week-8 mouse (*A*, antisense riboprobes; *B*, sense riboprobes). *C*, Expression of *Mecp2* in the brain of wild-type postnatal week-8 mouse. *D*, Western-blot analysis showing the absence of the 75-kDa Mecp2 protein in the brain of the *Mecp2* mutant male mouse, confirming that a null mutation has been generated by deleting the sequence coding the transcription-repression domain and the C-terminal of the protein (G. J. Pelka, C. Watson, T. Radziewic, M. Hayward, and P. P. L. Tam, unpublished data). *E* and *F*, Expression of *Stk9/Cdkl5* in the brain of postnatal week-8 *Mecp2*$^{-/Y}$ mutant male mouse (*E*, antisense riboprobes; *F*, sense riboprobes). In situ hybridization was performed on parasagittal slices of the mouse brain. Abbreviations for brain regions: bg = basal ganglia; cb = cerebellum; cc = cerebral cortex; cp = caudate-putamen; hf = hippocampal formation; ic = inferior colliculi; ob = olfactory bulb; sc = superior colliculi; th = thalamus.

identified in two patients with severe X-linked infantile spasms and MR (Kalscheuer et al. 2003), and a C-terminal deletion has also been described in a patient with epilepsy and retinoschisis (Huopaniemi et al. 2000). This led Kalscheuer et al. (2003) to propose *CDKL5* as the second locus for X-linked infantile spasms (ISSX) syndrome, features of which were present in both families 1 and 2 in the present study. In family 1, a single base-pair deletion was identified in the affected individuals, at nucleotide 183 of the coding sequence (c.183delT) (fig. 3). This mutation was not observed in unaffected family members. The c.183delT mutation leads to a frameshift and early truncation of the protein at amino acid 75. Combined, these findings suggest that this is a pathogenic mutation causing a number of clinical phenotypes in this family, including early-onset seizures and a clinical phenotype that overlaps RTT, autistic disorder with mild-to-moderate intellectual disability, and the phenotype in the affected male with profound intellectual disability and seizures. The finding that the mother does not carry the mutation was not entirely surprising, since she herself was clinically unaffected. Since mutation screening was performed on blood and buccal DNA, it is likely that she is a gonadal mosaic, carrying the mutation in her germ cells.

After identification of the *CDKL5* mutation in family 1, *CDKL5* sequencing was performed on a cohort of 44 other patients with RTT and no identified pathogenic *MECP2* mutation, including one proband from each of the four familial cases reported by Villard and colleagues (2001). A splice-site mutation was observed in intron 13 (IVS13-1G→A) in a single patient with a phenotype characterized by the early onset of seizures and features that overlap RTT (fig. 2). This patient had a half sister with classic RTT, but neither the sister nor their parents appeared to carry the *CDKL5* mutation.

This phenomenon is not without precedent, since the presence of a *MECP2* mutation in one but not all affected members of a family unit (who may each show variable clinical features) has been reported elsewhere (Gill et al. 2003). Our results from haplotype analysis of *CDKL5* and *MECP2* loci in the family further support the hypothesis of different causes of the RTT phenotype in the half sisters.

The IVS13-1G→A mutation changes the canonical 3′ acceptor splice site of intron 13 from AG to AA. Such a 3′ acceptor splice site has not been detected in eukaryotic genomes (Burset et al. 2000). It would, therefore, be highly unlikely for it to be recognized by the splicing machinery and thus be likely to cause abnormal splicing. We demonstrated that the consequence of the IVS13-1G→A change is an activation of a cryptic splice site (81.34% consensus strength) just 1 bp downstream of the original site (96% consensus strength) (Shapiro and Senapathy 1987). We also showed that the IVS13-1G→A nucleotide change leads to the production of *CDKL5* mRNA with a deletion of the G at position 2047 (c.2047delG; see fig. 4). This would lead to production of a truncated form of the STK9 protein and is the most likely cause of the clinical presentation of early-onset seizures and the phenotype that overlaps RTT observed in family 2 (patient II:1).

It is interesting to note that the seizures seen in the affected individuals reported in this study and those mentioned in other published reports of *CDKL5* gene defects (Huopaniemi et al. 2000; Kalscheuer et al. 2003) were early in their onset and were generally difficult to control with anticonvulsant therapies. Early-onset seizures, usually more severe, have been described elsewhere in patients with RTT, and these seizures may sometimes precede the appearance of the more recognizable RTT features (Steffenburg et al. 2001). Steffenburg et al. (2001) found an onset of seizures before a patient's first birthday in 7 (18%) of 53 cases in their series, although none were actually classified as infantile spasms. In our Australian cohort with RTT (excluding family 1 in the present study), 18 (7%) of 258 patients with verified RTT had seizures in the 1st year of life, and an *MECP2* mutation was found in only 3 of 12 individuals in whom mutation screening had been performed (H.L., unpublished data).

The twins from family 1 were confirmed as identical by microsatellite mapping of the X chromosome and HLA typing but were discordant for their phenotype. Since the twins are genetically identical, their phenotypic differences may only be attributable to epigenetic or environmental differences. Although rare, discordance between identical twins for the RTT phenotype has been described elsewhere (Migeon et al. 1995; Ishii et al. 2001). The most likely explanation for the discordance in these instances was thought to be differential skewing of X inactivation, as was observed by Hoffbuhr et al. (2001), although the development of a mutation in one but not the other postzygotic embryo remains an untested possibility in these studies predating the identification of *MECP2* as the major causative gene in RTT. However, both of the twins in family 1 had similar levels of skewing of the X chromosome (18:82 and 23:77), and this appeared to favor the same chromosome. As such, skewing of X inactivation does not explain their phenotypic differences, unless some discordance between the brain, as the primarily affected tissue, and blood is speculated. Such differences in X-inactivation patterns between different tissues have been described elsewhere (Tan et al. 1993; Gale et al. 1994). Moreover, variability in X inactivation across different brain regions in individual heterozygous *Mecp2*$^{308/X}$ mutant mice has been reported (Young and Zoghbi 2004), and this, too, could contribute to differences in the severity of the clinical phenotype.

It should be noted that another group has recently identified *CDKL5* missense mutations in two patients with diagnoses of RTT. These patients had early-onset seizures and severe psychomotor retardation—characteristics of the early-onset–seizure RTT variant—as well as a number of other typical clinical features associated with RTT, but they did not have the very noticeable intense eye gaze that is characteristic of patients with classical RTT (Tao et al. 2004 [in this issue]). Patients with atypical RTT, including those with the early-onset–seizure variant, are less likely to have *MECP2* mutations (Hammer et al. 2002) and so should be considered as strong candidates for *CDKL5*-mutation screening.

STK9 is a putative serine/threonine kinase of unknown function (Montini et al. 1998; Brunner et al. 1999). The mechanism by which mutations in *CDKL5* produce phenotypes overlapping those seen in patients with *MECP2* mutations is, therefore, a puzzle, although the most likely hypothesis is that they operate in the same developmental pathway. Little work has been done on *CDKL5,* but sequence comparisons have indicated that its protein product is most closely related to p56 KKIAMRE (the protein encoded by *CDKL2*) and p42 KKIALRE (the protein encoded by *CDKL1*), which share homology with the mitogen-activated protein (MAP) kinase family of cyclin-dependent protein kinases (Montini et al. 1998).

It has recently been reported that phosphorylation of MeCP2 causes it to be released from a target promoter, resulting in transcriptional reactivation (Chen et al. 2003). It is unknown at this stage whether MeCP2 and STK9 interact directly with each other, with MeCP2 possibly being a phosphorylation target for STK9; whether they operate via a common developmental pathway; or whether they might exert their effects through unrelated mechanisms. Future work to identify

the functional role of STK9, its relation to MeCP2, and the mechanisms regulating both will be essential in understanding the biological processes involved in the etiology of RTT and of related clinical phenotypes. Dissection of this developmental pathway may also identify further RTT candidate genes and potential targets for therapy.

Together, our results suggest that *CDKL5* mutations can give rise to a phenotype that strongly resembles RTT. However, given the variant phenotypes seen in family 1 and in the three other reported cases with *CDKL5* mutations (Huopaniemi et al. 2000; Kalscheuer et al. 2003), screening of a larger cohort of patients with atypical RTT—particularly those with early-onset seizures—and individuals with overlapping phenotypes, including severe intellectual disability with autistic features, may lead to the identification of further mutations in this gene and may clarify the contribution of *CDKL5* to the pathogenesis of RTT and related disorders.

In summary, we have identified another gene that may cause a phenotype that shows considerable overlap with RTT. Mutations in *CDKL5* may also be associated with the clinical phenotype of autistic spectrum disorder with MR and complex seizure disorders in females and the phenotype of severe neonatal-onset neurological abnormalities in males. It remains to be determined whether *CDKL5* mutations may account for a large proportion of cases showing significant overlap with RTT and in which no pathogenic *MECP2* mutations have been found. There is a need for a large-scale multicenter collaborative study using, when feasible, cases sourced on a population basis, to investigate the role of *CDKL5,* and possibly other genes from the same pathway, in the pathogenesis of RTT and related disorders.

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Electronic-Database Information

The URLs for data presented herein are as follows:

Cedar Genetics Map, http://cedar.genetics.soton.ac.uk/pub/chromX/gmap (for map of the X chromosome and genetic map distances)

Ensembl Human Genome Browser, http://www.ensembl.org/Homo_sapiens/mapview?chr=X (for MapView and genetic map distances)

Genome Database, http://gdbwww.gdb.org/ (for microsatellite information)

Integrated X Chromosome Database, version 2.3, http://ixdb.molgen.mpg.de/ (for genetic map distances)

Mouse Genome Informatics (MGI), http://www.informatics.jax.org/ (for details of the mouse *Cdkl5* sequence)

NCBI, http://www.ncbi.nlm.nih.gov/ (for BLAST searches and sequence information)

Online Mendelian Inheritance in Man (OMIM), http://www.ncbi.nlm.nih.gov/Omim/ (for *CDKL5*)

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