

ASPM is a major determinant of cerebral cortical size

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One of the most notable trends in mammalian evolution is the massive increase in size of the cerebral cortex, especially in primates. Humans with autosomal recessive primary microcephaly (MCPH) show a small but otherwise grossly normal cerebral cortex associated with mild to moderate mental retardation^{1–4}. Genes linked to this condition offer potential insights into the development and evolution of the cerebral cortex. Here we show that the most common cause of MCPH is homozygous mutation of *ASPM*, the human ortholog of the *Drosophila melanogaster* abnormal spindle gene (*asp*)⁵, which is essential for normal mitotic spindle function in embryonic neuroblasts⁶. The mouse gene *Aspm* is expressed specifically in the primary sites of prenatal cerebral cortical neurogenesis. Notably, the predicted *ASPM* proteins encode systematically larger numbers of repeated 'IQ' domains between flies, mice and humans, with the predominant difference between *Aspm* and *ASPM* being a single large insertion coding for IQ domains. Our results and evolutionary considerations suggest that brain size is controlled in part through modulation of mitotic spindle activity in neuronal progenitor cells.

MCPH exhibits genetic heterogeneity associated with mutations in five recessive loci that cause clinically indistinguishable disorders^{2,3,7–11}. Of these, mutations in *MCPH5* (1q31; ref. 2) are the most prevalent, present in 24 of 56 consanguineous Northern Pakistani families affected with MCPH¹². *MCPH5* is also the only locus associated with MCPH known to be affected in multiple ethnic groups^{2,3}. Brains of individuals with *MCPH5* mutations show a substantial reduction in size of the cerebral cortex without evidence of abnormal neuronal migration or abnormal architecture (Fig. 1a–d).

We adopted a positional cloning strategy to identify the gene *MCPH5* (Fig. 2), using 24 consanguineous Northern Pakistani families (including 61 affected individuals aged 4–35 years) affected with MCPH linked to the *MCPH5* locus. We assembled a complete electronic contig of the previously defined 8-Mb region of 1q31 containing *MCPH5* (flanked by GATA135F02 and DIS1678) by overlapping BAC clones and transcripts where sequences were available in the public domain. We designed new polymorphic microsatellite markers and used them to locate crossovers in three families with more than one affected individual (Fig. 2a), including the family in which we previously defined the locus². Each of these families had a two-point lod score >3 for probability of linkage to the *MCPH5* locus. This reduced the gene region for *MCPH5* from 8 Mb to 2.2 Mb (Fig. 2b). We then used more polymorphic microsatellite markers at a density of roughly one per 100 kb to determine the genotypes of all 24 families. We identified a region of 600 kb in which one haplotype was shared between three different families and a second haplotype was shared by three other families, indicating common ancestors carrying mutations in *MCPH5* (ref. 13). This region contained four potential genes identified from genetic databases (Fig. 2c). We eliminated one, *CRB1*, because mutations in that gene cause blindness due to Leber congenital amaurosis without microcephaly¹⁴. The other three transcripts, which are expressed in fetal brain, included a homolog of the *KIAA1089* gene on chromosome 9q34, a zinc-finger gene and a number of transcripts

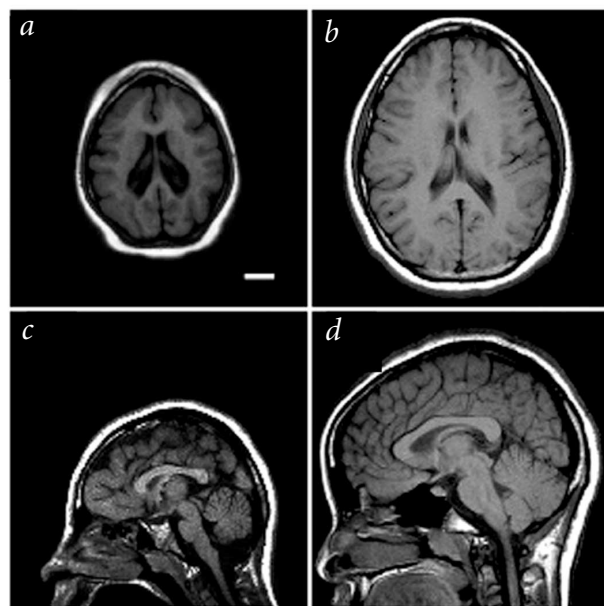
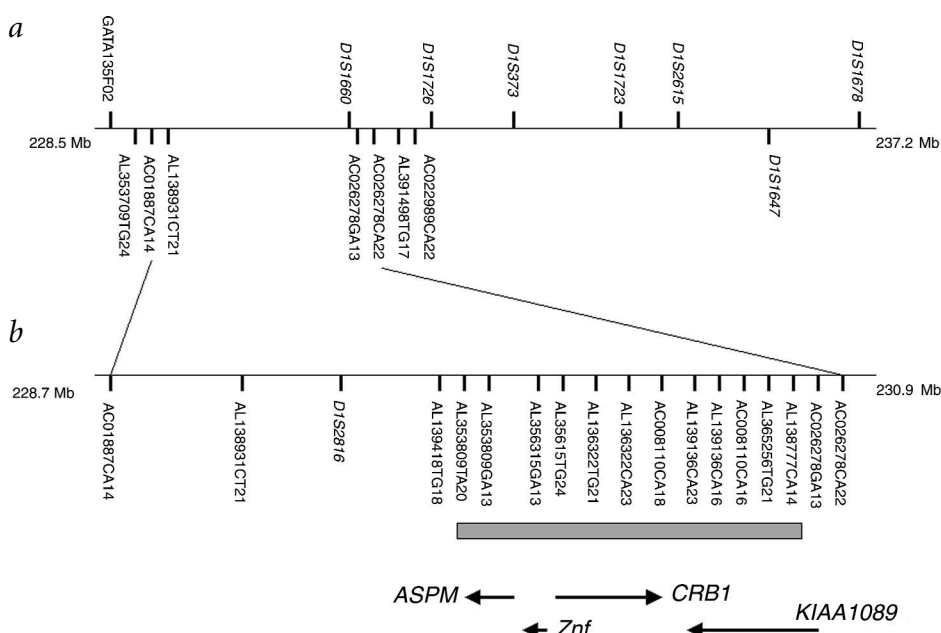


Fig. 1 Magnetic resonance images of brains of individuals with an *MCPH5* mutation. **a**, Axial T1-weighted image from a 13-year-old female with an *MCPH5* mutation showing reduced size of cerebral cortex and some simplification of gyral pattern. **b**, Axial T1-weighted image from an unaffected 11-year-old female control. **c**, Sagittal T1-weighted image from a 13-year-old female with an *MCPH5* mutation showing striking reduction in the size of the cerebral cortex, prominent sloping of the forehead and preserved midline structures. **d**, Sagittal T1-weighted image from an unaffected 11-year-old female control. Scale bar = 2 cm.

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Fig. 2 Overview of the mapping of the *MCPH5* locus and identification of *ASPM*. **a**, Uppermost markers define the original 8-Mb *MCPH5* region on chromosome 1q13, flanked by markers GATA135F02 and *D1S1678* (ref. 2). We used new polymorphic markers (below the line) to refine this region by the identification of crossovers in three large families. Markers AC01887CA14 and AC026278CA22 flank the smallest common homozygous region. **b**, The region of common haplotypes between families (filled box) identified by genotyping additional polymorphic markers. **c**, We identified four candidate genes (5' to 3' orientation) by bioinformatics within the region of common haplotype.



with similarity to *asp*. We determined the complete sequences of these three genes and sequenced them in the four largest families mapping to *MCPH5*, each of which had a distinct haplotype.

We identified a homozygous mutation introducing a premature stop codon into the predicted *ASPM* (abnormal spindle-like microcephaly associated) open reading frame in each of the four affected families. The four mutations are 719–720delCT (exon 3), causing a frameshift leading to a premature termination 15 codons downstream (Fig. 3a); 1258–1264 delTCTCAAG (exon 3), causing a frameshift leading to a premature termination 31 codons downstream; 7761T→G (exon 18), producing immediate truncation; and 9159delA (exon 21), causing a frameshift leading to a premature termination 4 codons downstream (Fig. 3b). Each mutation segregated with the disease as expected within the families, and was not found in 200 normal control chromosomes. We were unable to distinguish between the phenotypes of the four families in which mutations were found.

We confirmed the predicted 10,434-bp open reading frame of *ASPM* (Fig. 4a) in overlapping segments of cDNA from fetal brain and colon. The Kozak start and polyadenylation sites are consensus sequences. Exon 4 and large segments of exons 3 and 18 were not found in previously reported human expressed sequence tags (ESTs) or mRNA. The gene contains 28 exons and spans 62 kb of genomic sequence on the BAC RP11-32D17.

Interspecies comparisons of the predicted abnormal spindle proteins indicated that they are notably conserved overall, but show a consistent correlation of greater protein size with larger brain size. Both the putative amino-terminal microtubule-binding region of *asp* and a putative calponin-homology domain¹⁵ were conserved in *Aspm* and *ASPM* (Fig. 4b). Most of the remainder of the protein encodes a series of repeated calmodulin-binding IQ domains¹⁶, and the number of these repeats varies markedly between species. We found that the closest potential *Caenorhabditis elegans* homolog encoded 2 IQ repeats, whereas *asp* encoded 24 repeats, *Aspm* contained 61 repeats and *ASPM* 74 repeats, which is the largest number of IQ domains reported in any protein. The size difference between *Aspm* and *ASPM* principally reflected an inser-

tion of roughly 900 bp into exon 18 (Fig. 4a), resulting in a large exon of 4.7 kb and adding ten IQ domains to the predicted protein. BLAST analysis of this inserted sequence showed that its closest match was to an upstream region of exon 18, suggesting that the human insertion may have arisen from a duplication. There were other, smaller potential insertions that distinguished *ASPM* from *Aspm*, predominantly involving the two largest exons, 3 and 18.

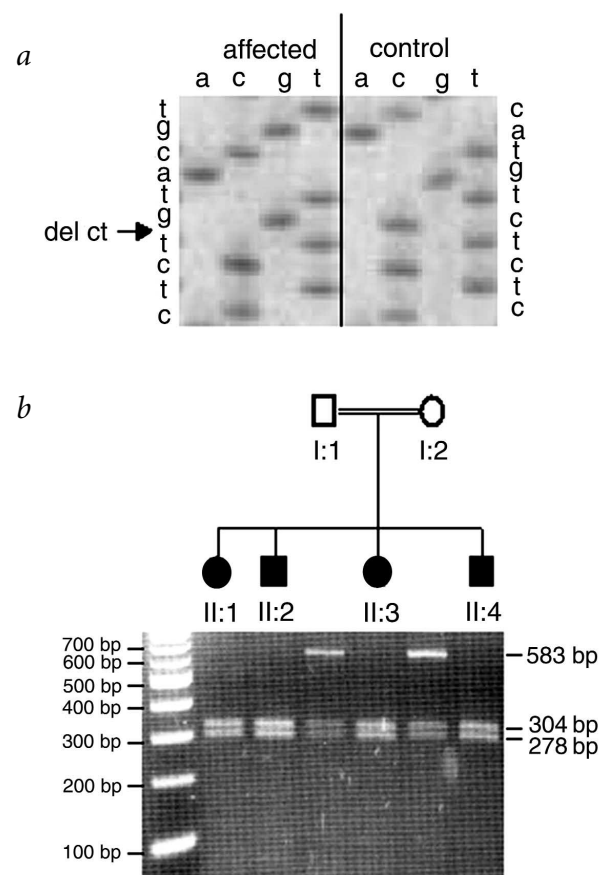


Fig. 3 Mutations in *ASPM*. **a**, 719–720delCT shown by direct sequence analysis with mutated sequence on the left and wildtype on the right. **b**, *AlwNI* restriction digest for 9159delA. Parents (obligate carriers) show the normal allele (583 bp) and the digested mutated allele (bands of 304 and 278 bp), and the four affected children are homozygous for the mutated allele.

Although the function of the repeated IQ domains is not known, loss of the terminal 425 amino acids of ASPM (mutation 9159delA), which encompass six IQ domains and the carboxy-terminal region, was sufficient to cause MCPH, suggesting that this region is essential for normal brain growth.

Northern-blot analysis of *Aspm* showed that it was highly expressed at embryonic day (E) 11–17 (Fig. 4c) and encoded on a large transcript (>9.5 kb). *In situ* hybridization of an antisense probe to *Aspm* showed preferential expression during cerebral cortical neurogenesis (Fig. 5a–h), specifically in the cerebral cortical ventricular zone at E14.5 (Fig. 5a) and E16.5 (Fig. 5b). We also observed expression in the proliferative region of the medial and lateral ganglionic eminence (MGE and LGE) at both E14.5 and E16.5, and in the ventricular zone of the dorsal diencephalon at E14.5, with weak hybridization in this region at E16.5. Other regions of the dorsal diencephalon showed *Aspm* expression at E14.5, but by E16.5, expression was increasingly limited to telencephalic structures. We observed little or no hybridization in ventral diencephalon and more caudal parts of the brain (data not shown). *Aspm* expression was quite intense (Fig. 5c) at E14.5, when there are many progenitor cells in the cortical ventricular zone, but had already begun to decrease in intensity by E16.5 (Fig. 5d). Fetal *Aspm* expression was overall greatest in the ventricular zones, which contain the progenitor cells for cerebral cortical pyramidal neurons¹⁷. *Aspm* was also highly expressed in the MGE and LGE, where up to one third of cerebral cortical neurons, predominantly interneurons, are formed and subsequently migrate non-radially from the ventral telencephalon into the cerebral cortex^{18,19}. Expression of *Aspm* was greatly reduced by postnatal day (P) 0 (day of birth), when neurogenesis in the cortical ventricular zone is completed and gliogenesis is increasing (Fig. 5e), suggesting that *Aspm* was preferentially expressed in progenitors that produce neurons rather than glia. By P9, expression was limited to rare scattered cells in the neocortex (Fig. 5f), though expression continued in regions of the brain that show

persistent postnatal neurogenesis, such as the dentate gyrus^{20,21} (Fig. 5g) and the subventricular zone²² of the rostral migratory stream leading to the olfactory bulb (Fig. 5h). These data suggest that *Aspm* has a preferential role in regulating neurogenesis before and after birth, and that the pattern of brain malformation associated with mutations in *MCPH5* (primarily reduction in size of the cerebral cortex) closely parallels the overall distribution of *Aspm* expression.

In *D. melanogaster*, *asp* is involved in meiosis and mitosis²³, both in the organization and binding together of microtubules at the spindle poles (which causes abnormal spindles when aberrant^{5,6}) and in the formation of the central mitotic spindle (whose perturbation leads to disruption of the contractile ring machinery and failure of cytokinesis²⁴). Mutations in *asp* cause dividing neuroblasts to arrest in metaphase, leading to reduced central nervous system development⁶. The neuronal progenitor cells of the mammalian cerebral cortical ventricular epithelium^{25,26} have a specific pattern of mitotic activity, as do neuroblasts in the fly: symmetric cell divisions with mitotic spindles in the plane of the neuroepithelium yield two progenitor cells or two neurons, whereas asymmetric divisions yield spindles oriented perpendicular to the neuroepithelium and produce one neuron and regenerate one progenitor cell^{27–29}. Mutations in *ASPM* associated with MCPH suggest that regulation of mitotic spindle orientation may be an important evolutionary mechanism controlling brain size³⁰.

Methods

Subjects and diagnosis. For this study, we defined MCPH as a congenital disorder in which an affected individual has microcephaly of at least –4 s.d. (measured as occipital frontal head circumference and corrected for age and sex) and mild to moderate mental retardation, without any generalized disorder of growth, abnormal neurological findings, fits or dysmorphic features, and in which other syndromic, neurological, chromosomal, metabolic and maternal or environmental diagnoses have been sought and

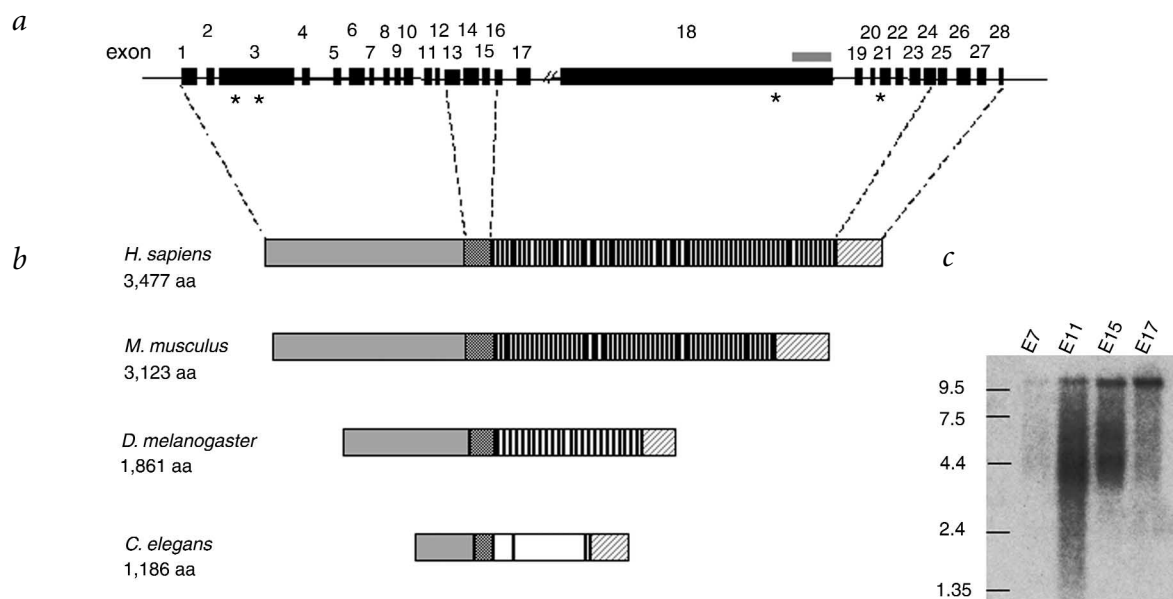
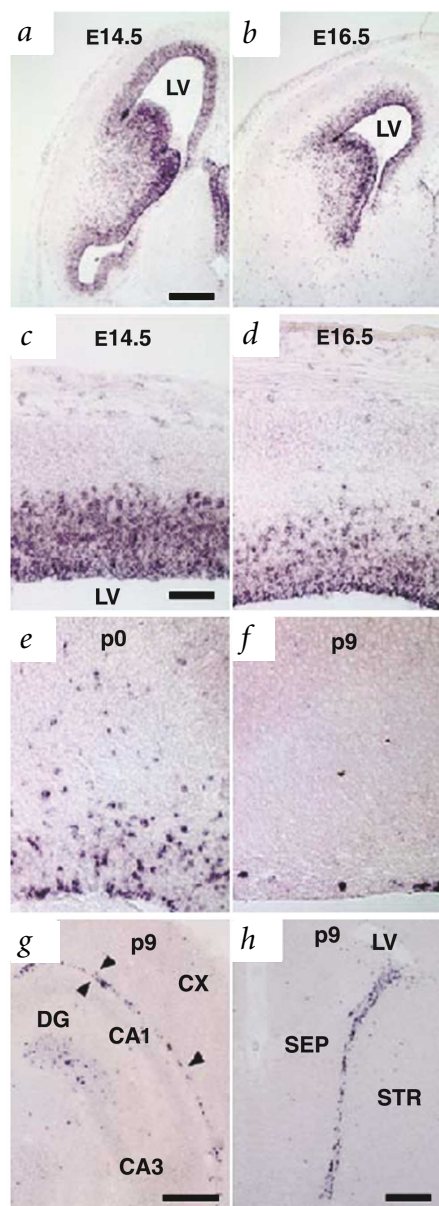


Fig. 4 Genomic structure of *ASPM*; phylogenetic comparison of the primary structure of *asp*, *Aspm* and *ASPM*; and northern-blot analysis. **a**, Genomic structure of *ASPM*, containing 10434 bp and 28 exons. The four premature stop codons that cause protein truncations in individuals with MCPH are marked with asterisks. The primary mouse–human difference is marked by a gray box. Dotted lines mark the exons comprising each domain of the *ASPM* protein. **b**, The predicted sizes and domains of abnormal spindle proteins in *Homo sapiens*, *Mus musculus*, *D. melanogaster* and *C. elegans* (*M. musculus* and *C. elegans* protein sequences are predictions). The putative microtubule-binding domain (gray box), calponin-homology domain (hatched box), multiple IQ calmodulin-binding domains (filled bars) and terminal region (diagonal striped box) are shown for each. **c**, Northern-blot analysis showed that *Aspm* was highly expressed at E11–17.

Fig. 5 Expression of *Aspm* in developing mouse brain shown by hybridization to antisense probe. Coronal sections of the forebrain of mouse embryos at **a**, E14.5 (LV, lateral ventricle) and **b**, E16.5 showed expression in the cerebral cortical ventricular zone, the proliferative region of the MGE and LGE and the ventricular zone of dorsal diencephalon. Higher magnification views of the cortical ventricular zone at **c**, E14.5, **d**, E16.5, **e**, P0 and **f**, P9 showed a progressive decrease in *Aspm* expression, and by P9, expression was limited to rare scattered cells in the neocortex. At P9, *Aspm* was persistently expressed in **g**, the dentate gyrus (CA1 and CA3, hippocampal regions; CX, cortex; DG, dentate gyrus; arrowheads, ventricular zone of the cortex) and **h**, the anterior subventricular zone leading to the olfactory bulb (SEP, septum; STR, striatum). Scale bar is 500 μ m for **a**, **b**, **g** and **h** and 100 μ m for **c**–**f**.



eliminated. Consanguineous affected families showing linkage of the disorder to *MCPH5* (age of affected individuals ranged from 4 to 35 years) were all of Northern Pakistani origin. Family members gave blood samples from which DNA was obtained by standard methods.

Ethical and licensing considerations. The study was approved by the Ethical Committee of the Combined Leeds Health Care Trusts. We obtained informed consent from all subjects involved in the study and, for those under 18 years of age, also from their parents. Mouse studies were approved by the Institutional Review Board (IRB) of the Beth Israel Deaconess Medical Center.

Electronic contig assembly. We assembled a substantially complete contig of the common haplotype region through iterative searches and comparisons of human DNA sequences, primarily using BLAST and ENTREZ.

New polymorphic markers. We identified all new polymorphic markers in the genomic sequence using Tandem Repeat Finder, NCBI and the Human Genome Browser. We designed primers with Primer3 and checked for specificity using BLAST. Primer sequences are available from the authors on request.

Gene elucidation. We predicted the mRNA sequences for *ASPM* (10,434 bp, 28 exons), a zinc-finger gene (2,001 bp, 10 exons) and the homolog of *KIAA1068* (2,203 bp, 27 exons) by iterative assessment and construction of mRNAs, ESTs, homology comparisons and gene predictions using BLAST, ENTREZ and GENSCAN. Although a mouse homolog of *asp* has been previously reported as *Calmbp1* (calmodulin-binding protein 1; ref. 15), this was considered to be a truncated 3' derivative because of a stop codon. Analysis of the mouse genome database revealed that the open reading frame containing the reported start codon for calmodulin-binding protein 1 extended further upstream without an in-frame stop codon. We constructed the predicted full *Aspm* gene by direct homology comparison of *ASPM* to mouse genome databases using BLAST, ENTREZ and the mouse genome database. The *asp* gene is documented in FlyBase. Protein analysis was done primarily by PIX.

Mutation detection. We detected all mutations by bi-directional sequence analysis of genomic DNA using standard methods. We designed primers using Primer3, BLAST and the sequence of RP11-32D17. Primer sequences are available from the authors on request. We confirmed each mutation as follows: 719–720delCT, amplification of a portion of exon 3 and fragment analysis on an ABI 377 Genescanner; 1258–1264delTCTCAAG, amplification of a portion of exon 3 followed by digestion with restriction enzyme *BsmI* (the mutation abolishes the single *BsmI* site within the fragment); 77761T→G, direct sequence analysis of a portion of exon 18; and 9159delA, amplification of exon 21 followed by digestion with restriction enzyme *AlwNI* (the mutation creates the single *AlwNI* site within the fragment).

ASPM mRNA sequence verification. We converted enriched fetal brain mRNA (Clontech Laboratories) to cDNA using standard RT–PCR methods. We obtained three specific fragments from the fetal brain cDNA by standard PCR methods using Pfu Turbo (Stratagene). A fourth fragment specific to the 5' region of *ASPM* was similarly obtained from the adenocarcinoma cell line COLO 205. Combined with two pre-existing neuronal precursor cell mRNA sequences from GenBank, these cDNAs confirmed the total *ASPM* sequence by overlap.

Mouse northern-blot analysis. We carried out northern-blot analysis of mRNA from total mouse embryos using standard methods (Clontech Laboratories). The probe represented a fragment of 654 bp, derived from an EST clone by restriction digestion with *KpnI*, that encodes the *Aspm* mRNA.

Mouse in situ hybridization. A partial transcript of the mouse ortholog of *asp* had previously been reported and designated *Calmbp1* (ref. 15). We carried out non-radioactive *in situ* hybridization using digoxigenin-labeled cRNA probes. We generated antisense and sense probes from a mouse EST clone, and hybridized and visualized frozen sections by standard methods.

URLs. The mouse genome database is available at <http://www.cebml.org>. PIX is available at <http://www.hgmp.mrc.ac.uk/>. FlyBase is available at <http://flybase.bio.indiana.edu/>.

GenBank accession numbers. The *MCPH5* region contained four candidate genes with the following accession numbers: *Calmbp1* (NM_009791), *KIAA1089* (AK000006), a zinc finger gene (T05514) and transcripts exhibiting homology to *asp* (AK001379, AK001380 and AK001411). AY101201,

AY099891 and AY099890 are the accession numbers for the ASPM-specific fragments generated from fetal brain cDNA, and AY099892 from COLO 205. AF509326 and AF533752 are for the full-length ASPM and *Aspm* mRNA sequences, respectively. Mouse EST clone AW558815 was used for mouse northern blots and mouse *in situ* hybridization experiments. NP492467 is a *C. elegans* homologue of *asp*.

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Competing interests statement

The authors declare that they have no competing financial interests.

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