

## Refinement of a 400-kb Critical Region Allows Genotypic Differentiation between Isolated Lissencephaly, Miller-Dieker Syndrome, and Other Phenotypes Secondary to Deletions of 17p13.3

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Deletions of 17p13.3, including the *LIS1* gene, result in the brain malformation lissencephaly, which is characterized by reduced gyration and cortical thickening; however, the phenotype can vary from isolated lissencephaly sequence (ILS) to Miller-Dieker syndrome (MDS). At the clinical level, these two phenotypes can be differentiated by the presence of significant dysmorphic facial features and a more severe grade of lissencephaly in MDS. Previous work has suggested that children with MDS have a larger deletion than those with ILS, but the precise boundaries of the MDS critical region and causative genes other than *LIS1* have never been fully determined. We have completed a physical and transcriptional map of the 17p13.3 region from *LIS1* to the telomere. Using fluorescence in situ hybridization, we have mapped the deletion size in 19 children with ILS, 11 children with MDS, and 4 children with 17p13.3 deletions not involving *LIS1*. We show that the critical region that differentiates ILS from MDS at the molecular level can be reduced to 400 kb. Using somatic cell hybrids from selected patients, we have identified eight genes that are consistently deleted in patients classified as having MDS. In addition, deletion of the genes *CRK* and *14-3-3ε* delineates patients with the most severe lissencephaly grade. On the basis of recent functional data and the creation of a mouse model suggesting a role for *14-3-3ε* in cortical development, we suggest that deletion of one or both of these genes in combination with deletion of *LIS1* may contribute to the more severe form of lissencephaly seen only in patients with MDS.

### Introduction

Classical lissencephaly (LIS) is a severe brain malformation characterized by absent or reduced gyration of the cerebral surface and a thickened and simplified four-layered cortex resulting from incomplete neuronal migration during early brain development. Patients with LIS usually have mental retardation, intractable epilepsy, spasticity, and reduced longevity (Dobyns et al. 1993). The clinical severity generally correlates with the degree of agyria and cortical thickening. The complete LIS spectrum includes agyria (LIS grade 1), mixed agyria-pachygyria (LIS grades 2 and 3), and pachygyria alone (LIS

grade 4) (Dobyns et al. 1999). The genetic basis of LIS has now been elucidated for most patients, with two main patterns evident. LIS more severe posteriorly than anteriorly is generally secondary to abnormalities of the *LIS1* gene (*PAFAH1B1* [MIM 601545]) on 17p13.3. This form of LIS is seen in both isolated lissencephaly sequence (ILS) and Miller-Dieker syndrome (MDS [MIM 247200]). LIS more severe anteriorly than posteriorly is generally due to abnormalities of the *DCX* gene (or *doublecortin* [MIM 300121]) on Xq22.3–q23 (Pilz et al. 1998a).

ILS is characterized by LIS and minimal or no other dysmorphic features. MDS is associated with LIS, significant facial dysmorphism, and, occasionally, other congenital anomalies, such as renal, gastrointestinal, and cardiac defects (table 1). In addition, the severity of LIS seen in MDS is generally greater than that seen in ILS (Dobyns et al. 1991). MDS is seen only in patients with deletions of 17p13.3, detected either by high-resolution cytogenetics or by the use of specific FISH

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**Table 1****Clinical Manifestations Observed in Patients with ILS versus Patients with MDS**

AFFECTED REGION	MANIFESTATION IN PATIENTS WITH	
	ILS	MDS
Brain	Grade 2–4 LIS	Grade 1 LIS
Face	Bitemporal hollowing, mildly small jaw	Bitemporal hollowing, small jaw, microcephaly, tall square forehead, vertical forehead furrow, flattened midface, short upturned nose, thickened rounded upper lip, low-set posteriorly rotated ears, frontal bossing, prominent lateral nasal folds
Other	None	Cardiac defects, omphalocele, inguinal hernia, duodenal atresia, cryptorchidism, cystic kidney, pelvic kidney, polydactyly, clinodactyly, campylodactyly, transverse palmar creases

probes (Ledbetter et al. 1989; Pilz et al. 1998b). In contrast, ILS may be secondary to deletions of 17p13.3, intragenic mutations, or internal deletions of the *LIS1* gene (Chong et al. 1997; Lo Nigro et al. 1997).

The precise deletion boundaries and, therefore, the deleted genes differentiating ILS from MDS in patients with 17p13.3 deletions have never been adequately determined. Previous studies have shown that the telomeric border of 17p13.3 deletions in MDS is generally more distal than those in patients with ILS (Chong et al. 1996). The study of patients with MDS and ILS also raises the question as to why patients with MDS have more severe LIS than patients with ILS. It may be assumed that the differences between the ILS and MDS phenotypes (namely, the severity of LIS and the dysmorphic features) must be secondary to deletion of additional genes telomeric to *LIS1*. Haploinsufficiency of the *LIS1* gene alone is insufficient to explain the severe (grade 1) LIS seen only in MDS patients. This suggests that there are cortical development genes other than *LIS1* deleted in patients with MDS but not in those with ILS.

The aim of this study was to investigate the genotypic difference between patients with MDS and ILS secondary to 17p13.3 deletions and to use this data to identify candidate genes to explain the difference in the phenotype. We initially performed a detailed phenotypic characterization of 34 patients with a deletion of 17p13.3, including 4 patients without LIS. We constructed a physical and transcriptional map covering the most distal 2.8 Mb of the 17p13.3 region. We used BAC/PAC genomic clones in FISH experiments to size the deletions in a spectrum of MDS and ILS patients, which defined the telomeric critical region for MDS distal to D17S1574. In addition, we mapped the 17p13.3 deletion in the four patients without LIS and mild or no dysmorphic features, to refine this region to 400 kb. Within this “telomeric MDS critical region,” we used PCR analysis of somatic cell hybrids from patients with ILS and MDS to identify two candidate genes among

eight, which, when deleted together with *LIS1*, could be responsible for the more severe cortical phenotype seen in MDS.

## Subjects and Methods

### Patient Ascertainment

Subjects were ascertained through the Lissencephaly Research Project, which currently is based at The University of Chicago, from referrals from physicians and genetic counselors, The Lissencephaly Network, and inquiry from parents of affected children. We reviewed clinical data in all 34 patients and cranial computed tomography or magnetic resonance imaging scans in 27 of 30 patients with LIS and 2 of 4 patients without LIS. If the child could not be examined, photographs were used to document the facial phenotype.

### Clinical Ascertainment and Diagnosis

We selected 30 patients with ILS or MDS for study. All came to medical attention because of problems, including abnormal appearance, feeding problems, developmental delay, seizures, or a combination of these. All had LIS on brain imaging, and all had deletions of 17p13.3 by high-resolution cytogenetics or FISH. Deletions were visible in 9 of 30 patients, including 2 patients (LP90-007 and LP95-041) with unbalanced segregation of familial translocations involving chromosome 17p and 16p or 3p. Brain imaging studies were available for 27 of the 30 patients and were reviewed to confirm the diagnosis of ILS or MDS. Evaluation was performed by use of an LIS grading scale described elsewhere (Dobyns et al. 1999).

We also studied four unrelated subjects with a deletion of the 17p13.3 region but without LIS. Of these subjects, two (96.583 and 97.513) had a telomeric 17p deletion identified by cytogenetic analysis (Martin et al. 2002). The other two subjects carried a deletion of the 17p13.3 region due to an unbalanced translocation between 17p

**Table 2****Primer Pairs Used to Amplify Genes Localized in the Telomeric MDS Critical Region**

Gene	Forward Primer (5'→3')	Reverse Primer (5'→3')
ABR exon 1	GCAGTGGCTCCTTGATACAAA	AACACTATGGGCTCCACTGAG
14-3-3 $\epsilon$ exon 6	TGGCTGCCATAGCCTAATGTA	TCAGTGACAATGGGGAGTTTC
CRK exon 2	CAGACTCCGAATAGGAGATCA	CCAAAGCCAAGGCTGTCTTGT
CRK exon 3	TAGTGGTCGTGCCCTGTGTGTA	AACAGTCTGTGCCATTGTGAT
MYO1C exon 2/3	GACTGTGTACCGAGCACTG	AGCACCGGGTTGCTCTGTAG
SKIP exon 9	CATCCTGTGGAGGCTGAAG	TACGTCATGTGGCTGCTGTAG
PITPN $\alpha$ 3' UTR	AGTGGTCAACAGGAAGACCC	AATGGATCGGAACACAATGG
SREC 3' UTR	AAGGCAGGAGGAACCTGAGTA	CAGGACAGCAGAGCAAAAAGTC
RILP exon 6	AGAGGGACAGTACAAAGGGTTG	CATCAGGCTCAGCAGAATGAG
PRP8 exon 41/43	CAAGATCATGGCTGACAACC	CAAAGTCTGAATGTCAGCG
SERPINF1 exon 5	TTGACCGGAAGCATGAGTATC	GGTGACTTCGCCTTCGTAAC
RPA1 3' UTR	CATCGGTAGGCAAAGGAAAA	ACAGAAGCCTGAAGGCAGAG
LIS1 exon 2	CATTACAGCCAAGATGGTGCT	AGCACCATCTTGGCTGTAATG

and 8p (patient DR00-063a1) or between 17p and 5p (patient DR01-001) (Mutchinick et al. 1999).

We examined 22 of the 30 patients with LIS and reviewed photographs of all but 1. We also examined and reviewed photographs of patients DR00-063a1 and DR01-001. We used a simple facial malformation grading system, in which facial changes were graded as normal (0), intermediate (1), or typical of MDS (2) by each of three experienced clinical geneticists (J.A., D.T.P., and W.B.D.). The three examiner scores were added together, giving overall scores of 0–6. Patients with scores of 0–2 were classified as having a normal facial appearance (ILS), patients with scores of 3–5 as having intermediate facial changes (ILS+), and patients with a score of 6 as having severe and characteristic facial abnormalities (MDS).

#### *Identification and Characterization of BAC and P1-Derived Artificial Chromosomes (PAC)*

Thirteen BAC clones from the RPCI-11 human library, nine BAC clones from Caltech human libraries B and D, and five PAC clones from RPCI-4 and -5 human libraries were selected to cover the most distal 3 Mb of chromosome 17p13.3, which corresponds to the presumed telomeric critical region for MDS. The vast majority of the clones were selected from human chromosome 17 database resources, such as the Sanger Institute and the Weizmann Institute, as well as genome resources, such as the National Center for Biotechnology Information (NCBI), for their content of STSs, ESTs, or genes described previously in the 17p13.3 region (Stack et al. 1995; Plummer et al. 1997; Hoff et al. 2000; McHale et al. 2000). All genomic clones were purchased from Research Genetics. Genomic DNA from BAC and PAC clones was isolated by use of an automated DNA isolation system (AutoGen 740, Integrated Separation

Systems). The BACs and PACs were further characterized by PCR to analyze the presence or absence of STSs and by FISH to confirm their cytogenetic positions. Two clones, PAC95H6 and RPCI5-59D14 (GenBank AC006435), were selected on the basis of published physical and transcriptional maps of 17p13.3 (Chong et al. 1997; McHale et al. 2000), and GSS-68F18 was selected as a telomeric probe (Knight et al. 2000).

#### *In Silico Identification and Mapping of STSs and ESTs*

For our BAC/PAC contig, the complete or unfinished sequence for all the clones was available from the NCBI databases. We systematically analyzed these sequences to identify and map STSs and new ESTs or to confirm the mapping of known genes, using the NIX genomic sequence analysis program and the BLASTN algorithm using the NCBI databases against STS or EST databases.

#### *STS and Gene Mapping by PCR*

PCR reactions were carried out in a total volume of 50  $\mu$ l containing 25–50 ng of DNA as the template, 1  $\times$  PCR buffer with 1.5 mM MgCl<sub>2</sub>, 0.2 mM of each dNTP, 1  $\mu$ M of each primer, and 2.5 U *Taq*Gold polymerase (PE Applied Biosystems). Amplifications were performed in a PE480 thermal cycler (Perkin Elmer-ABI), with a 5-min 94°C denaturation step followed by 35 cycles of 94°C for 35 s, 35 s of annealing at the appropriate temperature, and 40 s of elongation at 72°C. The last cycle was followed by a final extension at 72°C for 10 min. PCR products were separated on 2% agarose gels. Primer sequences and annealing temperatures for the previously assigned STSs were obtained by use of the CEPH-G n thon database, the Whitehead/MIT Center for Genome Research database, the NCBI UniSTS database, and the HUGO Genome database (GDB), as well as data published elsewhere (Hoff et al. 2000; McHale

et al. 2000). The primer pairs used to amplify all genes localized between D17S1574 and D17S1840 are listed in table 2.

#### *FISH Analyses*

Slide, probe preparation, and hybridization were completed using methods described elsewhere (Chong et al. 1997). For three-color FISH experiments, probes were directly labeled with DEAC (Diethylaminocoumarin-5-dUTP, Perkin Elmer Life Sciences), Spectrum Orange-dUTP, and Spectrum Green-dUTP (Vysis). The DEAC probe signals were pseudocolored with magenta, because of the color similarities between DEAC and DAPI. FISH slides were analyzed by use of a Zeiss Axiophot microscope with single pass filters (Chroma Technology). Images were collected using a CCD camera (Nu 200, Roper Scientific) and Smart Capture 2 software program (Digital Scientific).

#### *Somatic Cell Hybrids*

Somatic cell hybrids containing deletions of the 17p13.3 region were derived from two MDS patients (hybrid cell lines CA2 and KCB4 from patients LP82-002 and LP84-001, respectively) and from three ILS patients (hybrid lines DB, BR8, and SG2 from patients LP86-003, LP87-001, and LP90-017, respectively) and have been described elsewhere (Van Tuinen et al. 1987; Ledbetter et al. 1989; Ledbetter et al. 1992). DNA was extracted from somatic cell hybrids by use of a Puregene DNA isolation kit (Gentra Systems), according to the manufacturer's protocol. We ensured that the primers were human specific by using total genomic DNA from mouse and hamster as a negative control.

## **Results**

#### *Detailed Phenotypic Analysis of 34 Selected Patients*

To establish a strong genotype-phenotype correlation between patients with deletions of 17p13.3, we performed a detailed phenotypic analysis on 34 selected patients (see the "Subjects and Methods" section). Among the 34 patients, by comparison with normal MRI images (fig. 1I and 1J), 30 patients had LIS and 4 had no LIS. Of the 30 patients with LIS, 10 had consensus ILS with a normal facial appearance, except for the minor changes typical of this condition (table 1; fig. 1A); 9 patients had intermediate facial abnormalities and were classified as ILS+ (fig. 1C); and 11 had a consensus MDS facial appearance (table 1 and fig. 1E and 1G). Patients with ILS always had LIS grade 3 or 4 (fig. 1B), whereas those with ILS+ had LIS grade 2 or 3 (fig. 1D). In contrast, all patients with consensus MDS had LIS grade 1 or diffuse agyria (fig. 1F and 1H) and the characteristic facial features (table 1). Thus, we found a

strong correlation between the most prominent dysmorphic features and most severe brain abnormalities.

Of the four subjects who did not have LIS, two (96.583 and 97.513) were phenotypically normal (Martin et al. 2002), and the other two (DR00-063a1 and DR01-001) were mentally retarded. Regarding the latter, DR00-063a1 had a normal MRI scan, and DR01-001 had epilepsy and microcephaly, with an MRI scan showing a thin corpus callosum and frontal lobe hypoplasia (fig. 1L). DR01-001 had mild facial dysmorphism and has been reported elsewhere (Mutchinick et al. 1999) as having a facial phenotype consistent with MDS (fig. 1K). On consensus review for this study, we concluded that she had an abnormal facial appearance that did not meet our criteria for typical MDS.

#### *Physical and Transcriptional Map from D17S1845 to the 17p Telomere*

To identify which telomeric genes could participate with *LIS1* in producing the more severe phenotype seen in MDS, compared to that in ILS, we constructed a physical and transcriptional map from the proximal marker D17S1845 (close to *LIS1*) toward the telomere. A BAC/PAC contig was created by linking newly characterized BAC and PAC clones obtained from numerous databases with data published elsewhere (see the "Subjects and Methods" section). We used an STS-based approach combined with computational methods to construct a BAC/PAC contig spanning ~2.8 Mb (fig. 2). Each BAC or PAC clone was tested by FISH analysis on normal human metaphase chromosomes to verify its unique localization to 17p13.3. BAC407I21 (GenBank accession number AF322450), for which sequence is available at the NCBI database, was previously mapped on 17p13.3 by Gu and colleagues (Shanghai Cancer Institute).

The completed contig consists of 23 BAC and 6 PAC clones (fig. 2). It includes three regions covered only by a single BAC clone, which corresponds to the clones RPCI-11 135N5 (GenBank accession number AC015799), RPCI-11 676J12 (GenBank AC087392), and RPCI-11 818O24 (GenBank AC032044). For the latter region, the *CRK* gene (GenBank NM\_005206 and NM\_016823) helped us to link the clone RPCI11-818O24 with BAC407I21. The overlap between these two BACs, not previously identified as being overlapping clones, corresponds to exon 2 and intron 2 of the highly conserved *CRK* gene, which we estimate to be at least 12 kb by comparison with the mouse genome sequence (cf. clone RPCI23-78H4 [GenBank AL669897] and clone RPCI23-190H20 [GenBank AL663094]). In addition, the telomeric minicontig could not be joined with the main contig (fig. 2). On the basis of the size estimation of this region by the Weizmann Institute database, we expect that the gap between PAC RPCI5-

1029F21 (GenBank AC015853) and BAC CTD-2578J3 (GenBank AC108006) is no more than 150 kb in length. Using the physical clone contig as a resource, we assigned 23 known genes, 11 ESTs or unknown genes, and 1 pseudogene to 17p13.3, all of which were described in the Unigene database. We also assigned 24 genetic markers or anonymous STSs and 15 BAC/PAC end clones to this region (fig. 2).

#### *Characterization of the “Telomeric Critical Region for MDS”*

To further define the “telomeric MDS critical region,” we used a selection of BAC/PAC clones from our map as probes (fig. 2, clones noted in red) for FISH analysis to size and determine the boundaries of the deletion in our 34 selected patients (fig. 3). In addition, we used two BACs centromeric to D17S1845 as controls: RPCI11-713H12 (AC025518) contains the *NUDEL* gene (NM\_030808), which interacts with *LIS1* (Sasaki et al. 2000), and was not deleted in any of the 34 patients (fig. 3). Probe RPCI11-305G1 (AC032038) contains the *profilin1* gene (NM\_005022) and was deleted in only 4 of 34 patients (fig. 3). For one patient with MDS (LP84-001), PAC95H6 showed partial deletion of the *LIS1* gene (fig. 3), which was confirmed by deletion of the telomeric overlapping clone RCP111-135N5, which contains exons 1 and 2 of the *LIS1* gene (data not shown). Comparing the size of the deletions identified in patients with MDS with the size of the deletions in patients with ILS + and ILS, we found that BAC RPCI11-107N19 (AC006405) was deleted in three patients with ILS+ (LR01-118, LP99-086, and LP86-003) and all patients with MDS (fig. 3). Next, we compared the size of the deletion among patients with MDS and found that, for one MDS patient (LP82-002), the deletion did not extend beyond BAC RPCI11-818O24 (figs. 2 and 3). Therefore, our FISH studies demonstrated that the “telomeric critical region for MDS” is contained within the two BACs RPCI11-107N19 and RPCI11-818O24, which means that the critical region is distal to D17S1574, which is contained in RPCI11-4F24 (AC007873), and proximal to D17S1582, which is located at the telomeric end of RPCI11-818O24 (figs. 2 and 3).

This result is also supported by sizing of the deletion in the four subjects who did not have LIS. For the two phenotypically normal individuals (96.583 and 97.513), FISH analysis revealed a deletion of the 17p telomeric region, which for 97.513 included the *ABR* gene (fig. 3). Our data confirmed the previous cytogenetic analysis (Martin et al. 2002), which revealed satellites on the short arm of one chromosome 17 and indicated that the most distal genes are not dosage sensitive. Next, we mapped the breakpoint on the deleted chromosome 17

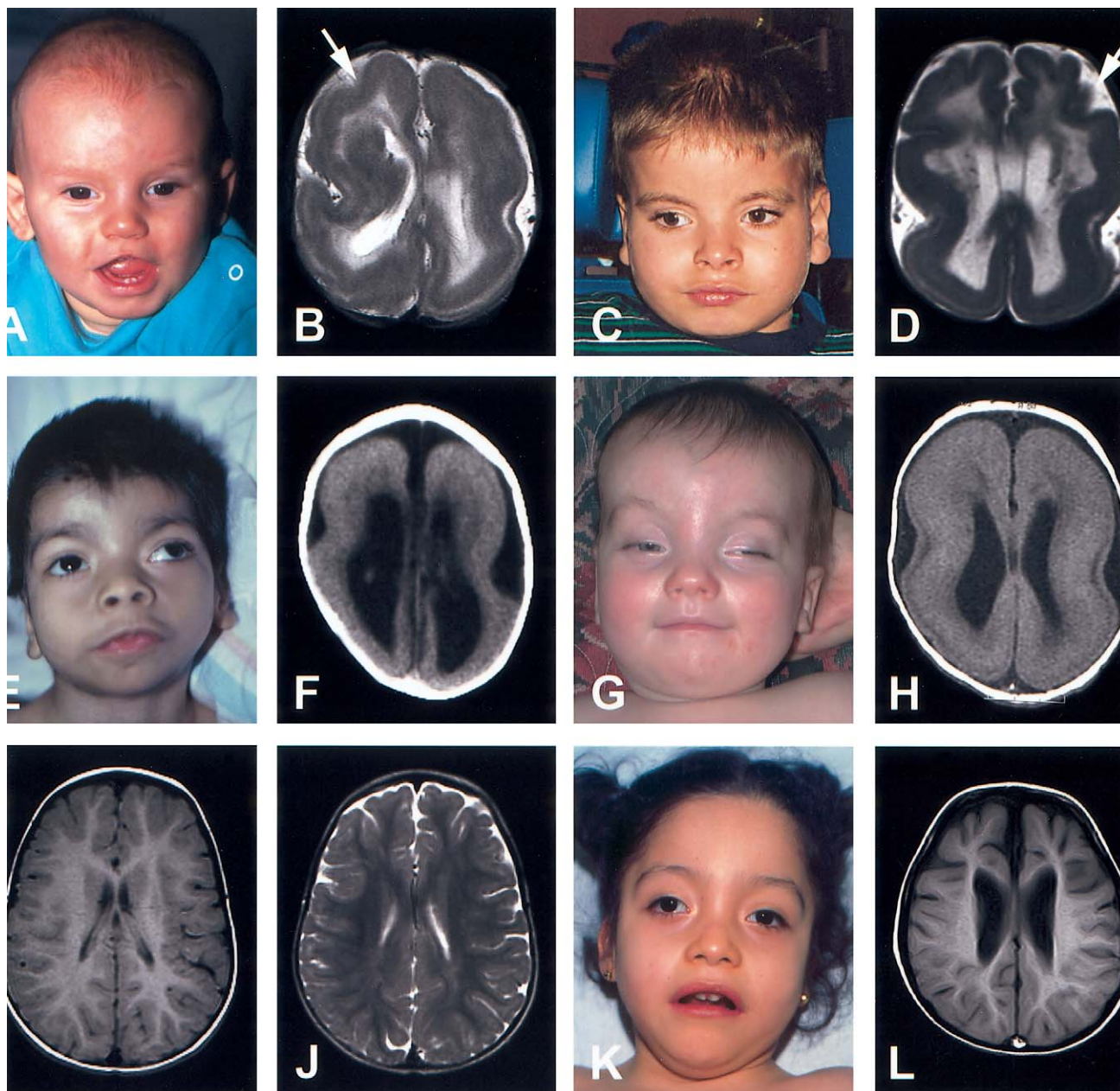
of the two mentally retarded patients (see the “Subjects and Methods” section) as being around marker SHGC-32238 for patient DR00-063a1 and D17S1574 for DR01-001 (figs. 2 and 3). DR00-063a1, with the smallest deletion, had mild mental retardation and normal facial appearance, as did her mother (data not shown). DR00-001, with the largest deletion, had severe mental retardation and mild facial dysmorphism not considered typical of MDS (fig. 1K and 1L). However, patient DR01-001 also has several specific phenotypic traits characteristic of the 5p trisomy due to significant additional material added to the deleted 17p as a result of the translocation with 5p. Specific phenotypic characteristics of the trisomy 5p present in patient DR01-001 are a depressed nasal bridge, long fingers, and bilateral talipes equinovarus (Mutchinick et al., 1999). In these two patients, we identified a region that may contain a gene involved or responsible for mental retardation, which seems to overlap with the “telomeric critical region for MDS” previously defined.

Finally, this data has enabled us to design a three-color FISH assay using PAC95H6, BAC RPCI11-818O24, and BAC68F18 (fig. 3) as a diagnostic screen for patients with 17p13.3 deletions, and the results showed a robust correlation with the clinical phenotype (fig. 4). Deletion of PAC 95H6 alone (fig. 4B) was associated with ILS (fig. 1C and 1D). Deletion of PAC95H6 in combination with one or both of RPCI11-818O24 or BAC68F18 (fig. 4C) was associated with MDS (fig. 1G and 1H). Deletion of RPCI11-818O24 and BAC68F18 but not PAC95H6 (fig. 4D) was not associated with a LIS phenotype (fig. 1K and 1L).

#### *Deletion of CRK and 14-3-3ε Is Seen Only in Patients with the Most Severe Form of LIS*

With the telomeric MDS critical region defined, we wanted to determine which genes within that region (in addition to *LIS1*) may contribute to the MDS phenotype when deleted. We used somatic cell hybrids established from five selected patients with LIS and deletions involving the telomeric MDS critical region. Among these five patients, three had an intermediate ILS+ phenotype with mild dysmorphic features and grade 3 LIS for SG2 (LP90-017) or grade 2 LIS for BR8 (LP87-001) and DB (LP86-003). The two others, CA2 (LP82-002) and KCB4 (LP84-001) had a consensus MDS phenotype with distinct dysmorphic features and grade 1 LIS (fig. 3).

By PCR, we tested all known genes in the telomeric MDS critical region between D17S1574 and D17S1582 (fig. 2 and table 2) (see the “Subjects and Methods” section). PCR results showed that, for hybrid SG2, amplification of the *PRP8* gene (GenBank XM\_028335) delineated a telomeric deletion boundary around the STS marker SHGC-32238 (figs. 2 and 5). For hybrids BR8



**Figure 1** Detailed phenotypic analysis of patients with deletions of 17p13.3, showing increasing severity of brain and face changes with larger deletions extending closer to the 17p telomere. LP95-108 has typical ILS with normal facial appearance (A) and LIS grade 3 with a posterior > anterior gradient (B) associated with a small submicroscopic deletion. LP99-086 has ILS with facial changes intermediate between typical ILS and MDS (C) and LIS grade 3 with a posterior > anterior gradient (D). LP82-002 (E and F) and LR01-167 (G and H) have consensus MDS (E and G) and severe LIS grade 1 (F and H) with no visible gradient. Both the brain and face abnormalities are more severe than in the patients with ILS shown (A–D). Normal T1 and T2 axial images are shown for comparison (I and J). The face and brain abnormalities are more subtle in subjects with deletions that do not include the *LIS1* gene. DR01-001 has a mildly dysmorphic facial appearance not specific for MDS (K) and mild frontal lobe hypoplasia without LIS (L).

and DB, their telomeric breakpoints are respectively localized between the *SREC* (GenBank XM\_008489) and *PITPN $\alpha$*  (GenBank NM\_006224) genes close to D17S1577 and between the *MYO1C* (GenBank XM\_028385) and *CRK* genes (figs. 2 and 5). Finally, deletions

in hybrids CA2 and KCB4 probably have the same telomeric boundary, which is localized around D17S1582, shown by amplification of only the *ABR* gene (NM\_021962 and NM\_001092) for both patients (figs. 2 and 5). In addition, for KCB4, we confirmed the partial de-



letion of the *LIS1* gene by no amplification of exon 2 (data not shown). Thus, in patients defined as having an MDS phenotype with the most severe LIS grade (CA2 and KCB4), we found exclusive deletion of only two genes (*CRK* and *14-3-3ε*) that were not deleted in ILS+ patients (SG2, BR8, and DB) (fig. 5).

## Discussion

Several syndromes—such as Williams syndrome (del 7q11), Prader-Willi and Angelman syndromes (del 15q11-q13), and DiGeorge syndrome (del 22q11)—are associated with hemizygous deletions at recurrent sites of a specific chromosome (Morrow et al. 1995; Osborne et al. 1997; Christian et al. 1999). These are classical contiguous gene-deletion syndromes, in which the inactivation of several unrelated genes is involved in producing a complex phenotype. MDS has long been recognized as a contiguous gene-deletion syndrome with variable breakpoints, but the relationship between the genes deleted within chromosome 17p13.3 and the MDS phenotype has never been elucidated. By mapping the extent of deletions of the 17p13.3 region, which result in either ILS or MDS, we previously described a common critical region containing the *LIS1* gene responsible for both disorders (Chong et al. 1997). Our previous data also suggested that considerable overlap exists between the deleted region in patients with ILS and MDS, with the deletion in patients with MDS extending more telomeric on 17p13.3 (Ledbetter et al. 1989; Chong et al. 1997).

We have previously shown a relationship between genotype and phenotype in a large number of patients with ILS secondary to intragenic *LIS1* abnormalities, revealing a correlation between LIS severity and the location and type of *LIS1* mutation (Cardoso et al. 2000; Leventer et al. 2001). In that group of patients—even in those with a completely deleted or nonfunctional *LIS1* gene—there were areas of simplified brain folds (pachygyria), rather than completely absent folds (agyria), as seen only in patients with MDS. There has never been an adequate explanation as to why patients with MDS have the most severe LIS phenotype. One hy-

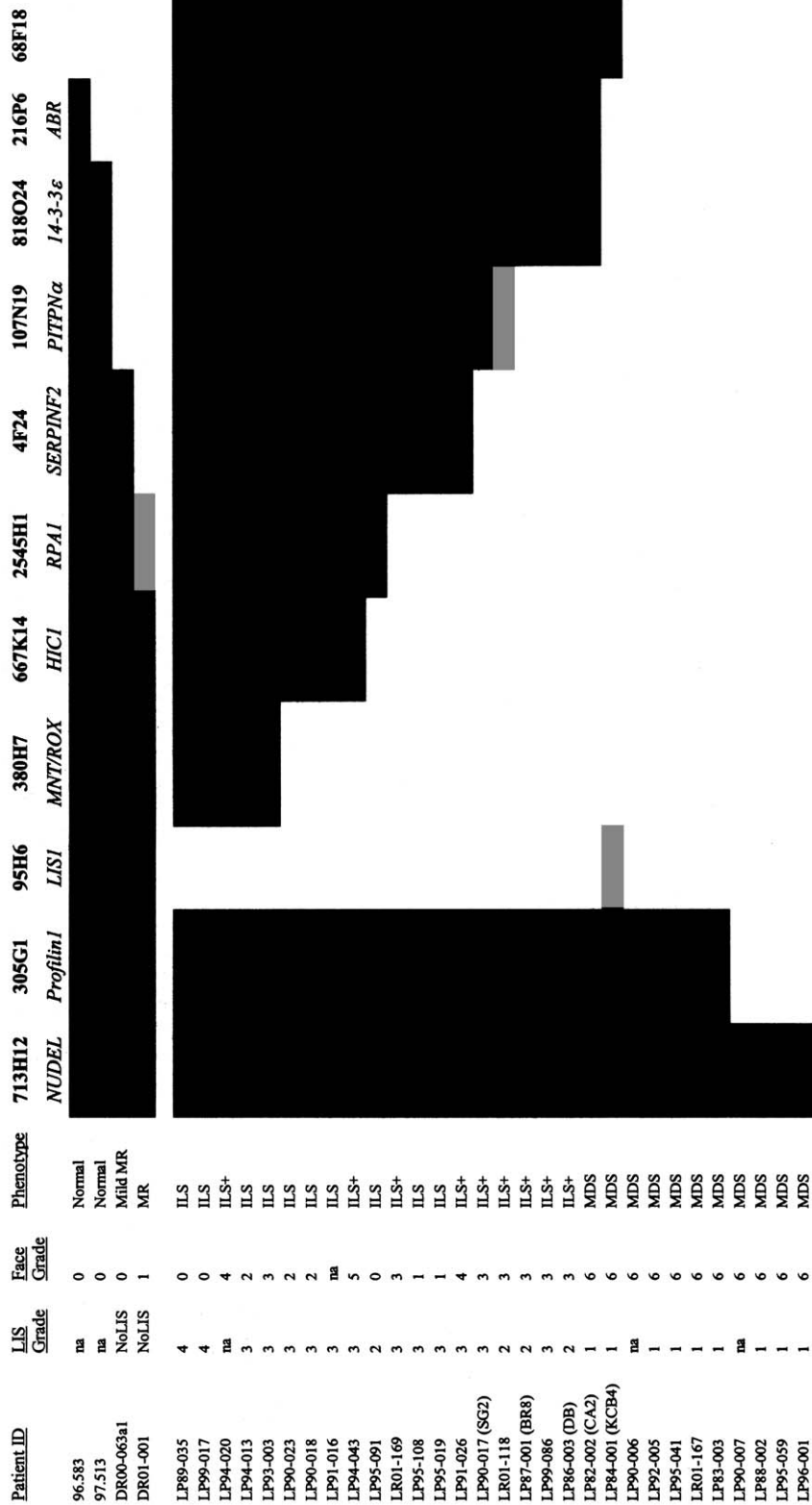
pothesis is that they are hemizygous for one or more genes, other than *LIS1*, that are involved in cortical development. To address this question, we initially constructed a detailed physical and transcriptional map of the 17p13.3 region. Next, we used this map to compare and contrast the boundaries of the deletions in a large number of patients with ILS and MDS. In addition, we studied four patients with terminal deletions involving 17p13.3 but without LIS. As hypothesized, we have shown that patients with MDS have a larger telomeric deletion than patients with ILS, and the larger deletions show a more severe grade of LIS. We have also shown that a deletion from *ABR*, toward the telomeric end of chromosome 17p, is associated with no apparent phenotype. These data proved very helpful in allowing us to define the distal boundary of the telomeric MDS critical region. We were able to narrow this region to 400 kb by comparing the deletions in ILS and MDS patients. In this interval we identified eight genes deleted exclusively in patients with MDS. These data clearly suggest that genes other than *LIS1* may participate in producing the MDS phenotype and support for the first time the concept that MDS truly is a contiguous gene-deletion syndrome.

The identification of eight genes (*PRP8*, *RILP*, *SREC*, *PITPNα*, *SKIP*, *MYO1C*, *CRK*, and *14-3-3ε*) in our newly defined telomeric MDS critical region is a first step in our goal to characterize which additional genes distal to *LIS1* may be responsible for the clinical features that distinguish MDS from ILS. Existing functional information regarding the proteins encoded by these genes show that *PRP8* is a highly conserved spliceosomal protein, which provides important catalytic and regulatory functions for pre-mRNA processing (Kuhn et al. 1999). Rab7-interacting lysosomal protein (or *RILP*) controls lysosomal transport by inducing the recruitment of functional dynein-dynactin motor complexes (Jordens et al. 2001). The scavenger receptor expressed by endothelial cells (or *SREC*) mediates the selective uptake of modified low-density lipoprotein (LDL) such as acetylated-LDL and oxidized-LDL into endothelial cells (Adachi et al. 2002). Phosphatidylinositol transfer protein alpha (*PITPNα*) is a member of a diverse set of

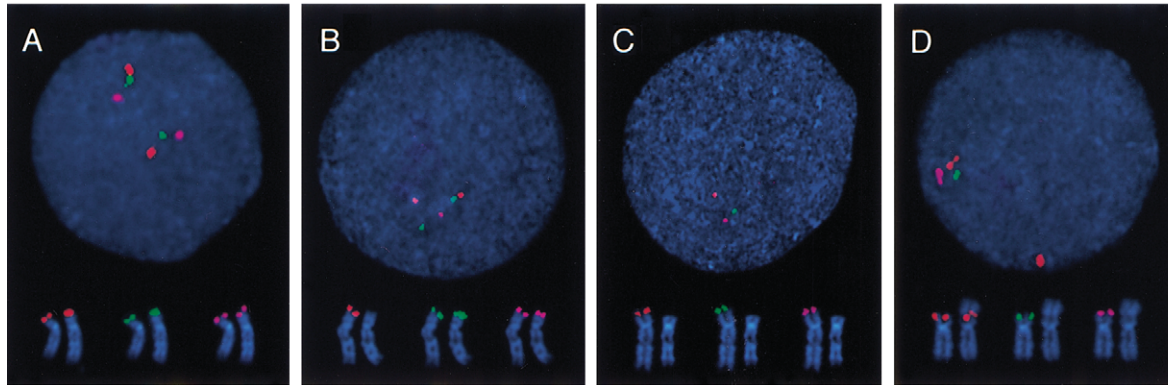
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**Figure 2** Physical and transcriptional map of the interval from D17S1845 to the telomere. *A*, The chromosomal orientation of the BAC/PAC contig covering the most distal 2.8 Mb of chromosome 17 in the region p13.3 is indicated. STS markers, genes, and BAC ends used for mapping are depicted by vertical lines at the top of the figure. A gray or red circle signifies the presence of an STS, a gene, or a BAC end on an individual PAC or BAC clone. All PAC clones are underlined, and BAC clones used in FISH analysis are indicated in red. The broken line indicates the gap between the main contig and the telomeric minicontig. Known genes mapped previously in this region are noted in blue, and the direction of transcription is indicated by an arrow. A pseudogene is denoted by a black box. The cDNA clones corresponding to unknown genes are noted in green. All known and new genes are placed exactly below the genomic clones that contain them. *B*, Schematic representation of deletions of the 17p13.3 region identified in patients with ILS, MDS, or other phenotypes without LIS. The solid lines below the map represent nondeleted genomic BAC/PAC clones. The dotted lines represent deletions associated with the patient number listed above. For patients with ILS or MDS, the dashed line indicates the largest deletion identified in each group of patients. The telomeric boundaries of 17p13.3 deletion of hybrids SG2, BR8, DB, CA2, and KCB4 are indicated by orange circles.





**Figure 3** Extent of deletion in 12 patients with MDS, 18 patients with ILS, and 4 patients without LIS. Black boxes denote the presence of the probe on both copies of chromosome 17, and white boxes denote the presence of the probe on only one chromosome 17. Gray boxes denote a partial deletion of the probe on one chromosome 17. na = Data not available. BAC ID numbers (*boldface*) and gene symbols (*italics*) are listed across the top of the figure from centromere (*left*) to telomere (*right*).



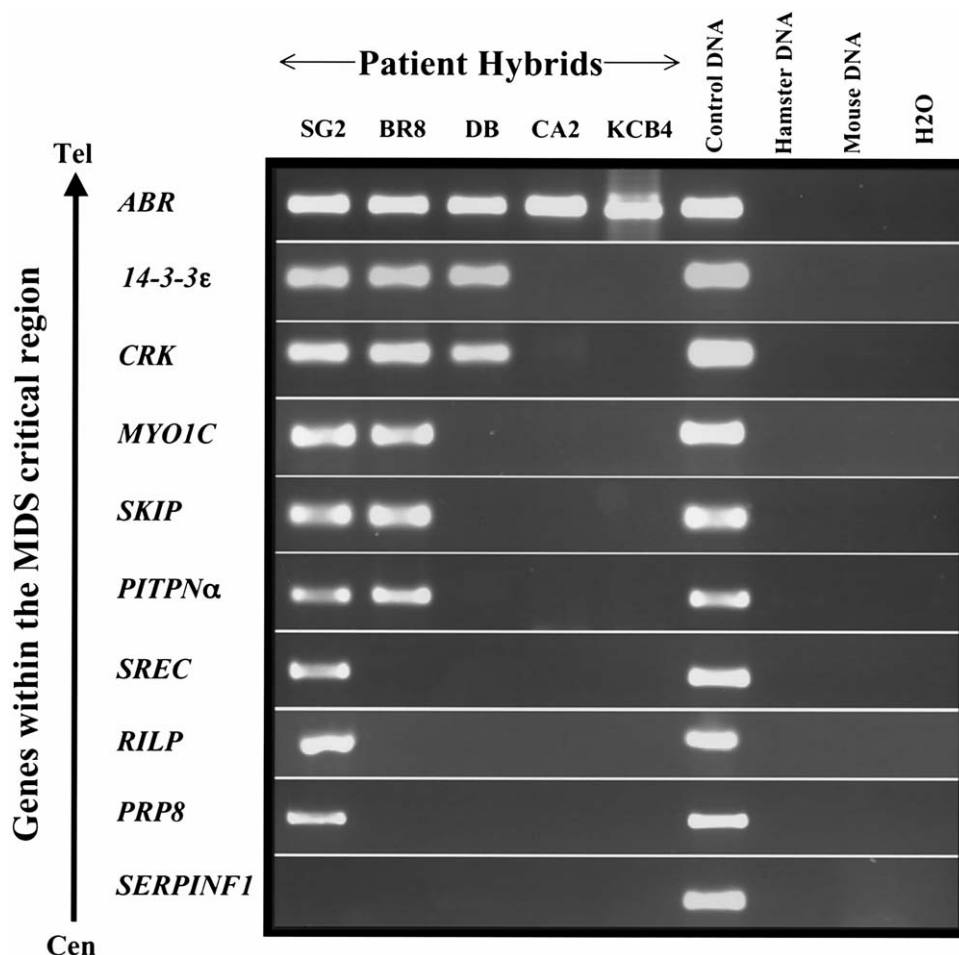
**Figure 4** Three-color FISH analysis showing the variability of the deletions of the 17p13.3 region. Three probes—PAC95H6 (red), BAC818O24 (green), and BAC68F18 (magenta)—from the 17p subtelomeric region were used in these FISH experiments to size the deletion of the 17p13.3 region in various patients (B, C, and D) compared with a normal control (A). B, FISH on patient (LP99-086) who has LIS (fig. 1C and 1D) showed the absence of PAC95H6 in red (containing the *LIS1* gene). C, In patient LR01-167, who has MDS (fig. 1G and 1H), there is absence of signal of all three probes on one chromosome 17. D, Patient DR01-001 does not have all typical MDS facial features or LIS, but does have an abnormal brain MRI (frontal lobe hypoplasia and partial agenesis of the corpus callosum) (fig. 1K and 1L). Her FISH study shows the presence of PAC95H6 on both chromosome 17s and absence of the probes BAC818O24 and BAC68F18 from one chromosome 17.

cytosolic phospholipid transfer proteins that are distinguished by their ability to regulate the interface between signal transduction, membrane-trafficking, and lipid metabolic pathways in eukaryotic cells (Liscovitch and Cantley 1995). SKIP (Ski-interacting protein) is a partner of the oculopharyngeal muscular dystrophy gene *PABP2* and stimulates directly or indirectly muscle-specific gene expression (Kim et al. 2001). *MYO1C* is a motor protein and participates in the slow component of adaptation by hair cells, which are the sensory cells of the inner ear (Holt et al. 2002). The *CRK* gene exists in two splice variants, *CRK I* (28 kDa) and *CRK II* (42 kDa), of which *CRK II* encodes an additional N-terminal SH3 domain (Reichman et al. 1992). The product of *CRK* is an adaptor signaling protein that is mostly composed of SH2 and SH3 domains, and has been shown to play a pivotal role in cell proliferation, differentiation, and migration (Feller et al. 1998). The 14-3-3 $\epsilon$  protein is a member of a large family of signaling molecules defined as discrete phosphoserine/threonine binding modules. The 14-3-3 family of proteins play critical roles in cell signaling events that control progress through the cell cycle, transcriptional alterations in response to environmental cues, and programmed cell death (Yaffe et al. 2002).

On the basis of this functional data, many of these genes may be considered good candidates to be involved in the more severe phenotype seen in patients with MDS. Our PCR mapping results of somatic cell hybrids from patients with MDS who have interstitial deletions has allowed us to further reduce the number of candidate genes likely to be involved in the brain phenotype MDS.

When we compared the severity of LIS in the patients in this study, only those with MDS had complete agyria (grade 1). We know from previous studies that deletion of *LIS1* alone is not sufficient to cause this severe form of LIS (Cardoso et al. 2000). The patients in our study with complete agyria uniformly had the largest deletions (fig. 3). Gene-by-gene mapping of these deletions in selected patients by PCR showed that two genes (*CRK* and 14-3-3 $\epsilon$ ) were exclusively deleted only in those patients with the most severe form of LIS (fig. 5). Thus, we conclude that deletion of one or both of these genes in addition to *LIS1* may contribute to the severe LIS phenotype seen only in MDS. To take this hypothesis further, it must then be assumed that one of these genes has a role in cortical development and specifically in neuronal migration.

The 14-3-3 $\epsilon$  gene product is probably the best candidate, on the basis of a mouse knock-out phenotype that supports a role for 14-3-3 $\epsilon$  in cortical development (K. Toyo-oka, A. Shionoya, M. J. Gambello, C. Cardoso, R. J. Leventer, H. L. Ward, R. Ayala, L. H. Tsai, W. B. Dobyns, D. H. Ledbetter, S. Hirotsune, and A. Boris-Wynshaw, unpublished data). As in human, heterozygous loss of both *Lis1* and 14-3-3 $\epsilon$  result in more severe cortical and hippocampal developmental defects (Hirotsune et al. 1998; Toyo-oka et al., unpublished data). Thus, the phenotypes in both human and mouse support the hypothesis that 14-3-3 $\epsilon$  is a gene that may contribute to the severe migration defects seen in patients with MDS. *CRK* should also be considered as a candidate to participate in the LIS phenotype seen in MDS. It has been shown that *CRK* may have a role in



**Figure 5** Extent of deletion in the telomeric MDS critical region. PCR analysis was performed on somatic cell hybrid cell lines derived from lymphoblasts of three patients with ILS—SG2 (LP90-017), BR8 (LP87-001), and DB (LP86-003)—and from two patients with MDS—CA2 (LP82-002) and KCB4 (LP84-001)—whose deletion breakpoints have been characterized by FISH analysis. Primers used specially amplified all 10 human genes (see the “Subjects and Methods” section) that extend from the centromere (*SERPINF1*) to telomere (*ABR*) that are present within the telomeric MDS critical region. DNAs from hamster, mouse, and human were used in these experiments to show the specificity of the primers.

cell proliferation and migration by regulating the connection between integrins and the cellular cytoskeleton in areas named “focal adhesion” (Tsuda et al. 2002). However, even if *CRK* is involved in cell growth and motility, there is no evidence as yet that haploinsufficiency of this gene can cause a brain anomaly, and the creation of a mouse knockout of the *CRK* gene is required. In view of the lack of functional information on the *CRK* gene, and evidence that *14-3-3ε* is involved in brain development, we propose that, of these two genes, *14-3-3ε* is the better candidate for the more severe LIS phenotype seen in patients with MDS compared with patients with ILS.

In conclusion, we propose that MDS is truly a contiguous gene-deletion syndrome, caused by haploinsufficiency of at least nine critical genes, including *LIS1*,

that contribute to the facial and brain phenotype seen in patients with MDS. Furthermore, we suggest that the deletion of *14-3-3ε* and/or *CRK* in addition to *LIS1* is the factor responsible for the most severe form of classical LIS seen only in patients with MDS.

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## Electronic-Database Information

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

CEPH-Généthon Database, <http://www.cephb.fr/>  
 GenBank, <http://www.ncbi.nlm.nih.gov/entrez/>, (for RPCI4-765005 [accession no. AL137038], RPCI5-59D14 [accession no. AC006435], RPCI5-1029F21 [accession no. AC015853], RPCI5-1037N22 [accession no. AL450226], RPCI5-1127L24 [accession no. AC008087], RPCI11-4F24 [accession no. AC007873], RPCI11-22G12 [accession no. AC016292], RPCI11-74E22 [accession no. AC005696], RPCI11-107N19 [accession no. AC006405], RPCI11-135N5 [accession no. AC015799], RPCI11-216P6 [accession no. AC015884], RPCI11-356I18 [accession no. AC036164], RPCI11-380H7 [accession no. AC021705], RPCI11-411G7 [accession no. AC027455], RPCI11-433M14 [accession no. AC068936], RPCI11-667K14 [accession no. AC090617], RPCI11-676J12 [accession no. AC087392], RPCI11-818O24 [accession no. AC032044], CTB-11O23 [accession no. AC002316], BAC407I21 [accession no. AF322450], CTB-145P4 [accession no. AC002093], CTD-2326F1 [accession no. AC109339], CTD-2348K1 [accession no. AC108004], CTD-2507J6 [accession no. AC107911], CTD-2545H1 [accession no. AC099684], CTD-2573J8 [accession no. AC108006], CTD-3086F13 [accession no. AC099721] and yRM2111 (17pTEL) [accession no. AF240580], RPCI23-78H4 [accession no. AL669897], RPCI23-190H20 [accession no. AL663094], RPCI11-305G1 [accession no. AC032038], RPCI11-713H12 [accession no. AC025518], *NUDEL* [accession no. NM\_030808], *profilin1* [accession no. NM\_005022], *KIAA0664* [accession no. XM\_03478], *LIS1* [accession no. XM\_034770], *MNT* [accession no. XM\_012601], *SRR* [accession no. NM\_021947], *KIAA0397* [accession no. AB007857], *C17orf31* [or *KIAA0732*] [accession no. XM\_029470], *KIAA1401* [accession no. AB037822], *HIC1* [accession no. NM\_006497], *DPH2L* [accession no. NM\_001383], *RPA1* [accession no. NM\_002945], *SERPINF2* [accession no. NM\_000934], *SERPINF1* [accession no. NM\_002615], *KIAA1936* [accession no. XM\_056082], *PRP8* [accession no. XM\_028335], *RILP* [accession no. NM\_031430], *SREC* [accession no. XM\_008489], *PITPN- $\alpha$*  [accession no. NM\_006224], *SKIP* [accession no. NM\_016532], *MYO1C* [accession no. XM\_028385], *CRK III* [accession no. NM\_005206 and NM\_016823], *14-3-3 $\epsilon$*  [accession no. NM\_006761], *ABR* [accession no. NM\_021962 and NM\_001092], *TIMM22* [accession no. XM\_033715], *NXN* [accession no. XM\_033714], *HC90* [accession no. AF177344], *CGI-150* [accession no. NM\_016080], *ELP* [accession no. AF229804], *HCS6* [accession no. AF177341], *FLJ22282* [accession no. NM\_024792], *FLJ32270* [accession no. AK056832], *FLJ10979* [accession no. NM\_018289], *FLJ31761* [accession no. AK056323], *RPH3AL* [accession no. NM\_006987], and *DOC2B* [accession no. NM\_003585])  
 HUGO chromosome 17, <http://gdbwww.gdb.org/hugo/chr17/>  
 Lissencephaly Network, <http://www.lissencephaly.org>

NCBI UniGene, <http://www.ncbi.nlm.nih.gov/UniGene/>  
 NCBI UniSTS, <http://www.ncbi.nlm.nih.gov/80/entrez/query.fcgi?CMD=DB=UniSts>  
 NIX application, <http://www.hgmp.mrc.ac.uk/Registered/Webapp/nix/>  
 Online Mendelian Inheritance in Man (OMIM), <http://www.ncbi.nlm.nih.gov/Omim/> (for MDS, PAFAH1B1/LIS1, and DCX)  
 Sanger Institute, [http://www.ensembl.org/Homo\\_sapiens/Weizmann\\_Institute](http://www.ensembl.org/Homo_sapiens/Weizmann_Institute), <http://bioinformatics.weizmann.ac.il/chr17/>  
 Whitehead Institute, [http://www-genome.wi.mit.edu/cgi-bin/contig/sts\\_by\\_chrom?chrom=Chr17](http://www-genome.wi.mit.edu/cgi-bin/contig/sts_by_chrom?chrom=Chr17)

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