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A Novel CNS Gene Required for Neuronal Migration and Involved in X-Linked Subcortical Laminar Heterotopia and Lissencephaly Syndrome

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Summary

X-SCLH/LIS syndrome is a neuronal migration disorder with disruption of the six-layered neocortex. It consists of subcortical laminar heterotopia (SCLH, band heterotopia, or double cortex) in females and lissencephaly (LIS) in males, leading to epilepsy and cognitive impairment. We report the characterization of a novel CNS gene encoding a 40 kDa predicted protein that we named Doublecortin and the identification of mutations in four unrelated X-SCLH/LIS cases. The predicted protein shares significant homology with the N-terminal segment of a protein containing a protein kinase domain at its C-terminal part. This novel gene is highly expressed during brain development, mainly in fetal neurons including precursors. The complete disorganization observed in lissencephaly and heterotopia thus seems to reflect a failure of early events associated with neuron dispersion.

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Introduction

Development of the six-layered neocortex depends on precisely orchestrated proliferative, migratory, and maturational events (Allendoerfer and Shatz, 1994; McConnell, 1995). During embryogenesis, neocortical cells arise from a proliferative neuroepithelium, the ventricular zone (VZ) adjacent to the lateral ventricle; then, waves of cells migrate long distances through changing environments to reach their final destination and settle in an inside-to-outside order within the developing cortex (reviewed in Rakic, 1988). Investigations of the pattern of cortical cell dispersion suggest that radial migration along radial glia (Rakic, 1972; Luskin et al., 1988) and tangential migration are both involved in cortical specification (Walsh and Cepko, 1992; Tan and Breen, 1993; O'Rourke et al., 1995). Intrinsic to the neuronal migratory process are decisions about the initiation of migration, the path to be taken, locomotion itself, and final position in the appropriate cortex layer. Although none of these processes are understood in detail, there is now cumulative evidence that several classes of molecules, including adhesion molecules, ion channels/receptors, and intracellular cytoskeletal proteins, may all be involved (reviewed in Hynes and Lander, 1992; Rakic and Caviness, 1995; Huttenlocher et al., 1995).

Defects in neuronal migration are believed to be implicated in a large heterogenous group of genetic disorders associated with cortical dysgenesis or gray matter heterotopia (Raymond et al., 1995). These cortical malformations, revealed mainly by the recent widespread clinical use of magnetic resonance imaging (MRI), are increasingly implicated as a major cause of intractable epilepsy and cognitive impairment (Kuzniecky et al., 1993; Harding, 1996). Among these cortical dysgenesis conditions two major distinct malformations of genetic origin have been described: lissencephaly (LIS) or agyria-pachygyria, and subcortical laminar heterotopia (SCLH) or band heterotopia, also referred to as "double cortex" (DC) syndrome. SCLH consists of bilateral plates or bands of gray matter located beneath the cortex and ventricle but well separated from both, hence the descriptive term double cortex. True cortex appears normal in lamination while neurons within the band are scattered with apical dentrites oriented either toward the cortex or inverted (Harding, 1996). Clinical manifestations are mainly epilepsy and mental retardation (Palmini et al., 1991). Skewed sex ratio toward females (51 out of 54 patients) among sporadic patients with SCLH (Dobyns et al, 1996) suggests the involvement of X-linked mutations. Lissencephaly denotes an absence of gyri (agyria) or a reduced number of broadened gyri (pachygyria) and an abnormally thick cortex. The main clinical features associated with lissencephaly are profound mental retardation, intractable epilepsy, feeding problems, and shortened lifespan (Aicardi, 1991). The most characteristic histological appearance is an absence of the clear neuronal lamination of normal six-layered cortex. Instead, it can be roughly demarcated into four-layered cortex overlying a thin periventricular

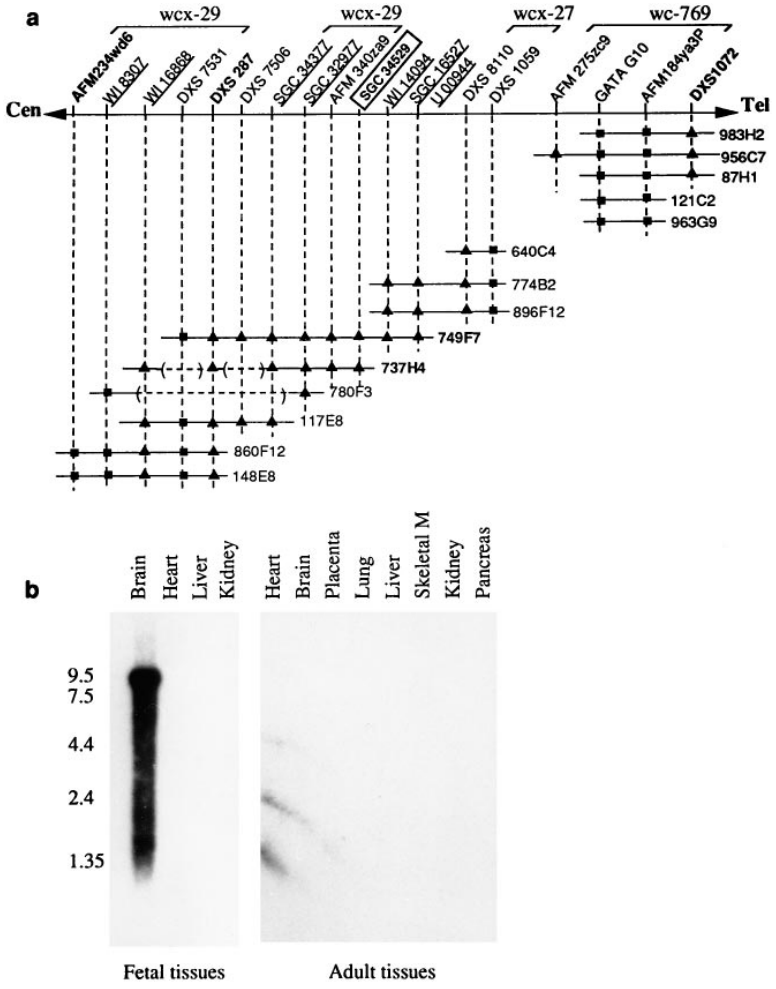


Figure 1. Physical Map of the *X-SCLH/LIS* Genomic Region within Bands Xq22.3-q23

(a) Schematic presentation of YAC contig (14 clones) within bands Xq22.3-q23 between markers DXS287 and DXS1072 (in bold). Upper line indicates YAC contigs reported by the Whitehead Institute/MIT Center database. The order of these contigs is represented according to our data. STSs and ESTs (underlined) used for YAC clones ordering and ESTs mapping were amplified by PCR on YAC DNA. Some were confirmed by hybridization of HindIII-digested YAC DNA blots (triangles). Markers order within contig wc-769 remains unknown. EST *SGC34529*, part of the *doublecortin* gene, is boxed.

(b) Fetal and adult multiple-tissue Northern blots hybridized with *SGC34529* probe. A strong and unique tissue-specific signal was detected in fetal brain after 12 hr exposition at −80°C.

rim of white matter in which are numerous gray heterotopias. The deep, abnormally thick neuronal layer, which may break up into bands or cells descending into the white matter, suggests an arrest of neuronal migration (Houdou et al., 1990; Harding, 1996; Ross et al., 1997). SCLH and lissencephaly can be observed as sporadic cases or inherited together in a single pedigree. Several families have been recognized in which affected hemizygous males have lissencephaly and heterozygous females have SCLH, suggesting the involvement of an X-linked gene (Pinard et al., 1994; Dobyns et al., 1996). Recent genetic mapping studies (des Portes et al., 1997; Ross et al., 1997) localized the gene responsible for X-SCLH/LIS syndrome in Xq22.3-q23. This region was further defined by physical mapping of an X;autosome translocation in a girl with lissencephaly (Ross et al., 1997).

Here we report the cloning of a novel CNS gene highly expressed in developing brain and mainly in neuronal cells that encodes for a 40 kDa predicted protein called Doublecortin. Mutations in the *doublecortin* gene were identified in four unrelated SCLH/LIS cases. Our data provide convincing evidence that the *doublecortin* gene is responsible for X-SCLH/LIS syndrome and suggest an involvement of this gene in early events associated with neuronal migration.

Results

Yeast Artificial Chromosome (YAC) Contig of the *X-SCLH/LIS* Critical Region and Physical Mapping of ESTs

According to our previous genetic study (des Portes et al., 1997), DXS1072 was identified as the distal recombinant marker of the *X-SCLH/LIS* genetic locus; also, Ross et al. (1997) mapped the breakpoint of the X;2 translocation associated with lissencephaly, distal to DXS287. Therefore, the critical region of the *X-SCLH/LIS* gene was identified, extending from DXS287 to DXS1072. To generate a YAC contig covering the region of interest, data available in the Whitehead Institute/MIT Center database were used as a basis, and YAC clones previously localized within bands Xq22.3-q23 from DSX1210 to DXS1072 were requested. Overlaps between clones were analyzed by PCR amplification of 14 STS and hybridization of HindIII-digested YAC DNA blots, using STSs as probes. Thus, a reliable contig with only one gap between contigs wcx-27 and wc-769 was constructed (Figure 1a). Fifteen ESTs roughly localized on radiation hybrid panels within the Xq22.2-q24 region by the Human Gene Map consortium (Schuler et al., 1996) were fine mapped by PCR amplification and hybridization on the YAC contig. Only eight ESTs were localized on the

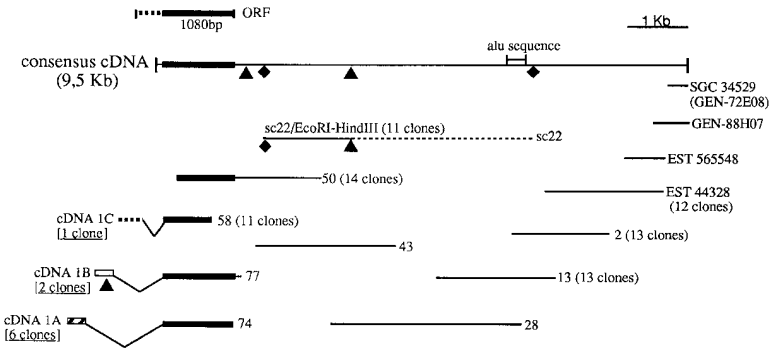


Figure 2. *doublecortin* cDNA Contig and Schematic Presentation of the 9.5 kb Consensus Transcript

A reliable consensus map was obtained by investigating 10 ESTs and 52 cDNA phage clones. This figure shows a summary of this investigation, and only four ESTs and the minimal set of eight overlapping cDNA clones (out of 52 positive clones) are shown. Five cDNA clones, numbered 2, 13, 50, 58 and EST 44328 and the EcoRI–HindIII fragment isolated from *sc22* genomic subclone were used for successive screenings of fetal brain cDNA library. The number of purified and analyzed positive clones isolated at each step is indicated in brackets. In the 5′ region, three types of clones (cDNA 1A, 1B, and 1C) were detected after screening with cDNA 58; for each type, the number of identical clones is indicated in square brackets and underlined. Open reading frames (ORF) are shown: bold line corresponds to the common ORF, bold dotted line corresponds to the additional in-frame ORF present only in cDNA 1C. The EcoRI (diamonds) and HindIII (triangles) restriction sites and the Alu sequence are indicated on the consensus cDNA. *Sc22* clone (dotted line) is an EcoRI genomic subfragment isolated from cosmid 9 (see Results section and Figure 3b). This cosmid contains part of the coding region and the 3′ UTR of the *doublecortin* gene.

constructed YAC contig. Their expression was studied by hybridization of EST probes to fetal and adults multiple tissue Northern blots. One EST (*SGC34529*), showed a strong signal, corresponding to a 9.5 kb long transcript and present only in fetal brain (Figure 1b). The localization of this EST in the region of interest (distal to DXS287 and proximal to DXS1072) and its high level of expression in fetal brain led us to consider the gene corresponding to this EST as a candidate for X-SCLH/LIS condition.

Isolation and Characterization of the Full-Length (9.5 kb) Candidate Transcript

Taking into account overlapping EST sequences available in GenBank data bases (Figure 2), a preliminary cDNA contig (2.5 kb long) was set up. Then, two clones of this contig (ESTs *565548* and *44328*) were used to screen a human fetal brain cDNA library. Seven walks were required to clone the full-length transcript (Figure 2). At each screening, inserts of purified positive clones were amplified by PCR and their ends sequenced. The first three walks and sequences of the corresponding cDNA clones did not identify any potential open reading frame (ORF); in addition, the presence of an Alu sequence in several cDNA clones and the colinearity between the consensus sequence of the cDNA and genomic DNA sequence, assessed by hybridization and sequence identity, suggested a large 3′ UTR. This latter region is included within the HindIII fragment of about 9 kb and the overlapping EcoRI *sc22* genomic fragments (Figures 2 and 3b). These fragments were generated from a cosmid clone (ICRF coordinates: c104J0516Q8, also called cosmid 9 in Figure 3b) that was isolated from the flow-sorted human X-specific cosmid library by ESTs *565548* and *44328*. We then decided to use the genomic EcoRI/HindIII fragment of the *sc22* subclone to screen the cDNA library, which enabled us to reach the coding part of the cDNA (Figure 2). Localization in Xq22 critical region of the cDNA and genomic fragments described in this study was performed by hybridizations on Southern blots containing HindIII-digested DNA of YAC clones covering the critical region (data not shown). Also, to confirm this localization, we have used DNA of somatic hybrids containing either the whole human X chromosome or a translocated der12 chromosome that has retained most of the long arm of the X chromosome (Bienvenu et al., 1997). At each walk in the cDNA library and after confirmation of the overlapping between the clone used as a probe and the new clones, the insert of at least one new clone was used to probe the above described Southern blots and the fetal and adult multiple tissue Northern blots.

The sequence of both ends of the large number of positive clones obtained after each screening of the cDNA library (52 clones in total) allowed us to generate 85 kb of sequence and a reliable consensus sequence of about 9.5 kb representing the full-lengh cDNA, therefore bypassing any further subcloning of cDNA clones. The consensus sequence of the cDNA showed a single ORF of 1080 bp starting from a putative translation initiation codon (CAAAAT**ATG**G) in good agreement with the Kozak consensus sequence (Kozak, 1986). This ORF encodes a predicted protein of 360 amino acids. Sequence analysis of the cDNA clones corresponding to the 5′ end of the transcript showed three divergent types of sequences: cDNA 1A (8 clones), cDNA 1B (2 clones), and cDNA 1C (1 clone) (Figure 2). The sequence of cDNA 1C (represented by only one clone) showed an additional ORF encoding for 42 amino acids that are in frame with the downstream ATG (Figures 2 and 3a). This additional in-frame ORF starts also at an ATG flanked by a good consensus sequence (Figure 3a). In order to define the genomic structure of this gene, we constructed and investigated a cosmid/phage contig that covers the gene (Figure 3b). Determination of exon–intron boundaries was performed through sequence comparison between cDNA clones and genomic DNA, which led to the identification of nine exons (Figure 3b). The common ORF is encoded by exon 2 to exon 6 and the initial 54 nucleotides of the last exon. The identified splice junction sequences (data not shown) exhibit close adherence to the 5′ and 3′ consensus sequences (Senapathy et al., 1990).

The structure of this gene is unusual in that only 16% of its sequence is coding and the 3′ UTR, which is contained in only one exon, is 7.9 kb long. This latter striking

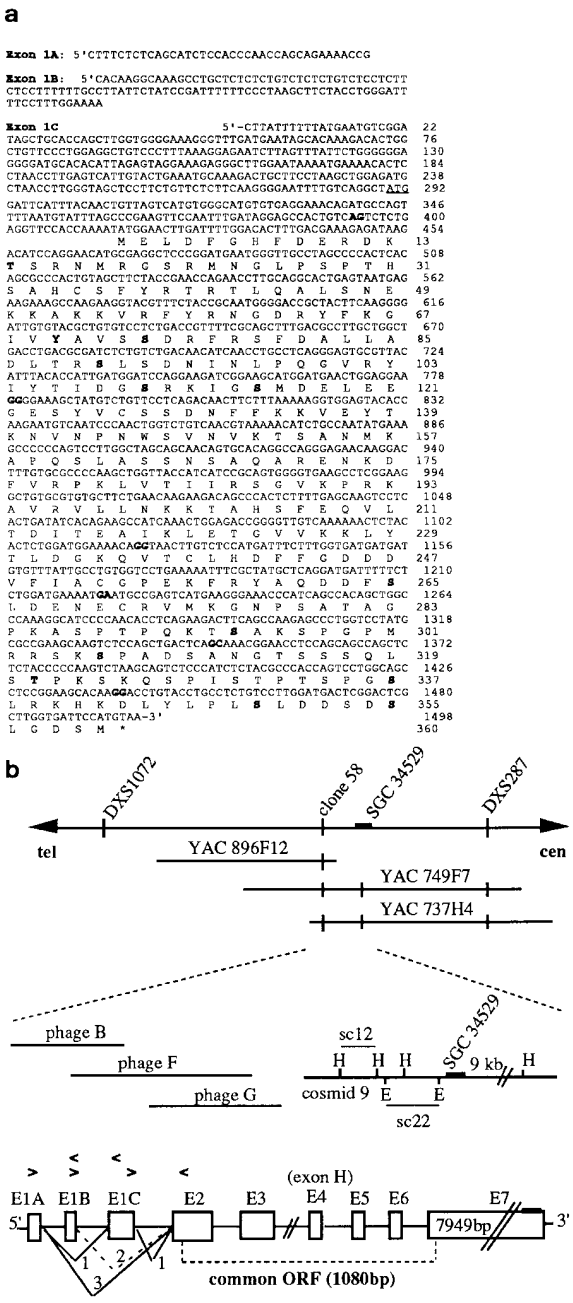


Figure 3. cDNA and Predicted Protein Sequences and Genomic Organization of *doublecortin*

(a) Coding *doublecortin* cDNA and Doublecortin amino acid sequences. Nucleotide sequences of exons 1A, 1B, 1C, and common ORF are shown. The predicted common ATG with the first in-frame stop codon (TAA) yields an ORF of 1080 bp that encodes a predicted protein of 360 amino acids, named Doublecortin. An additional codon (GTA) at exon 6–exon 7 boundaries (between amino acids 342–343), leading, therefore, to a predicted protein of 361 amino acids, was identified on some RT–PCR products. The upstream in-frame ORF, starting at the ATG within exon 1C (underlined ATG), encodes for 42 additional amino acids. Amino acids indicated in bold correspond to potential phosphorylation sites. Nucleotides in bold correspond to exon–exon boundaries.

(b) Mapping, orientation and genomic structure of the *doublecortin* gene. YAC clones 749F7, 737H4, and 896F12 map in the Xq22 critical region and are positive with the cDNA corresponding to the *doublecortin* gene represented in this figure by cDNA clone 58 and EST SGC 34529. In addition to 749F7 and 737H4, cDNA clone 58 corresponding to the 5′ end of the gene hybridizes with the YAC clone 896F12. As this YAC extends toward the telomere (see Figure 1), transcription of the gene is therefore orientated toward the centromere. Telomere and centromere were drawn differently from Figure 1 to allow a representation of the gene 5′, on the left, and 3′, on the right.

The minimal set of genomic phage and cosmid clones covering all exons is shown. Exons are indicated by open boxes. HindIII (H) and EcoRI (E) restriction map of the 3′ UTR is indicated on cosmid 9. Sc12 and sc22 are plasmid subclones isolated from the cosmid 9 clone.

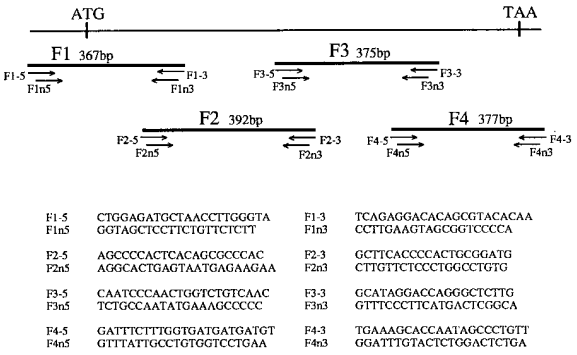
Position and orientation of primers used to study divergence at the 5′ ends of *doublecortin* transcripts are indicated by arrows. Primers are located in the three exons: 1A, 1B, 1C, and the first common exon. Forward (F) primers are oriented to the right and reverse (R) primers are oriented to the left. Sequences of the primers are as follows: 1AF, 5′-TTTCTCTCAGCATCTCCACCCAA-3′; 1BF, 5′-CAAAGCCTGCTCTCTCTGTC-3′; 1BR, 5′-CAAAGGAAAATCCCAGGTAGA-3′; 1CF, 5′-CTGGAGATGCTAACCTTGGGT-3′; 1CR, 5′-ATAGCCTGACAAAATTCCCCT-3′; ComR, 5′-CCTTGAAGTAGCGGTCCCCA-3′. Identified splicing events, corresponding to the three different transcript isoforms, are indicated with continuous and dotted lines.

feature was reported several times in the literature; for instance, the gene causing type 1 spinocerebellar ataxia has a 3′ UTR of about 7.2 kb (Banfi et al., 1994). The extensive 3′ UTR (data not shown) contains two AU-rich elements (AREs) defined by AUUUA motifs, which are present in the 3′ UTR of many labile mRNAs thought to be involved in the regulation of mRNA stability (McCarthy and Kollmus, 1995).

In order to clarify the divergence of the 5′-end sequences, we cloned the corresponding genomic region, characterized three exons (1A, 1B, and 1C) at the 5′ end of the candidate gene, and performed RT-PCR experiments using different combinations of primers (Figure 3b). The absence of a consensus splice site at the 5′ end of exon 1A suggested that this exon corresponds to the 5′ end of the 1A–transcript isoform. In line with this hypothesis is the presence in the genomic sequence upstream from exon 1A of a TATA box, 2 AP1 (Boyle et al., 1991), and 2 brn2/N-Oct3 (Li et al., 1993) consensus putative binding sites, reminescent of a promoter region (data not shown). It is worth noting that N-Oct3 is a highly expressed CNS-specific POU domain transcription factor (Schreiber et al., 1993). Results of RT-PCR experiments using human fetal brain RNA (at 21 weeks of gestational age) are represented in Figure 3b. In addition to the alternative splicing event concerning exon 1C (isoforms 1 and 3), it appears that exon 1B, which has a potential splice acceptor site, is spliced neither with 1C nor with 1A. Only RT-PCR products resulting from a splicing with the first common exon were obtained (Figure 3b, isoform 2), suggesting that transcripts containing exon 1B are expressed from a potential alternative promoter. These data suggest that alternative splicing events and potential alternative start sites of transcription are involved in the diversity of transcripts produced by this gene.

Identification of Mutations in Unrelated Patients with X-SCLH/LIS Syndrome

To prove that the isolated gene is reponsible for X-SCLH/LIS syndrome, five unrelated individuals were analyzed



F1-5	CTGGAGATGCTAACCTTGGGTA	F1-3	TCAGAGGACACAGCGTACACAA
F1n5	GGTAGCTCCTTCTGTTCTCTT	F1n3	CCTTGAAGTAGCGGTCCCCA
F2-5	AGCCCCACTCACAGCGCCCAC	F2-3	GCTTCACCCCACTGCGGATG
F2n5	AGGCACTGAGTAATGAGAAGAA	F2n3	CTTGTTCTCCCTGGCCTGTG
F3-5	CAATCCCAACTGGTCTGTCAAC	F3-3	GCATAGGACCAGGGCTCTTG
F3n5	TCTGCCAATATGAAAGCCCCC	F3n3	GTTTCCCTTCATGACTCGGCA
F4-5	GATTTCTTTGGTGATGATGATGT	F4-3	TGAAAGCACCAATAGCCCTGTT
F4n5	GTTTATTGCCTGTGGTCCTGAA	F4n3	GGATTTGTACTCTGGACTCTGA

Figure 4. Primer Sequences and Sizes of Expected Nested RT–PCR Products

External primers were used for the first round of RT–PCR and internal primers for the second round of PCR (see Experimental Procedures). Amplified overlapping fragments cover the coding sequence that starts at the common ATG. F1-5 and F1n5 are located in exon 1C.

for the presence of mutations: affected males of three previously mapped X-SCLH/LIS families (two caucasian and one black from Guadeloupe [des Portes et al., 1997]) and two caucasian female sporadic cases (patient OD with SCLH and patient MJ with pachygyria and corpus collosum agenesia). The strategy involved amplification by nested RT-PCR (Figure 4) and direct sequencing of the few copies, also called illegitimate (Chelly et al., 1989) or ectopic (Sarkar and Sommer, 1989), of the *X-SCLH/LIS* candidate transcript present in total lymphoblastoid cell line RNAs. The complete coding sequence was sequenced on both strands in the 5 patients.

The sizes of nested PCR products analyzed on 2% Nusieve gel were normal in all patients except patient MJ. In this patient, analysis of fragment 3 revealed an additional band shorter than the 375 bp expected length. Sequence analysis of the abnormal cDNA fragment showed a deletion of 103 bp corresponding to the complete exon 4 (Figure 3b), also called exon H in Figure 5d. The cause of this abnormal exon skipping was identified by the analysis of the genomic sequences flanking the skipped exon, which revealed a heterozygous point mutation, GT to AT, at the invariant dinucleotide GT of the 5′ donor site (Figure 5d). This exon skipping causes a frameshift and premature termination four residues downstream of the aberrant splicing (Figure 5d). This splice site mutation is a new mutation as it was not found in genomic DNA of the two healthy parents (data not shown).

The nucleotide sequence of the three familial cases revealed the presence of independent missense point mutations. However, no sequence abnormality was detected in the remaining sporadic case of SCLH (patient DO). Pedigrees, MRI images, and corresponding mutations are shown in Figures 5a, 5b, and 5c. Positions of the mutations are summarized in Table 1. Amino acid substitutions generated by these missense mutations change either the neutral–polar or acid–base nature of the amino acid residues involved. Cosegregation of the mutations with the disease was confirmed in all three families on genomic DNA using either restriction enzymes (families 1 and 2 showed in Figures 5a and 5b) or allele specific amplification (family 3 showed in Figure 5c). In the latter family, identification of the mutation allowed reassessment of the genotype of all members of the large pedigree, as reported in des Portes et al. (1997), and excluded the involvement of the mutation in the ambiguous brain MRI abnormalities observed in two females (cousins of affected cases). The presence of the four mutations (missense mutations and splice mutation) was systematically tested in a control population; none of the mutations was detected among 100 control X chromosomes. Control individuals are mainly (90%) of caucasian origin. These data demonstrate that this candidate gene, which we named *doublecortin*, is responsible for X-linked subcortical laminar heterotopia (or double cortex) and lissencephaly syndrome.

Expression of *doublecortin* Gene

As shown in Figure 2, it appears that the highly expressed 9.5 kb *doublecortin* transcript is exclusive to fetal brain. As X-SCLH/LIS syndrome is believed to result from an arrest of neuronal migration, we used in situ hybridization to examine *doublecortin* expression in developing human cerebral cortex. Coronal sections of a human cerebral cortex at 21 weeks of gestational age were hybridized with *doublecortin* antisense (Figure 6b) and sense (Figure 6a) probes. Autoradiograms suggest a strong labeling of the ventricular zone (VZ) and cortical plate (CP) and a moderate labeling of the intermediate zone (IZ). At higher magnification, it appears that *doublecortin* is expressed in the majority of cells of the CP, IZ, and VZ. Rare negative cells were identified in the three zone. In the IZ, labeled cells are organized as oriented chains (Figure 6f) reminiscent of migrating neurons.

In order to confirm the expression in neuronal cells, we cloned the mouse homologous gene, derived appropriate primers, and investigated its expression by RT-PCR procedure (1) in mouse brain at embryonic day (E) 15, postnatal days (P) 1 and 60; and (2) in primary cultures of mouse neuronal and astro-glial cells derived from fetal brains at E15 and newborn mouse brains, respectively. Figure 6h shows the results after 25 cycles of RT-PCR amplification of the mouse *doublecortin* mRNA and *GDI-1* mRNA (*rab GDP-dissociation inhibitor*, Shisheva et al., 1994) known to be expressed in developing brain (Bächner et al., 1995) and used here as a control. In addition to the expected decrease of *doublecortin* expression after birth, Figure 6h shows that in primary cultures of neuronal cells a significant level of *doublecortin* expression is observed, whereas it is not detected in glial cells. This latter result was also obtained after 35 cycles of amplification.

Although we have not excluded a low level of in vivo expression in astro-glial cells and in other tissues during development, our results indicate that the *doublecortin* gene is mainly expressed during early brain development in neuronal cells including VZ precursors and migrating neurons. Further investigations should allow precise determination of whether *doublecortin* expression is restricted to cortical neurons or whether it also takes place in other structures of the developing brain.

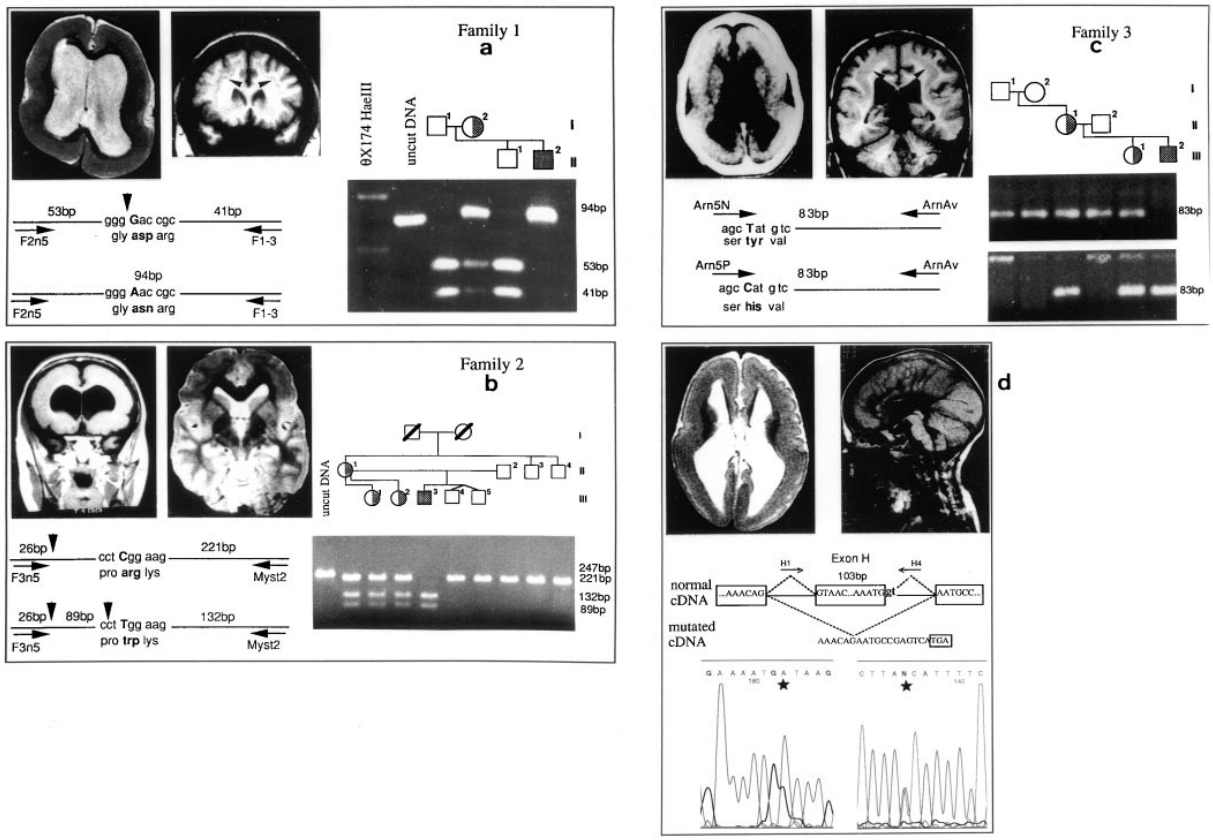


Figure 5. Identification of Mutations in *doublecortin* and Their Segregation in X-SCLH/LIS Families

(a) Family 1. MRI images of affected mother (I-2) and her son (II-2) show bifrontal SCLH (arrows, right coronal image) and generalized agyria (left axial image), respectively. The G-to-A mutation was detected in individual II-2 patient. As the mutation disrupts an AvaII site (lower sequence), genomic PCR products using primers F2n5 and F1-3 (previously tested on genomic DNA) were digested and analyzed on 2% Nuesieve gel for all members of family 1. DNA from the affected son remains uncut, while expected products (53 bp and 41 bp) were obtained for the healthy son and the father. The heterozygous female (I-2) line shows three bands, confirming the presence of both alleles.

(b) Family 2. Three affected children are born to the same affected mother but to three different fathers. Axial MRI image of one affected female (III-2) shows extended SCLH (right) and coronal MRI image of her brother (III-3) shows bifrontal agyria–pachygyria (left). As the C-to-T mutation creates an StyI restriction site, genomic PCR product (with primers F3n5 and Myst2) from the affected son is digested in three fragments (132, 89, and 26 bp undetectable on the gel); only two products (221 and 26 bp) were obtained for the healthy males. Both alleles are shown for the three heterozygous females. Myst2 sequence is: 5′-GTTTTCCATCCAGAGTGTAGAG-3′.

(c) Family 3. Coronal MRI image (right) of affected daughter (III-1) and axial CT scan (left) of affected son (III-2) show extended and thick SCLH and agyria, respectively. The T-to-C mutation was detected in individual III-2. Allele-specific genomic PCR was performed. Using normal forward primer (Arn5N, 5′-AACTGGAGGAAGGGGAAAGCT-3′; upper gel), an expected PCR product (83 bp) was obtained for all individuals except the affected boy, while using a 3′-mutated one (Arn5P, AACTGGAGGAAGGGGAAAGCC-3′; lower gel), a specific PCR product was exclusively amplified with DNA of the heterozygous females and the affected male. ArnAv sequence: 5′-GTTGGGATTGACATTCTTGGTG-3′.

(d) Sporadic case (JM) with abnormal skipping of exon 4 (initially named exon H). Axial (left) and sagittal (right) MRI images show extended agyria-pachygyria and complete corpus callosum agenesia, respectively.

A shorter nested PCR product of fragment 3 was amplified in this patient (data not shown). The cDNA sequence exhibited a skipping of exon H (103 bp) that induces a frameshift and a premature stop codon five residues downstream of the abnormal splice.

Lower, electrophoregrams of the genomic DNA sequence of intron–exon H junctions using forward primer H1 (left) and reverse primer H4 (right) showed a heterozygous G-to-A mutation indicated by an asterisk at the donor splice site. (H1, 5′-ATGGATAGACAATGGTACTCAG-3′; H4, 5′-ACAGGAGAAAGACCAACATTAT-3′).

doublecortin Gene Encodes a Novel Polypeptide

The ORF starting at the ATG common to all types of transcript encodes a predicted protein of 360 amino acids (Figure 3a). However, if we take into account the alternatively spliced in-frame exon 1C, the *doublecortin* gene product would be composed of 402 amino acids. Hydropathicity analysis (Kyte and Doolittle, 1982) of the deduced amino acid sequence did reveal the presence of neither signal peptide nor hydrophobic segment reminescent of transmembrane domains and suggested that Doublecortin is hydrophilic and probably intracellular. Based on consensus protein kinase phosphorylation site motifs (Kemp and Pearson, 1990; Songyang et al., 1995), several potential phosphorylation sites for protein kinase C and casein kinase II and one potential site for Abl at tyrosine residue 70 were identified in the deduced protein (see Figure 3a). Comparison with nucleotide and protein sequences in databases using BLAST and FASTA indicated that Doublecortin has no significant homology to any protein of known function, except with

Table 1. Summary of Mutations in XLIS/SCLH Patients

Patient	Type of Mutation	Nucleotide Position	Effect of Mutation
Family 1	G to A	599	Asp to Asn (62)
Family 2	C to T	989	Arg to Trp (192)
Family 3	T to C	788	Tyr to His (125)
JM case (Sporadic)	G to A donor splice site	exon–intron junction +1 from 1223	aberrant splicing, frameshift, and stop codon 1236

a gene product of 729 amino acids (GeneBank accession number AB002367, gene called *KIAA0369*) reported in a recent large-scale study of brain cDNA clones (Nagase et al., 1997). This similarity of about 75% starts at the N-terminal end of both proteins and extends over 340 amino acids (Figure 7). It is noteworthy that BLAST searches concerning the remaining C-terminal part of the 729–amino acid protein showed a significant homology with calcium calmodulin–dependent (CaM) kinases type II. The highest score, 97% identity, was observed with the *rattus norvegicus* CaM-kinase cpg16 (GenBank accession number U78857). These data suggest that the polypetide of 729 amino acids has two major segments: an N-terminal domain of about 340 amino acids homologous to Doublecortin and a C-terminal part of 389 amino acids bearing an extensive homology with protein kinases. Doublecortin also showed homology over a short segment of 30 amino acids (position 312 to 342) with the N-terminal domain (position 8 to 38) of the *rattus norvegicus* CaM-kinase cpg16 (Figure 7).

The expression of the *KIAA0369* gene was analyzed by Northern blot hybridization, which showed the presence of a major transcript of about 7.5 kb expressed at a high level in fetal brain and at a lower level in adult brain (data not shown).

Discussion

Several lines of evidence strongly suggest that defects in the *doublecortin* gene herein reported are responsible for the X-linked lissencephaly and subcortical laminar heterotopia, or double cortex, syndrome. First, *doublecortin* maps to the potential genetic locus in Xq22 identified by linkage analyses (des Portes al., 1997; Ross et al., 1997). The mapping of the cDNA in Xq22 was ascertained by several hybridizations using cDNA clones as probes on genomic Southern blots containing YAC DNA covering the critical region and DNA from two somatic hybrids containing either the whole human X chromosome or a translocated derivative chromosome that has retained most of the long arm of the X chromosome. Second, the *doublecortin* gene is expressed in early embryonic brain neurons. Third, missense mutations in Doublecortin leading to drastic amino acid changes, and cosegregating with the phenotype, were identified in three unrelated families. In each family, the same mutation was identified in hemizygous males affected with lissencephaly and heterozygous females affected with SCLH, confirming the common genetic origin of these two apparently different phenotypes. Fourth, an additional screening for mutations, by denaturing gradient gel electrophoresis (DGGE) and direct sequencing, in sporadic cases of SCLH enabled us to identify eight new mutations, including two nonsense mutations. Our study provides convincing evidence that *doublecortin* is the causative gene for X-SCLH/LIS syndrome. These data are supported by Walsh's group (Gleeson et al., 1998 [this issue of *Cell*]), who identified in the *doublecortin* gene four inherited and three sporadic mutations.

The difference in phenotypes between males and females can be explained as follows: in hemizygous males with mutations in *doublecortin*, absence of functional protein in all cells of the developing brain will lead to a generalized abnormal organization of the neocortex resulting in lissencephaly or pachygyria. In contrast, in females with SCLH, functional Doublecortin is absent only in cell populations that inactivate the X chromosome bearing the normal allele, leading, therefore, to a less severe neocortical dysgenesis. Despite normal cortical histogenesis and cellular connections (Harding, 1996), it appears that heterotopic cells are not rescued by neighboring cells. This is in line with our data suggesting a neuronal intracellular localization of Doublecortin.

The hypothesis that SCLH and LIS phenotypes result from a loss of function of the *doublecortin* gene is supported by the identification of nonsense and frameshift mutations (Gleeson et al., 1998) and a de novo mutation at one of the invariant dinucleotides GT of the 5′ donor site resulting in an exon-skipping event with a frameshift (MJ case). This latter mutation was detected in an atypical sporadic case as it concerns a female affected with extended SCLH and pachygyria and corpus callosum agenesia. In view of this association, it will be of interest to determine whether mutations in the *doublecortin* gene contribute to other agenesia or cortical dysgeneses such as focal cortical dysplasia. Investigation of *doublecortin* transcripts carried out in one additional sporadic female case affected with SCLH did not reveal any abnormality. One can expect a poor efficiency of RT–PCR-based approaches in heterozygous mutations leading to a significant reduction of the abnormal transcript. In this latter situation, the PCR will favor the amplification of the normal transcript. Alternatively, other loci may be involved and the *KIAA0369* gene could be considered as a good candidate.

Expression of the *doublecortin* gene during brain development, assessed by Northern blot, in situ hybridization, and RT-PCR, suggests that the transcript is present at a very high level in fetal brain and especially in neurons and is then gradually down-regulated and reaches an undetectable level (by Northern blot) in adult brain. This high and diffuse expression of the *doublecortin* gene in fetal neurons, including precursors, supports the requirement of Doublecortin in the remarkable organization of normal six-layered cortex.

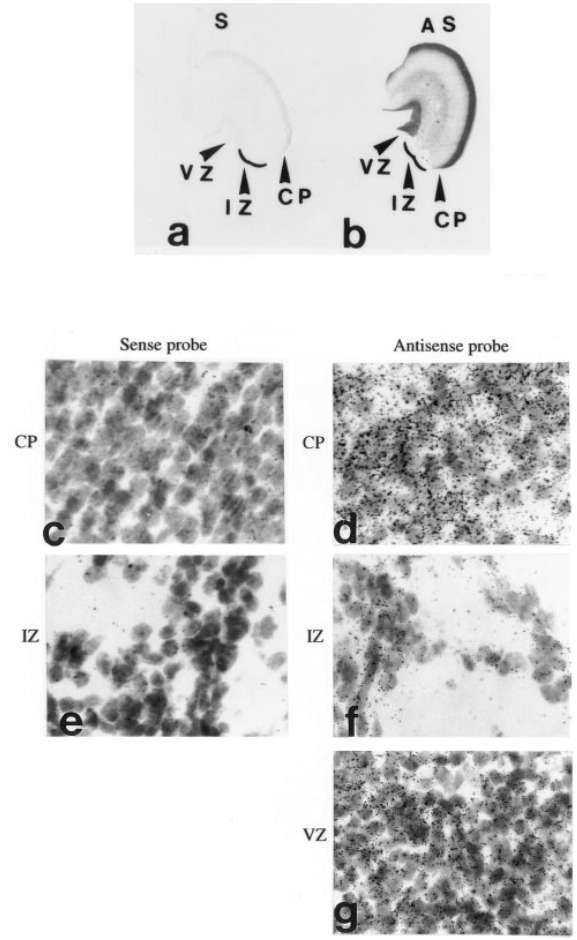


Figure 6. *Doublecortin* Expression

(a-g) In situ hybridization analysis of *doublecortin* expression. ^{32}P-labeled sense (S) and antisense (AS) probes were hybridized to coronal sections of human fetal brain (parieto-frontal cortex).

(a-b) Autoradiograms of hybridized sections. Strong signal was observed in the ventricular zone (VZ) and cortical plate (CP) and moderate signal in the intermediate zone (IZ) with the antisense probe; no significant signal was detected with the sense probe.

(c-g) Higher magnification (40×) of the same sections showing accumulation of silver grains within cells of the CP, IZ, and VZ. In the IZ, cells are organized as oriented chains. There was no specific labeling using the sense probe as control (c and e).

(h) Expression study by RT–PCR of the *doublecortin* gene in mouse brain and in neuronal and glial cultured cells. *mGDI-1* was used as control. The structure of the 5′ region of mouse gene is similar to the human one. Nucleotide sequences homology (calculated for 360 coding bases in the common exon) is 89%, and amino acid sequence identity (for the first 120 residues downstream of the common ATG) is 99% (data not shown). Mouse 1AF (5′-TTTCTCTCAGCA TCTCCACCCAA-3′) and mouse CoR (5′-CCTTGAAGTAACGGTCC CCA-3′) primers used to amplify the mouse *doublecortin* transcript are in the exons equivalent to exon 1A and the first common exon. The amplified fragment results from the alternatively spliced transcript lacking exon 1C. Primers used to amplify the mouse *GDI-1* transcript are: forward, 5′-GAGGCCTTGCGTTCTAATCTG; reverse, 5′-TGAGGATACAGATGATGCGA (Shisheva et al., 1994).

(E), embryonic; (PN), postnatal; (D), days of culture. Number of days for neuron culture is indicated on the figure.

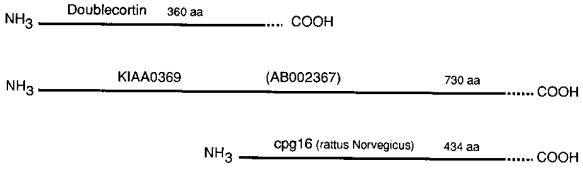


Figure 7. Position of Amino Acid Sequence Homology between Doublecortin and Two Protein Kinases

Doublecortin shares homology only with the N-terminal part of the *KIAA0369* gene product. The C-terminal part of the *KIAA0369* gene product and the *rattus norvegicus* cpg16 (GenBank U78857) share significant homology with many calcium calmodulin–dependent protein kinases. Dotted lines indicate divergent sequences.

Although the precise function of Doublecortin is unknown, its coexpression in fetal brain with the *KIAA0369* gene might suggest that Doublecortin could regulate the function of KIAA0369 protein either via a competitive interaction with upstream and downstream effectors or a modulation of KIAA0369 kinase activity. A similar pattern of kinase activity regulation was reported for c-Src protein kinase and its homologous protein v-Crk, which has Src homology sequences (SH 2 and 3) without intrinsic protein kinase activity (Reichman et al., 1992). Although v-Crk shows no protein kinase activity, coexpression of c-Src and v-Crk demonstrated an increase of c-Src kinase activity (Sabe et al., 1992).

The potential implication of Doublecortin, a new CNS protein, in the regulation of neuronal protein kinase activities is an attractive molecular basis for the pathological mechanism underlying lissencephaly and SCLH. One could imagine that Doublecortin participates, via its regulatory function, in a signaling pathway that is crucial for neuronal interactions before and during migration.

With the cloning of *doublecortin*, at least four genes implicated in cortical dysgenesis disorders can be proposed to function in the migration and assemby of neurons during cortical histogenesis. (1) The *reelin* gene, involved in the autosomal recessive mouse reeler phenotype, encodes for a protein that resembles extracellular matrix molecules. It acts at a late step during the arrest of locomotion of the migrating neuron along glial fibers and formation of neuronal layers (D'Arcangelo et al., 1995; Hirotsune et al 1995). (2) The mouse *disabled*

gene (*mdab*), involved in *scrambler* and *yotari* mouse mutations (Sheldon et al., 1997; Ware et al., 1997) that exhibit a phenotype identical to *reeler*, encodes for a phosphoprotein that appears to function as an intracellular adaptor in protein kinase pathways. This phosphoprotein seems to participate in a common signaling pathway with *reelin* that controls the final positioning of neurons (Howell et al., 1997; Sheldon et al., 1997). (3) The *LIS1* gene, involved in another form of lissencephaly phenotype, Miller-Dieker syndrome (MDS), and isolated lissencephaly sequence (ILS) (Reiner et al., 1993; Lo Nigro et al., 1997), encodes for the 45 kDa noncatalytic subunit of the brain isoform of platelet-activating factor (PAF) acetylhydrolase (Hattori et al., 1994). It acts at an early stage probably via the infuence of PAF levels on Ca^{2+} fluxes (Kornecki and Ehrlich, 1988), which could affect neuron ability to initiate or continue migration (Komuro and Rakic, 1992, 1993). (4) The *doublecortin* gene encoding for a novel 40 kDa predicted protein and expressed in fetal neuronal cells, including precursors, seems to be required for initial steps of neuronal dispersion and cortex lamination.

Experimental Procedures

Family Material

Clinical data and diagnosis concerning the three X-SCLH/LIS families analyzed in this study were described previously (des Portes et al., 1997). The sporadic case JM is a five-year-old female born to nonconsanguinous healthy parents (both have normal MRI). She has had seizures since 9 months of age and severe developmental delay with severe cognitive impairment. MRI showed a diffuse, thick cortex with agyria and pachygyria associated with an extended atypical aspect of SCLH and an unexpectedly complete corpus callosum agenesia (Figure 5d). The second sporadic case (DO) is a 15-year-old female born to healthy parents. She has a severe mental deficiency and an intractable epilepsy; MRI shows thick subcortical laminar heterotopia (not shown).

YAC Clones, STS, and EST Analysis

YAC clones of the *X-SCLH/LIS* critical region were obtained from the UK HGMP Resource Centre. Preliminary YAC ordering data were available online (CEPH-Généthon and Whitehead Institute/MIT Center databases). Analyzed STSs and ESTs were selected according to the available physical and radiation hybrid maps on the World Wide Web site at http://www.ncbi.nlm.nih.gov/SCIENCE96/. Primer sequences corresponding to these STSs and ESTs were also available on the same World Wide Web site. YAC clones were grown in selective media, and DNA was prepared using standard protocols. YAC overlaps and EST mapping were confirmed by a combination of STS/EST amplification and hybridization approaches.

cDNA Isolation and Characterization

Approximately 1×10^6 recombinant clones of a λgt10 human fetal brain cDNA library (CLONTECH) were plated and screened following standard techniques (Sambrook et al., 1982). Library screening was performed using the IMAGE consortium cDNA clones *44328* (EST *H05397*), *565548* (EST *AA129714*), and further positive clones. Positive phages were plaque purified and their inserts were amplified by PCR using λgt10 primers flanking the cloning site. All inserts were digested with MboI and AluI to generate a consensus restriction enzyme map. Direct sequencing with λgt10 primers was also performed using purified inserts as templates.

Genomic DNA Analyses of Human and Mouse Cosmid and Phage Clones

Human cosmid clones were identified by screening the ICRF flow-sorted human X chromosome library (Lehrach et al., 1990) with ESTs *565548* and *44328* and obtained from the German resource center (RZPD). As no positive cosmid clones corresponding to the 5′ end of the gene were identified in the cosmid library, the YAC clone *737H4* was subcloned into EMBL3 phage. MboI partial digestion and EMBL3 BamHI-digested arms were used to construct the library. Screening of the library with the cDNA inserts and purification of phage DNA corresponding to positive clones were performed according to standard procedures (Sambrook et al., 1982). HindIII-digested phage DNA was subcloned into pBluescript SK (+) vector. Mouse phage clones were isolated by screening mouse genomic phage library (genomic DNA of 129 strain). Screening was performed at a low stringency using cDNA 1C. HindIII-digested DNA from positive clones were subcloned into pBluescript (+). Subclones corresponding to the 5′ end of the gene were sequenced with T3 and T7 primers and with human exonic primers.

Mutations Screening

Total RNA was extracted from EBV-transformed lymphoblastoid cell lines by the guanidium thiocyanate method using the RNA–BTM extraction kit (Bioprobe systems). First strand synthesis of cDNA using 2 μg of total RNA was carried out in a final volume of 40 μl according to a standard procedure. We performed 40 cycles of PCR (94°C, 30 s; 55°C, 30 s; 72°C, 1 min, in a PTC200 MJ Research machine) on 5 μl of the cDNA sample using one of four sets of primers (Figure 4) to obtain four overlapping fragments spanning the whole *doublecortin* coding sequence. Then a second round of PCR amplification with nested primers (Figure 4) was performed using 0.5 μl of the first PCR product. Both strands of nested PCR products were directy sequenced using the DyeDeoxy terminator cycle sequencing kit protocol (Applied Biosystems). Cosegregation of mutations with phenotypes was carried out on genomic DNA using appropriate restriction enzymes (see Figure 5).

Expression Studies

Fetal and adult mutiple-tissue Northern blots (Clontech) were hybridized with ESTs and cDNA clones and subsequently washed according to standard procedures. For RT–PCR experiments, total RNA samples were prepared from human fetal brain (21 weeks old) and embryonic (E15), newborn, and postnatal (P60) mouse brains. Cells were derived from brains of randomly bred Swiss mice. Glial cells were from newborn mouse cerebral hemispheres. Ninety-five percent of the cells were identified as type-1 astrocytes; neither neurons nor oligodendrocytes were detected in multiple screenings. Culture of neuronal cells was set up from single-cell suspension of fetal brains at 15 days of gestation. Cultures consisted predominantly of neurons (>95%) identified by surface labeling with tetanus toxin and intracellular labeling with antibodies to γ-enolase or neurofilament proteins. Amplification by RT–PCR was performed according to standard procedure. Products obtained after 25 and 35 cycles of PCR were analyzed by gel electrophoresis and ethidium–bromid staining. Primer sequences used for RT-PCR are described in the figure legends (Figure 6h).

For in situ hybridization, 8 mm thick coronal sections of fetal brain were fixed in 4% (w/v) paraformaldehyde, cryoprotected with 10% sucrose in phosphate buffer, frozen with isopenthan and stored at −80°C until sectioning. Briefly, hybridization of coronal brain sections (10–14 μm thickness) with sense and antisense α^{35}S-labeled RNA probes was carried out in a 50% formamide solution at 52°C. Sections were successively washed in 50% formamide, processed for digestion with RNase A and T1, and washed by successive passages in progessive stringent solutions. Final washing conditions are 0.1 SSC solution at 60°C. Sections were first exposed for three days in cassette with autoradiographic film, then slides were dipped in diluted Kodak NTB2 emulsion and exposed for 5 to 15 days. Emulsion autoradiographs were developed and sections were counterstained with toluidine blue, mounted in Eukitt, and examined under light microscope.

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EMBL Accession Number

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