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doublecortin, a Brain-Specific Gene Mutated in Human X-Linked Lissencephaly and Double Cortex Syndrome, Encodes a Putative Signaling Protein

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Summary

X-linked lissencephaly and "double cortex" are allelic human disorders mapping to Xq22.3-Xq23 associated with arrest of migrating cerebral cortical neurons. We identified a novel 10 kb brain-specific cDNA interrupted by a balanced translocation in an XLIS patient that encodes a novel 40 kDa predicted protein named Doublecortin. Four double cortex/X-linked lissencephaly families and three sporadic double cortex patients show independent *doublecortin* mutations, at least one of them a de novo mutation. Doublecortin contains a consensus Abl phosphorylation site and other sites of potential phosphorylation. Although Doublecortin does not contain a kinase domain, it is homologous to the amino terminus of a predicted kinase protein, indicating a likely role in signal transduction. Doublecortin, along with the newly characterized mDab1, may define an Abl-dependent pathway regulating neuronal migration.

Introduction

The cerebral cortex shows a precise layering of multiple neuronal types with distinct form and function, and this laminar organization is essential for the cognitive functions that define us as human. Remarkably, cortical neurons are not formed in the cortex itself but in specialized proliferative regions deep in the brain, so that postmitotic neurons must migrate as far as a 1000 cell body-lengths to reach the site of their final differentiation. The layers of cortical neurons are also formed in systematic fashion from deepest to most superficial ("inside-out"), so that each newly generated cohort of neurons must migrate past the previously formed neurons. Therefore, complex signaling mechanisms must exist to guide migrating neurons, yet virtually nothing is known about the molecular basis of this specialized targeted neuronal migration. The analysis of mutations that disrupt normal neuronal migration represents a unique avenue to identify molecules involved in this process.

Lissencephaly ("smooth brain") is one of the most severe malformations of the human cerebral cortex. It results in migrational arrest of virtually all cortical neurons short of their normal destination, and produces profound mental retardation and seizures (Dobyns and Truwit, 1995). One gene that causes lissencephaly when mutated was identified on chromosome 17p13 and is called *LIS1* (Dobyns et al., 1993; Reiner et al., 1993). Surprisingly, LIS1 was independently identified as a regulatory subunit of platelet activating factor (PAF) acetylhydrolase (Hattori et al., 1994). A potential role for PAF and PAF acetylhydrolase in neuronal migration remains undetermined.

Recently, substantial evidence has accumulated for the existence of a second lissencephaly gene on the X chromosome. Linkage analysis from two studies (des Portes et al., 1997; Ross et al., 1997) provides strong evidence that a single gene maps to Xq21–24, with a pooled multipoint LOD score of approximately 4.2 and informative recombination events that place the gene in an approximately 12 cM interval between markers DXS8020 and DXS1072. In affected pedigrees, males with X-linked lissencephaly (XLIS) display a phenotype essentially indistinguishable from lissencephaly caused by chromosome 17 mutations (Dobyns et al., 1996; Berg et al., 1997). Affected females heterozygous for the X-linked mutation show a milder phenotype, presumably representing a mosaic state caused by random inactivation of either the mutant or normal X chromosome. The brains of affected females (Figure 1) show a population of neurons that behaves normally and a population that migrates approximately halfway to the cortex and then arrests in the subcortical white matter, producing a band of neurons called subcortical band heterotopia or "double cortex" (DC) (Raymond et al., 1995; Harding, 1996). The majority of patients with DC are female, presumably representing mutations in the X-linked locus.

The mapping of double cortex/X-linked lissencephaly (DC/XLIS) to Xq21-q24 is intriguing because of a previously described female patient with lissencephaly associated with a de novo, apparently balanced X;2 (q22;p21) translocation within the candidate region (Dobyns et al., 1992). Such X;autosome translocations often result in females that exhibit the male phenotype, as the

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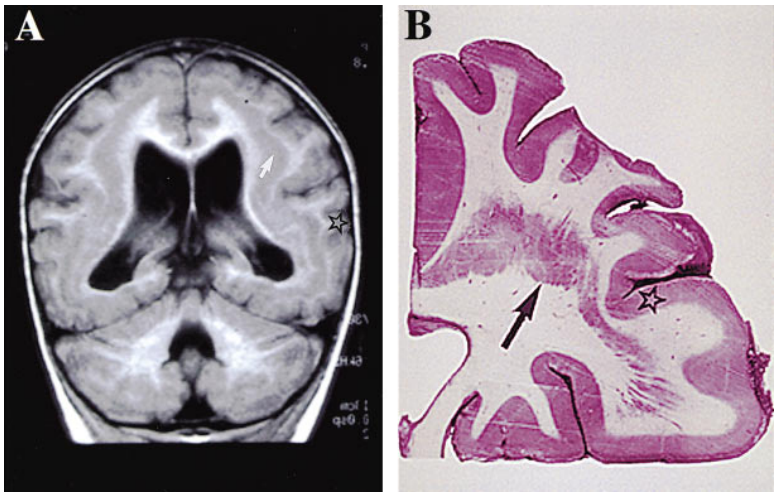

Figure 1. Double Cortex Phenotype

MRI from a female patient with double cortex syndrome is shown in (A). The image is taken in a coronal plane and shows the cortex and cerebellum. A postmortem specimen of a double cortex brain, in the same plane as the MRI in (A), is shown in (B). The outer cortex (star) is normal but with a band of gray matter (arrow) embedded within the white matter beneath the cortex, representing the "double" cortex (also referred to as band heterotopia, or subcortical laminar heterotopia).

normal X chromosome is preferentially inactivated to retain autosomal euploidy. Hence, the mapping data suggests that the translocation disrupts the function of the DC/XLIS gene. Here we report the positional cloning of a large brain-specific cDNA spanning this X;2 translocation breakpoint, which encodes a novel 40 kDa predicted protein called Doublecortin. Independent mutations in the *doublecortin* gene were identified in four pedigrees with DC/XLIS as well as in additional sporadic females with DC. Doublecortin contains a predicted Abl phosphorylation site and several other potential sites for phosphorylation by other kinase proteins, suggesting a tyrosine kinase signal transduction pathway may control neuronal migration.

Mapping of the X;2 (q22;p21) Breakpoint

Previous work (Ross et al., 1997) had identified marker DXS1105 as the closest published proximal (centromeric) marker and DXS1072 as the closest published distal (telomeric) marker to the translocation breakpoint. Further mapping of the breakpoint by PCR narrowed the candidate region to between DXS287 and DXS1072 based on the current X-chromosome physical map (Srivastava et al., 1995). In order to generate a physical map in the region of the translocation breakpoint, two YAC (yeast artificial chromosome) walks were initiated from DXS287 and AFM340za9 proximally, and from DXS1072 distally, with each YAC walk progressing centrally to attempt to fill the "hole" in between (K. M. A., J. G. G., and C. A. W., unpublished data). For each YAC isolated, the DNA at the termini of the YAC was amplified using linker-mediated PCR and sequenced. For each YAC end successfully sequenced, a new pair of primers was designed in order to amplify a sequence-tagged site (STS) within that YAC end. Each new STS was then used as a PCR reaction in (1) normal genomic DNA; (2) genomic DNA isolated from the somatic cell hybrid line JFA6 (Coriell Cell Repository #GM12515) derived from the X;2 (q22;p21) translocation patient and thus containing X chromosomal DNA only distal to the Xq22 translocation breakpoint (the derivative chromosome 2) (Ross et al., 1997); (3) genomic DNA isolated from a similarly prepared somatic cell hybrid BEN3B (Coriell Cell Repository #GM11172) derived from a different X;2 translocation containing X chromosomal DNA only distal to Xq21.3 (in order to verify that the STS maps to the telomeric q arm of the X chromosome); and (4) a further YAC library screen. YAC-end STSs, which mapped back to the X chromosome but were absent from JFA6, were interpreted as centromeric to the translocation breakpoint, and YAC ends present in JFA6 were interpreted as telomeric to the breakpoint.

Serial rounds of YAC identification and YAC-end isolation identified several YACs that showed one end present and one end absent in JFA6, indicating that these YACs spanned the translocation breakpoint. One such YAC (4X148D1), obtained from the ICRF library containing small average insert size, was subcloned to make a cosmid library. Cosmids were arrayed into a contig by restriction mapping, by PCR with previously identified YAC-end STSs, and by using new STSs corresponding to the end sequence of individual cosmids (Figure 2). In addition, two human BAC libraries were screened with AFM340za9, which identified two BACs.

Sequence of BAC and cosmid ends were obtained either by using linker-mediated PCR as for YACs, by creating internal deletions of the clone, or by sequencing the clone directly. STSs corresponding to cosmid and BAC ends were tested in JFA6 in order to identify cosmids (A10, C3, C12, D11) that contained one end present and one end absent in JFA6, suggesting that these smaller clones spanned the translocation breakpoint. Data for cosmid A10 is shown in Figure 3A. BACs and cosmids were organized into a single contig that crossed the breakpoint of the Xq22 translocation with greater than 3-fold coverage. Selected clones are depicted in Figure 2.

The PCR results using STSs designed to the ends of the cosmids suggested that several cosmids spanned the translocation but did not exclude the existence of a rearrangement or deletion at the site of the translocation in JFA6. Therefore, cosmid A10 was used as a probe for FISH in the parental X;2 translocation cell line containing the full complement of human chromosomes (Figure 3B). Hybridization signal from cosmid A10 was seen on the normal X chromosome and on the derivative

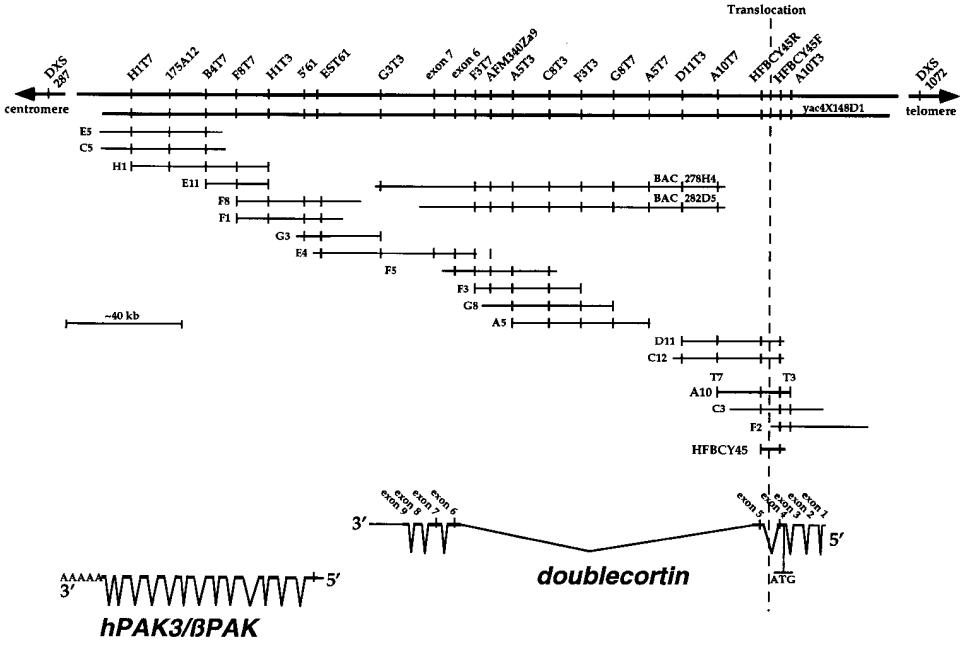

Figure 2. Physical Map of the X Chromosome in the Region of the X;2 Translocation Breakpoint Showing the Genomic Organization of the *doublecortin* and *hPAK3/*β*PAK* Genes

STSs are indicated on the top line, and the translocation breakpoint is indicated with a dashed line. Previously published markers include DXS287, DXS1072, and AFM340Za9. YAC 4X148D1 is represented by a line running the length of the figure. Cosmids are indicated by a well address; BAC's are indicated by BAC address (Genome Systems). The nine individual exons of *doublecortin* are indicated, with their location determined by mapping of STSs in the cosmid library. The fourteen exons of *hPAK3/*β*PAK* are also indicated.

X chromosome centromeric to the translocation breakpoint (it is not surprising that cosmid A10 did not show detectable hybridization signal distal to the translocation as sequencing data revealed that the majority of cosmid A10 lies proximal to the translocation breakpoint). PCR results demonstrating that part of cosmid A10 was present telomeric to the breakpoint and FISH results demonstrating that part of cosmid A10 was present centromeric to the breakpoint confirm that the translocation is not associated with a gross rearrangement or deletion and that cosmid A10 spans the translocation breakpoint in the X;2 lissencephaly patient.

Identification of a cDNA Interrupted by the X;2 Translocation

Cosmid A10 was subcloned into Bluescript and sequenced, forming a single DNA sequence contig >35 kbp. BLAST analysis of this sequence did not detect significant homology throughout any of its sequence to the coding region of known genes. Analysis of A10 with restriction enzymes that recognize CpG islands (AscI, MluI, NotI, NarI, SmaI) showed no more than one recognition sequence for each of these enzymes, suggesting that A10 contained very few genes. One fragment of DNA sequence from A10 was 98% identical to the full 436 bp of an EST (GenBank clone ID 417033) from the Soares human fetal liver spleen library. However, Northern analysis of this clone did not detect any transcript in fetal brain (data not shown) and this sequence was not analyzed further.

BLAST analysis of a second sequence from A10 detected >97% identity of three regions of sequence to a single 1814 bp human fetal brain EST called HFBCY45, suggesting that these three areas of homology represented putative exons of the HFBCY45 cDNA. Surprisingly, STSs designed to the three exons showed that two were present and one was absent from JFA6 (Figure 3C), suggesting that the HFBCY45 transcript spanned the translocation. Southern analysis with HFBCY45 in the parent cell line carrying the X;2 translocation showed two novel restriction bands with multiple restriction enzymes (Figure 3D), confirming that HFBCY45 indeed spanned the translocation breakpoint. Northern analysis showed HFBCY45 hybridizing to a single 10 kb message expressed exclusively in developing brain (Figure 4A).

Elimination of a *PAK* Gene As a Candidate for DC/XLIS

DNA sequence analysis of two short stretches (≈90 bp) from the end of another YAC 4X148D1-derived cosmid (H1) located approximately 160 kbp from the translocation breakpoint (Figure 2) showed a 92% match to the recently cloned rat β-*PAK* (Manser et al., 1995) and mouse *mPAK-3* genes (Bagrodia et al., 1995), suggesting that these two short stretches represented two exons of the human homolog of these rodent genes. We refer to the human homolog of these rodent genes as *hPAK3/*β*PAK*. The PAKs (p21-activated kinases) are a well-characterized family of signal transduction molecules that are involved in changes in cell morphology and actin organization (Sells et al., 1997), making *hPAK3/*β*PAK* an attractive candidate gene for a disease in which migration of neurons is defective. The full coding region for *hPAK3/*β*PAK* was identified within a 2.5 kbp cDNA

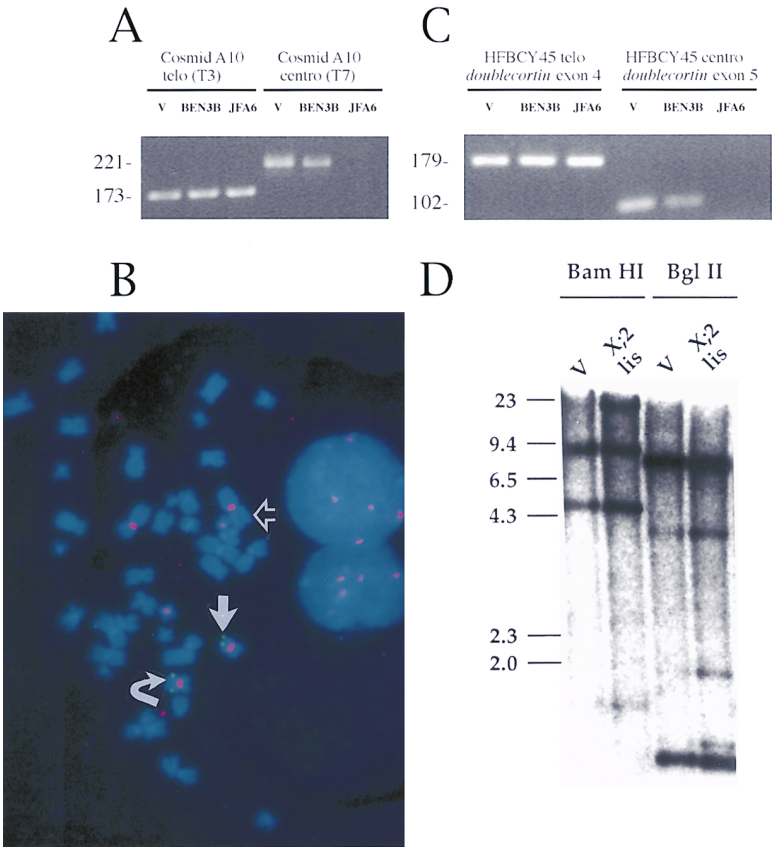

Figure 3. Evidence that Cosmid A10 and EST HFBCY45 Span the X;2 Translocation Breakpoint

(A) PCR amplification of STSs from both the T3 and T7 ends of cosmid A10. DNA is from (V), a normal volunteer; (BEN3B), the control somatic cell hybrid containing human DNA distal to Xq21.3; and [JFA6], the somatic cell hybrid containing DNA distal to the X;2 translocation associated with lissencephaly. Numbers on the left represent the bp size of product. Both STSs are present in (V) and (BEN3B), but (JFA6) shows a loss of the T7 marker.

(B) FISH using the parent cell line from the X;2 translocation probed with cosmid A10. Cosmid A10 hybridization signal (in green) is seen on the normal X chromosome (smaller curved arrow) and the derivative X chromosome containing the centromeric portion of X (larger straight arrow) at the site of the translocation. Signal is not detected on the derivative 2 chromosome containing the telomeric portion of X (open arrow), because the majority of the probe is located centromeric to the translocation. Centromere probes (in red) to chromosome X and 2 are also visible.

(C) PCR results of STSs derived from HFBCY45 (exons 4 and 5 of *doublecortin*). DNA is from (V), (BEN3B), and (JFA6). Numbers on the left represent bp size of products. The telomeric HFBCY45 marker (*doublecortin* exon 4) is present while the centromeric marker (*doublecortin* exon 5) is absent from JFA6.

(D) Southern blot analysis of DNA from a normal volunteer (V) and the parent cell line from the X;2 translocation (X;2 lis). Numbers on the left represent kbp size. The blot was probed with the full-length HFBCY45. There are two unique restriction bands produced with both restriction digests in the (X;2 lis) lanes. The 23 and 1.5 kbp BamH1 bands and the 1.9 and 1 kbp BglII bands in (X;2 lis) appear to be junction fragments, confirming that the translocation interrupts the HFBCY45 transcript (*doublecortin* exons 4 and 5).

contig using a combination of rapid amplification of cDNA ends (RACE) and cDNA library screening. Northern analysis of *hPAK3/*β*PAK* identified a 10 kb message present exclusively in the developing brain similar in size and expression to the band that hybridized to HFBCY45 (data not shown), suggesting initially that HFBCY45 might be part of the *hPAK3/*β*PAK* mRNA.

In order to evaluate *hPAK3/*β*PAK* as a candidate gene for DC/XLIS, we identified 13 of 14 exons from genomic DNA covering 95% (1564 of 1653 bp) of the ORF (open reading frame) (J. G. G., K. M. A., and C. A. W., unpublished data) and evaluated these 13 exons for mutations in 56 unrelated DC patients using single-stranded conformational polymorphism analysis (SSCP). No polymorphisms were found in any patients. Furthermore, Southern blot analysis of 23 unrelated DC patients probed with 500 bp of *hPAK3/*β*PAK* 5′ ORF did not reveal any polymorphisms, implying that *hPAK3/*β*PAK* is not causally related to DC/XLIS.

With the exclusion of *hPAK3/*β*PAK* as causative for DC/XLIS, we suspected that HFBCY45 and *hPAK3/*β*PAK* were, in fact, not part of a common mRNA. Initially, lambda screening, Northern analysis, and RT-PCR suggested that HFBCY45 and *hPAK3/*β*PAK* messages differ approximately 10-fold in abundance. Subsequent Northern analysis showed that HFBCY45 and *mPAK-3* probes recognize transcripts of distinct size and temporal expression pattern in mouse. The HFBCY45 transcript is 9.5 kb and maximally expressed at E11, while the *mPAK-3* message is 9 kb and expressed equivalently at E11, E15, and E17 (Figure 4B). These data strongly suggested that the two human cDNAs are encoded on distinct messages, implying that the *hPAK3/*β*PAK* mRNA is not interrupted by the X;2 translocation.

Identification of a Novel Gene, *doublecortin*, Interrupted by the X;2 Translocation

In order to further characterize the transcript interrupted by the X;2 translocation, HFBCY45 was used as an initial probe to screen a lambda fetal brain cDNA library with positive clones organized into a contig based upon their sequence. Sequences from the distal ends of the largest clones were used to generate probes for subsequent rounds of screening; each new probe was confirmed to be part of the HFBCY45 transcript by Northern analysis. After four rounds of screening and a single 3′ RACE reaction, a 9 kbp contig including a poly A tail was generated from over 350 positive clones, the entirety of which represents unique sequence. Subsequent BLAST search analysis identified several GenBank ESTs, including accession numbers AA129192, AA081738,

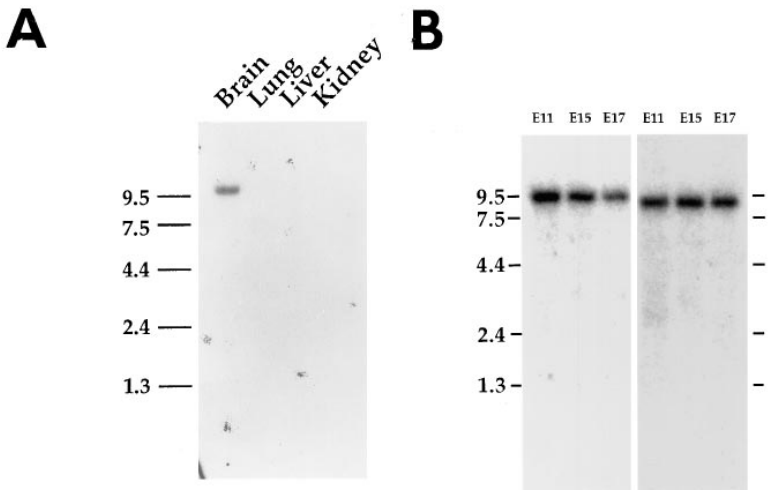

Figure 4. Northern Analysis of *doublecortin* and Demonstration of Distinct Size and Temporal Expression Patterns of *doublecortin* and *mPAK-3* in Mouse

(A) Northern analysis of *doublecortin* in multiple 20-week human fetal tissues. Doublecortin is encoded on a 10 kb brain-specific transcript.

(B) Northern analysis of *doublecortin* in fetal total mouse RNA. The *doublecortin* transcript is 9.5 kb in mouse and displays maximal expression at E11 (left panel), while the *hPAK3/βPAK* transcript is 9.0 kb in mouse and expressed equally at the three developmental time points (right panel).

T07153, and T31616, which share greater than 99% sequence identity to the 3′ end of the HFBCY45 contig.

Analysis of the new 9 kbp contig placed HFBCY45 near the 5′ end of the message and revealed that HFBCY45 partially defined a 1080 bp ORF, broken by the X;2 translocation (Figure 5A). This ORF encodes a novel 360 amino acid, 40 kDa predicted protein that we named Doublecortin.

The *doublecortin* transcript shows several interesting features. First, there are multiple alternative transcripts. Nearly half of the cDNA clones contained an additional 18 bases within the ORF, introducing 6 additional amino acids that do not alter the reading frame (displayed in Figure 5C). Also, there is alternative splicing at the exon 8/9 junction, with an additional single amino acid (valine) following amino acid K342 in half of the cDNA clones. In addition, three alternative splice forms of the 5′ UTR of *doublecortin* were identified and extensively characterized, none of which are likely to extend the ORF further (data not shown). The significance of these alternative splice forms is not known. Second, *doublecortin* is characterized by a short 5′ UTR (<500 bp), which we confirmed by RNAse H analysis (data not shown). *doublecortin* also has an unusually long 3′ UTR (>7000 bp) that shows no BLAST homology and contains repetitive sequences such as Alu elements and CA repeats. Finally, *doublecortin* is encoded over a large genomic segment. Mapping the *doublecortin* ORF back to genomic DNA showed that the first 1122 bp of message mapped to cosmid A10, while the remaining sequence mapped to cosmid E4, approximately 100 kbp away. Therefore, the *doublecortin* gene is a 1080 bp ORF encoded on a brain-specific, 10 kb message with six coding exons in a span of >100 kbp of genomic sequence with an unusually long 3′ UTR. The X;2 translocation occurs within an intron that separates the first two exons of the coding region. Sequence analysis of *doublecortin* intron–exon boundaries allowed screening of additional patients with DC/XLIS for mutations.

doublecortin Mutations in Patients with Double Cortex and X-Linked Lissencephaly

In order to confirm the involvement of *doublecortin* in DC/XLIS, we performed mutational analysis using DNA prepared from patients with DC/XLIS. Because of our concern about locus heterogeneity in lissencephaly and DC, we concentrated initially on families with a clear X-linked pattern of inheritance. Nine pedigrees were analyzed for mutations in *doublecortin* by SSCP; all six coding exons were analyzed in two pedigrees, while only the first two exons have been analyzed in the remaining seven pedigrees. Clear band shifts were identified in four pedigrees, three of which are presented (Figure 6). In each pedigree analyzed, the novel SSCP conformer cosegregated with the disease; males with lissencephaly displayed only the disease allele, while females with DC displayed both a normal and a disease allele, consistent with an X-linked dominant pattern of inheritance. Subsequent sequence analysis of the aberrant bands identified point mutations in all four pedigrees, each leading to a nonconservative amino acid substitution (Table 1). A limited screen for mutations in the first two exons of the *doublecortin* ORF in sporadic female DC patients identified band shifts in several additional patients. Subsequent sequence analysis from three patients identified frame shift mutations in each: a 2 bp insertion at position 36 from the ATG in patient 1, a 2 bp deletion at position 684–685 from the ATG in patient 2, and a 2 bp deletion at position 691–692 from the ATG in patient 3, each leading to premature protein termination. SSCP and sequence analysis from the unaffected parents of patient 3 demonstrated that this deletion represents a de novo mutation as neither parent harbors the mutant allele. Finally, a comprehensive SSCP screen of each of the six coding exons of *doublecortin* in 30 unaffected individuals yielded no polymorphisms. These data, as well as comparable data from des Portes et al. (1998 [this issue of *Cell*]), very strongly suggest that *doublecortin* is the causative gene for DC/XLIS.

doublecortin Encodes a Putative Signaling Molecule

BLAST analysis of Doublecortin showed 75% identity to a predicted protein named KIAA0369 (GenBank #AB002367) isolated from a human fetal brain library (Nagase et al., 1997). Although KIAA0369 is 73% identical at the nucleotide level to *doublecortin* over 825 bp,

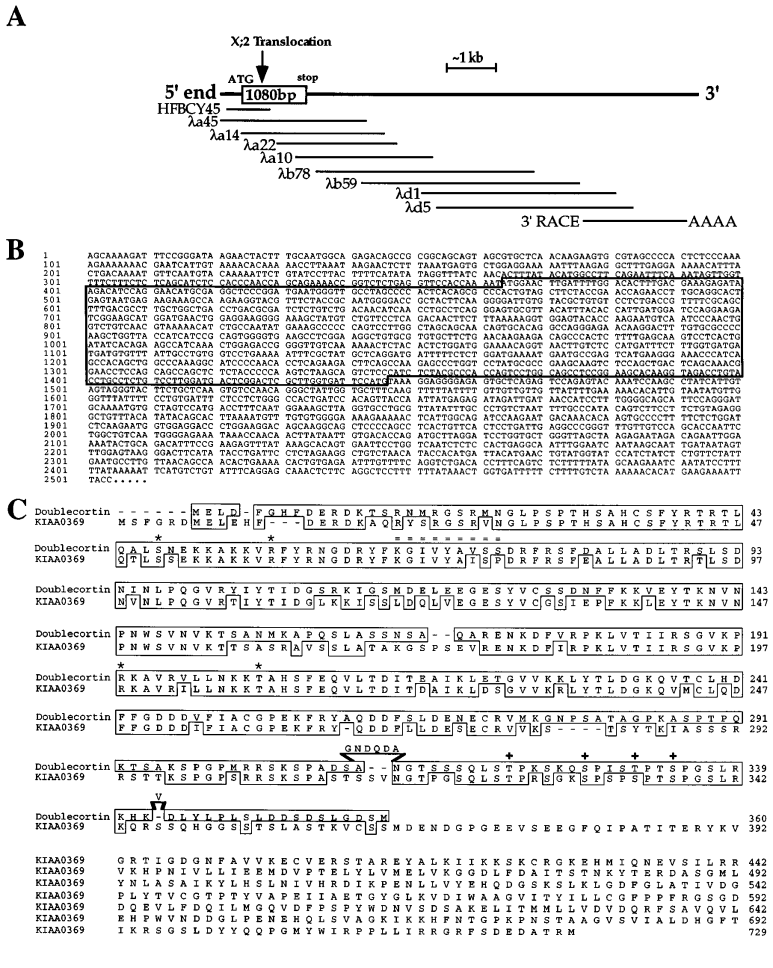

Figure 5. cDNA Contig and Sequence of *doublecortin*

(A) Several of the contiguous lambda clones and the EST HFBCY45. The contig is 9 kbp and includes the 1080 bp ORF and a 3′ RACE product defining the end of the message. The ATG, stop codon, and site of the X;2 translocation are represented.

(B) DNA sequence of the *doublecortin* ORF. The major 5′ splice variant is represented; other splice variants do not alter the start codon. Additional 5′ sequence and a portion of the cloned 3′ UTR sequence is listed.

(C) Comparison of Doublecortin with KIAA0369 and identification of Doublecortin motifs. The entire Doublecortin sequence is boxed; identical residues in KIAA0369 are also boxed. Nearly half of the *doublecortin* cDNA clones are alternatively spliced and encode a six amino acid insert (GNDQDA) or a single amino acid insert (V), which does not change the downstream ORF. Double dashed lines above the sequence indicate the potential Abl tyrosine phosphorylation site, and crosses indicate potential sites for phosphorylation by the MAP kinase family. Asterisks above the sequence indicate identified point mutations.

the ORF of KIAA0369 extends an additional 1104 bp in the 3′ direction (Figure 5C). The carboxyl terminus of KIAA0369, not present in Doublecortin, is strikingly similar at the amino acid level to the family of calcium calmodulin dependent kinases (CAM kinases) in the protein kinase ATP-binding domain and the serine-threonine kinase domain. The carboxyl terminus of KIAA0369 is 99% identical with CAM kinase CPG16 from rat (GenBank U78857). However, Doublecortin does not share homology with the CAM kinase family over a significant distance and does not encode a predicted kinase domain. Thus Doublecortin and KIAA0369 share substantial homology in their amino termini, but KIAA0369 likely functions as a kinase, possibly a CAM kinase.

Doublecortin has no apparent consensus signal peptide or transmembrane sequences, and a protein BLOCKS (Smith et al., 1996) search revealed no conclusive matches. Doublecortin was also used to search the Web site CANSEARCH 1.0, which tests putative proteins against the results of a combinatorial peptide library analysis for selectivity of phosphorylation by signal transduction kinases (Songyang et al., 1995; Nishikawa et al., 1997). Sequence analysis of Doublecortin revealed potential MAP kinase family phosphorylation sites at amino acids T321, S327, T331, and S334. Doublecortin also showed a high likelihood of being phosphorylated by Abl at tyrosine residue 70 (Figure 5C) with a specificity predicted to be within the top 1% of >100,000 peptides tested. It is therefore likely that Doublecortin functions as an intracellular signaling molecule, possibly as part of an Abl-dependent signal transduction pathway.

Discussion

In this report, we describe the characterization of a de novo balanced translocation in a patient with XLIS, the construction of YAC and cosmid contigs in the region of the translocation, the identification of a single EST that spans the translocation breakpoint, and the construction of a 9 kbp cDNA contig based upon this EST. Characterization of this message shows that it encodes a novel predicted protein named Doublecortin. *doublecortin* is transcribed on a 10 kb brain-specific message which is alternatively spliced. Mutational analysis in both DC/XLIS pedigrees and sporadic patients with DC has identified numerous deleterious mutations in *doublecortin* including nonconservative amino acid substitutions and frame shifts. No polymorphisms were detected in unaffected individuals. These data strongly suggest that *doublecortin* is the responsible gene for both DC and XLIS. Doublecortin is very similar to the putative brain protein KIAA0369 over nearly its entire sequence, although the carboxyl terminus of KIAA0369 extends further and appears to encode a potential CAM

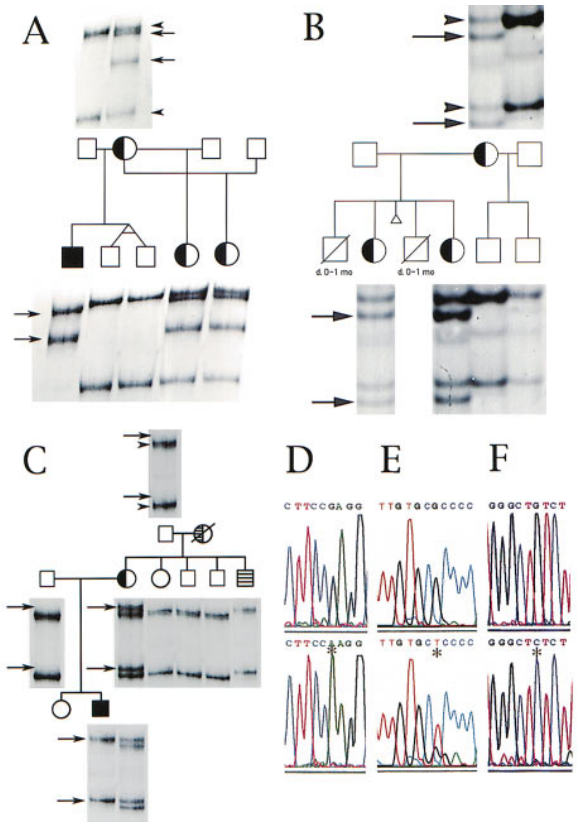

Figure 6. DC/XLIS Pedigrees Display Deleterious Point Mutations in *doublecortin*

(A–C) SSCP analysis of three DC/XLIS pedigrees with corresponding sequences of point mutations (D–F respectively). Unfilled characters are unaffected, half-filled circles are females with DC, filled squares are males with XLIS, the small triangle represents a miscarriage, and hashed figures are individuals with mental retardation and epilepsy who are likely affected but in whom a brain MRI scan has not been performed. (A) An affected female has kindred with three unaffected males, and with each partner there is one affected child with either DC or XLIS. (B) An affected female has kindred with two unaffected males, with two affected daughters, and three late term or neonatal deaths (death at 0–1 month), two of which are known to be male. (C) An affected female (mother) has an unaffected daughter and an affected son (with parallel heterozygous lane for comparison); mother has a male sibling who is likely affected and harbors the mutant allele. For each pedigree, unaffected individuals display the two normal SSCP bands, affected males display two novel bands, and affected females display four bands, both the normal and affected bands.

(D–F) Normal sequence (above) and sequence from the respective pedigree (below). Sequencing was performed with antisense oligonucleotides. (D) a G-to-A mutation (C-to-T) results in an R-to-W change at amino acid 192, (E) a G-to-T mutation (C-to-A) results in an R-to-L change at amino acid 59, (F) a G-to-C mutation (C-to-G) results in a T-to-R change at amino acid 203.

kinase. Doublecortin represents a likely substrate for Abl and a possible substrate for members of the MAPK family and may therefore serve to transduce signals critical for the migration of developing neurons.

doublecortin Mutations in DC/XLIS

Analysis of the DC/XLIS pedigrees and *doublecortin* mutations identified to date allows several interesting observations. First, it appears that the severity of the DC phenotype can be roughly correlated with the severity of the *doublecortin* mutation identified. The three sporadic patients who show frame shift mutations display very severe mental retardation, while the familial DC patients with single amino acid substitutions display mild or no mental retardation (J. G. G. and C. A. W., unpublished data).

It is also interesting to note that pedigree 2 appears to present a male lethal phenotype. This X-linked dominant pattern of inheritance with hemizygous male lethality has been observed in other human diseases including the neuronal migration disorder periventricular nodular heterotopia (PNH) (Eksioglu et al., 1996). The mutation associated with the phenotypic severity seen in pedigree 2 may define a more critical region of Doublecortin or may suggest that a significant contribution to the DC/XLIS phenotype is determined by the genetic background of the affected individual. This latter theory is supported by the observation that the X;2 translocation patient likely displays a full null allele yet has survived into early childhood.

Finally, a previous study (des Portes et al., 1997) suggested that DC/XLIS may show anticipation, with worsening phenotypes among females in subsequent generations. Mutational analysis in these families, however, does not support this claim. Anticipation is often seen in neurological diseases due to expanding trinucleotide repeats, but observations of anticipation are problematic in small pedigrees because of ascertainment and reproductive bias, since more severely affected individuals are less likely to have children. Moreover, variability in X chromosome inactivation patterns tend to enhance phenotypic variability for most X-linked mutations. One of the pedigrees previously used to support the claim of anticipation (Figure 6A) shows a simple amino acid substitution and no evidence of a worsening mutation in subsequent generations. While we cannot rule out that some pedigrees may show additional mechanisms of mutations other than simple alterations in the *doublecortin* coding region, altered patterns of X-inactivation may suffice to explain the majority of phenotypic heterogeneity.

doublecortin Defines a New Locus for Lissencephaly

The appearance of the cortex in the X-linked lissencephaly and the chromosome 17-linked lissencephaly are pathologically indistinguishable (Berg et al., 1997), suggesting that Doublecortin and LIS1 may lie on the same genetic pathway or even physically bind one another. Given the striking similarity of Doublecortin to the amino terminus of a kinase protein (KIAA0369), and given that both proteins are expressed in the developing nervous system, Doublecortin may regulate the activity of kinases such as KIAA0369 by competing with these kinases in binding to a regulatory protein. LIS1 is a candidate for this regulatory protein, since it is known to bind to the activated form of a different kinase, Syk, in the spleen (Brunati et al., 1996).

Tyrosine Kinase Signaling and Neuronal Migration

Recent data have strongly implicated signaling processes through nonreceptor tyrosine kinases as critical

Table 1. Summary of *doublecortin* Mutations in DC and XLIS Patients

	doublecortin Mutation	Pedigree Comments	Resultant Doublecortin Abnormality
Pedigree A	point mutation C to T at bp 574	affected mother, daughters, and son with mutation	R to W at aa 192
Pedigree B	point mutation C to A at bp 177	affected mother and daughters with mutation	R to L at aa 59
Pedigree C	point mutation C to G at bp 608	affected brother, sister, and son with mutation	T to R at aa 203
Pedigree D	point mutation A to C at bp 139	affected mother and son with mutation	S to R at aa 47
Patient 1	2 bp AG insertion at bp 36		protein termination at aa 24
Patient 2	2 bp CT deletion at bp 684		protein termination at aa 240
Patient 3	2 bp CT deletion at bp 691	unaffected parents without mutation	protein termination at aa 240

Mutations were identified in four pedigrees, and each represents a single amino acid substitution that alters charge. In each pedigree, affected males are hemizygous for mutations, while affected females are heterozygous. Mutations are also listed for three sporadic female patients with double cortex syndrome. Each of these represents an insertion or deletion of 2 bp that alters the translational reading frame, resulting in premature protein termination. The parents of patient 3, whose DNA was available for testing, do not display the mutation, indicating that the 2 bp deletion arose de novo in this patient.

to accurate neuronal migration to the cortex, and it appears that Doublecortin may function in this signaling pathway. The mouse *disabled* homolog 1 (*mdab1*) has very recently been identified as the gene mutated in the *scrambler* mouse (Ware et al., 1997), which is characterized by a severe abnormality of neuronal migration. mDab1 was identified by virtue of its physical binding to Src and also binds other nonreceptor tyrosine kinases such as Abl and Fyn (Howell et al., 1997). Although mutations in Src and Fyn appear to play a role in axon and neurite outgrowth (Beggs et al., 1994, 1997; Ignelzi et al., 1994), engineered mutations in *src* (Rusanescu et al., 1995) and *fyn* (Stein et al., 1992) have not been reported to affect the migration of cortical neurons.

Although engineered mutations in the *abl* gene have not been reported to cause neurons to migrate abnormally in the cortex (Schwartzberg et al., 1991; Tybulewicz et al., 1991), there are several lines of evidence that implicate Abl signaling in neuronal migration. Although mDab1 was identified based on its physical binding to Src (Howell et al., 1997), it is homologous to *Drosophila disabled*, which has been implicated in neuronal development through genetic interactions with the *Drosophila abl* (*Dabl*) gene (Gertler et al., 1993). Another gene identified based on its interaction with *Dabl*, *enabled*, has a mouse homolog, *mena*, that has recently been critically implicated in the migration of nonneuronal cells (Gertler et al., 1996). These genetic studies in *Drosophila* strongly suggest that physical interactions between mDab1 and other signaling proteins are necessary for normal process outgrowth and that Abl or close relatives of Abl may represent the likely route of mDab1 action. For this reason, the finding that Doublecortin encodes a putative Abl substrate with very high specificity suggests that the interaction of Doublecortin with other proteins may be regulated by Abl phosphorylation.

In the mouse, the *reeler* mutation has been the paradigm of abnormally targeted neuronal migration to the cortex. The *reeler* and *scrambler* mutations cause essentially the same phenotype (Gonzalez et al., 1997) and share gross similarity to lissencephaly and double cortex, since in all cases migrating cortical neurons seem to be unable to penetrate and migrate past a preexisting population of older neurons. The *reeler* gene encodes a large secreted polypeptide, called Reelin, with structural homology to extracellular matrix proteins such as Tenascin (D'Arcangelo et al., 1995). The identity of the *scrambler* gene as *mdab1*, encoding a protein evidently involved in signal transduction at the plasma membrane, is consistent with the view that the mDab1 protein is involved in Reelin receptor signaling, although mDab1 is unlikely to represent the receptor protein itself. Thus, the similarity of the mouse and human neuronal migration disorders may reveal a large number of interacting proteins (perhaps forming a large complex) that transduce a signal through Abl, mDab1, and Doublecortin from the plasma membrane to the cytoskeleton.

Given the possibility that mDab1 and Doublecortin may be part of a common signaling pathway, it will be interesting to determine whether engineered mutations in *doublecortin* cause a phenotype similar to *mdab1* mutations. Furthermore, there are likely to be lissencephaly loci in addition to Doublecortin and LIS1 (Dobyns et al., 1992), and the human homologs of *mdab1* and *reelin* may represent candidate loci for this type of developmental brain abnormality.

Experimental Procedures

Library Screening

YAC libraries (ICRF and ICI: HGMP, Cambridgeshire, UK, and CEPH YAC library, Research Genetics, Huntsville, AL) were obtained as pooled samples suitable for analysis using PCR. PCR was performed using standard techniques. A human BAC library was purchased from Research Genetics as PCR-ready pools and screened according to the manufacturer's instructions. A second BAC library

was screened commercially by Genome Systems using a hybridization-based strategy with AFM340za9. Cosmids were screened for the presence of STSs using PCR in microtiter arrays, with each well containing DNA from a single cosmid.

Ligation-Mediated PCR

In order to build a YAC and cosmid contig, the end sequence of individual clones was required. This was accomplished by end rescue using the vectorette method and "bubble PCR" by first digesting clones with blunt end restriction enzymes AluI, EcoRV, PvuII, and RsaI, and ligating the vectorette to the blunt ends (Ogilvie and James, 1996). Nested PCR yielded at least one strong band that was cut from the gel and sequenced in order to create an STS from the end of the YAC or cosmid.

Cosmid Cloning

ICRF YAC clone 4X148D1 was grown in AHC media, pelleted, and mixed with low-melt agarose. A partial 2 hr restriction digest with MboI was performed in agarose, the fragment size checked on a pulse field gel to select for fragments centered at 50 kbp. The SuperCos I vector (Stratagene, San Diego, CA) was prepared using manufacturer's instructions. YAC DNA was extracted with phenol-chloroform and ligated with vector DNA overnight using T4 DNA ligase. Ligation was checked on a 0.8% agarose gel for the presence of high molecular weight concatemers. The ligation reaction was packaged using the Gigapack II gold lambda kit and transfected into XL1-Blue MR host cells (Stratagene), titered, and the resultant clones lifted, fixed onto positively charged membranes, and hybridized with labeled total human DNA to identify clones with a human insert. Ninety-six positive clones were chosen and gridded into a microtiter plate.

FISH

Total cosmid DNA was labeled and used as a probe for FISH on the parental X;2 translocation cell line (Coriell Cell Repository #GM12514a). FISH was performed commercially (Genome Systems).

Shotgun DNA Sequencing

Several cosmids were chosen for shotgun sequencing. Cosmids were grown in LB with 100 mg/l ampicillin to late log phase. Cosmid DNA was isolated using columns (Qiagen, Chatsworth, CA) and sheared using an ultrasound sonicator. Fragments of 1–2 kbp were cut from a low-melt gel, extracted (GeneClean, Bio101, Vista, CA), and the ends polished using T4 DNA polymerase. Fragments were ligated into either Bluescript or PCR-script using manufacturer's recommendations (Stratagene) and checked for insert size by digesting with HindIII and EcoRV. Positive clones were prepared using either Qiaprep (Qiagen) or the Autogen P850 automatic mini-prep robot (Integrated Sequencing Systems, Natick, MA). Clones were sequenced using T3 and T7 primers.

cDNA Library Screening

The IMAGE library (Research Genetics) was screened to obtain the full ORF of *hPAK3/βPAK*. High density membranes containing the 270,000 IMAGE clones were screened with α-^{32}P-labeled probes and identified clones sequenced for identity. Full length clones of *doublecortin* were obtained by screening a human fetal brain library in lambda gt10 obtained from Clontech. The library was amplified once, arrayed onto filters, and screened radioactively using the manufacturer's directions. Positive clones were analyzed by PCR using vector and gene-specific primers to amplify and sequence the inserts.

Northern and Southern Blotting

Probes were labeled by incorporation of [α-^{32}P]dCTP by random priming. Commercial human multiple tissue or total mouse tissue Northern blots (Clontech) were blocked with 1× SSC/4% SDS at 50°C for 1 hr, hybridized with the probe using 20cc of ExpressHyb (Clontech) hybridization solution at 68°C for 2–4 hr, washed with 1× SSC/0.1% SDS at 25°C for 20 min and 0.1× SSC/0.1% SDS at 65°C for 45 min, and exposed to BioMax MS film (Kodak). DNA for Southern blots was prepared from human lymphoblasts or cell lines using Qiagen columns, digested to completion with restriction enzymes, precipitated, and 5 μg was loaded on a 1% agarose gel in TAE buffer. The gel was run overnight at 70 V, denatured with 0.5 M NaOH/0.6 M NaCl, neutralized with 0.5 M Tris–HCl/0.6 M NaCl for 20 min each, transferred neutrally to a Hybond N+ nylon membrane overnight, fixed with NaOH, then blocked and hybridized as above at 60°C for 2–4 hr.

SSCP

PCR reactions were run on a nondenaturing 6% acrylamide gel run at 30 W for 2–4 hr at 4°C, and the DNA was visualized using the silver staining technique (Promega, Madison, WI). Aberrant bands were excised and the DNA was eluted (in 0.1% SDS/0.5 M ammonium acetate/10 mM magnesium acetate/1 mM EDTA at 37°C for 1 hr), reamplified, and characterized by sequence analysis. For each patient who displayed an aberrant SSCP conformer, genomic DNA was reamplified and reanalyzed to confirm the suspected mutation.

DNA Sequence Analysis

DNA sequencing was performed using standard techniques on an ABI 377 automated sequencer, and DNA sequences were constructed, edited, and analyzed using the Sequencher program (Ann Arbor, MI). All DNA sequences were analyzed using the BLAST program to detect matches to previously known DNA sequence.

Patient Identification

Patients were recruited through their neurologists in accordance with protocols approved by the Committees on Clinical Investigation at Beth Israel Deaconess Medical Center, Children's Hospital, and Fairview-University Medical Center. All patients entered into the study had a brain MRI scan using standard clinical scanning sequences. MRIs from members of pedigrees were reviewed by one of us (J. G. G., C. A. W., W. B. D., M. E. R.). MRI reports from single sporadic cases were not always reviewed; thus, sporadic cases are likely to be somewhat genetically heterogeneous. Several asymptomatic parents of affected patients also had brain MRI scans when their disease status was not clear.

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