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A centrosomal mechanism involving CDK5RAP2 and CENPJ controls brain size

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Autosomal recessive primary microcephaly is a potential model in which to research genes involved in human brain growth. We show that two forms of the disorder result from homozygous mutations in the genes *CDK5RAP2* and *CENPJ*. We found neuroepithelial expression of the genes during prenatal neurogenesis and protein localization to the spindle poles of mitotic cells, suggesting that a centrosomal mechanism controls neuron number in the developing mammalian brain.

Brains of individuals with autosomal recessive primary microcephaly (MCPH) are characterized by a substantial reduction in size of the cerebral cortex and a generalized reduction in size of the remainder of the central nervous system, but with normal architecture[1]. In MCPH, microcephaly is evident at birth, with head circumference ranging between −4 and −12 s.d. from the mean and thereafter remaining proportionately small with age (**Fig. 1a**). Cognitive functions are reduced, but epilepsy and other neurological disorders or decline are rarely reported, and motor skills are preserved[2]. MCPH is hypothesized to affect neuronal precursor cells in the neuroepithelium, resulting in reduced production of functional neurons during fetal life[3,4].

MCPH is genetically heterogeneous; mutations in six loci (*MCPH1–MCPH6*) have been reported to cause clinically indistinguishable disorders[1,2]. Of these, mutations at *MCPH5* are the most common[2,5]. Mutations in the genes *MCPH1* (encoding microcephalin) and *ASPM* (encoding abnormal spindle–like microcephaly associated, ASPM) at loci *MCPH1* and *MCPH5*, respectively, have previously been identified[3,5,6]. Microcephalin has a role in the initiation of chromosome condensation during mitosis and DNA damage–induced cellular responses[7,8]. The *Drosophila melanogaster* ASPM

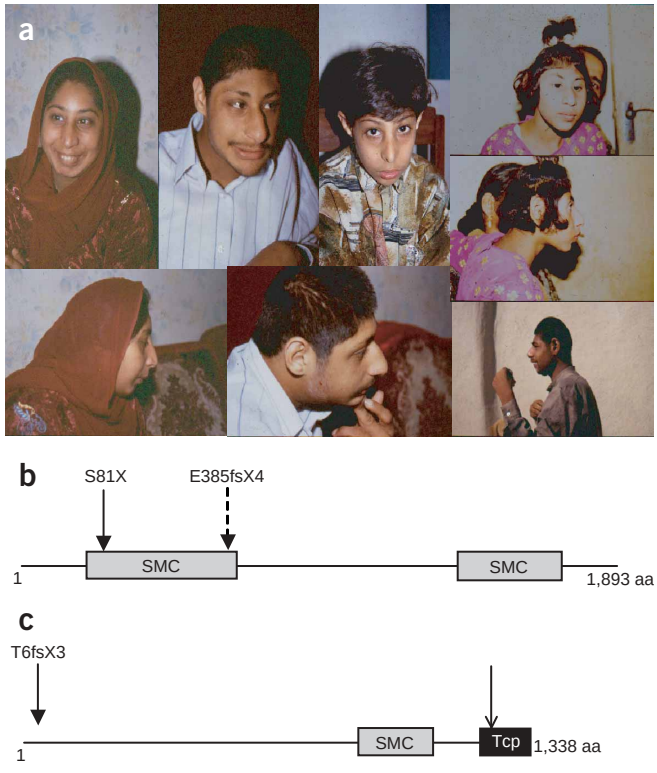

Figure 1 Clinical features of MCPH and the identification of pathogenic mutations in *CDK5RAP2* (at locus *MCPH3*) and *CENPJ* (at locus *MCPH6*). (**a**) Clinical features of Northern Pakistani individuals with mutations at *MCPH3* or *MCPH6*, showing the typical sloping forehead and reduced head circumference. (**b**) Cartoon representation of the domain structure of CDK5RAP2 showing the positions and effects of mutations that cause MCPH. Nonsense mutation is shown as a solid arrow; splicing mutation leading to a premature termination codon is shown as a dotted arrow with a filled arrowhead. Predicted chromosome segregation ATPase domains (SMC) are shown as gray boxes. aa, amino acids. (**c**) Cartoon representation of the domain structure of CENPJ showing the positions and effects of mutations that cause MCPH. Nonsense mutation is shown with a filled arrowhead; missense mutation is shown with an open arrowhead. Predicted chromosome segregation ATPase domain (SMC) is shown as a gray box; the defined T-complex protein 10 C-terminal domain (Tcp, pfam 07202) is shown as a black box. aa, amino acids.

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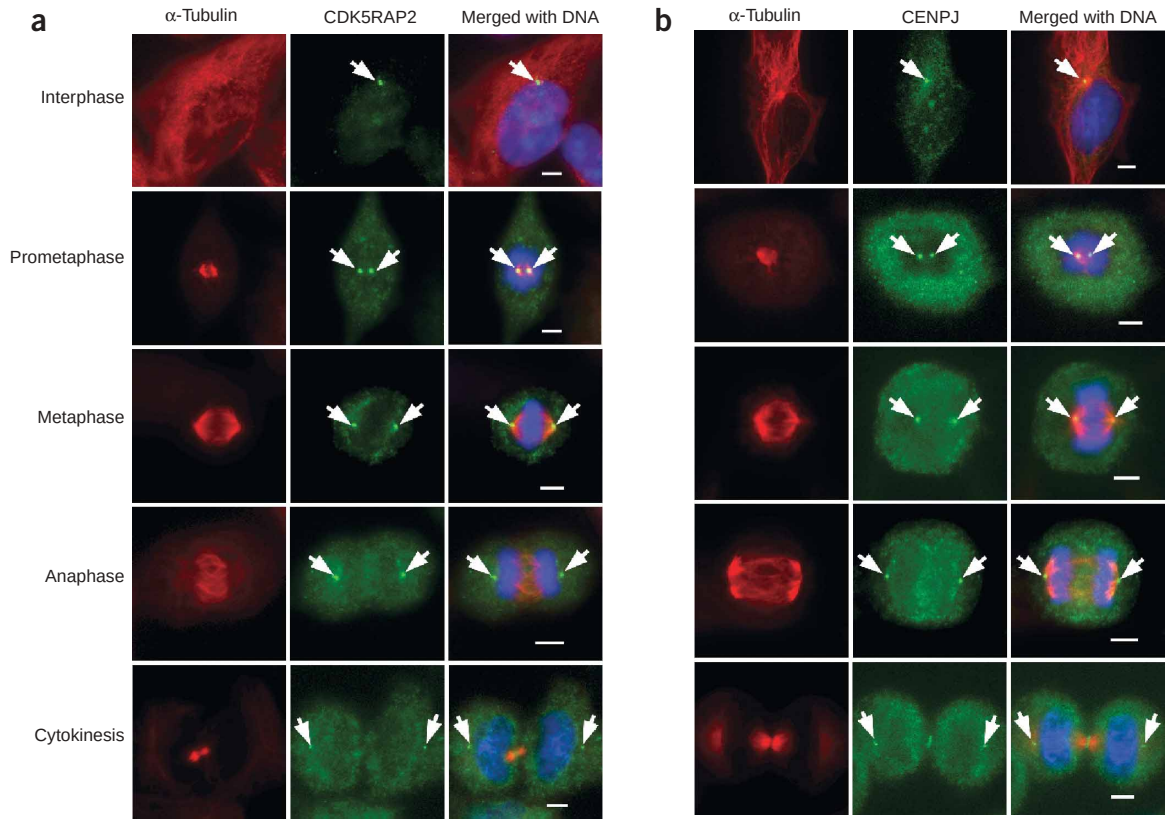

Figure 2 Expression profiles of CDK5RAP2 and CENPJ. (**a**) Confocal microscopy analysis of fixed HeLa cells at various stages of mitosis, showing centrosomal localization of human CDK5RAP2 (green), rat monoclonal antibody to α-tubulin (red) and DNA (blue) during mitosis. Scale bars, 5 μm. White arrows indicate CDK5RAP2 expression. (**b**) Confocal microscopy analysis of fixed HeLa cells undergoing mitosis confirming centrosomal localization of human CENPJ (green), rat antibody to α-tubulin (red) and DNA (blue) during mitosis. Scale bars, 5 μm. White arrows indicate CENPJ expression.

ortholog, Asp, focuses microtubules of the mitotic spindle onto the centrosome and may have a role in cytokinesis[9].

We used a positional cloning strategy to identify genes in loci *MCPH3* and *MCPH6* (**Supplementary Methods** online). We previously identified locus *MCPH3* on chromosome 9q34 and locus *MCPH6* on 13q12.2 using single consanguineous families with multiple affected individuals and autozygosity mapping[10,11]. We genotyped new polymorphic microsatellite markers in families with MCPH that had not previously been linked to a specific locus and identified one more family in whom the disease was linked to *MCPH3* and two additional families in whom the disease was linked to *MCPH6* (**Supplementary Figs. 1** and **2** and **Supplementary Table 1** online). Results of this analysis reduced the *MCPH3* region to 2.2 Mb and the *MCPH6* region to 3.1 Mb. We carried out bioinformatic analysis of the regions seeking candidate genes and identified cyclin dependant kinase 5 regulatory associated protein 2 (*CDK5RAP2*) in the *MCPH3* region and centromere associated protein J (*CENPJ*) in the *MCPH6* region[12,13]. We determined the sequences of these genes and sequenced genomic DNA in the relevant families. A homozygous mutation was present in each of the five families (**Supplementary Figs. 3** and **4** online). Each mutation was absent from 380 Northern Pakistani control chromosomes, showed the expected disease segregation in families and was not present in chimpanzee, gorilla, orangutan, gibbon, mouse or rat.

We identified mutations in the 34-exon gene *CDK5RAP2* in the two families with MCPH linked to *MCPH3*. In pedigree 1, we found a single homozygous base substitution in exon 4 (243T→A, resulting in the amino acid change S81X), and in pedigree 2, we found a homozygous mutation in intron 26 (IVS26–15A→G; **Fig. 1b**). Using a minigene splicing vector assay, we determined that IVS26–15A→G creates a new, superior splice acceptor site, leading to the addition of four new amino acids and a termination codon (E385fsX4; **Supplementary Figs. 5** and **6** online)[14].

In the 17-exon gene *CENPJ*, we identified a homozygous single-base deletion (17delC, resulting in the amino acid change T6fsX3) in pedigrees 4 and 5 and a substitution in the first base of exon 16 (3704A→T, resulting in the amino acid change E1235V) in pedigree 3 (**Fig. 1c**). We analyzed RNA from an affected child and one parent in pedigree 3 and found that 3704A→T did not affect splicing. With the exception of the missense mutation 3704A→T, all mutations causing MCPH have been nonsense mutations[3,5,6,7,8,15]. Multiple sequence alignments of the CENPJ protein sequences from diverse species indicate that Glu1235 is highly conserved; therefore, E1235V is probably an important change (**Supplementary Fig. 6** online). CENPJ was initially named centrosomal protein 4.1-associated protein (CPAP), as it is located in the centrosome throughout the cell cycle and interacts with the nonerythrocyte 4.1 protein 135 splice variant (4.1R-135)[13]. The E1235V mutation in pedigree 3 occurs in a Tcp10 domain of CENPJ, which has been shown to interact with 4.1R-135 (ref. 13).

We carried out *in situ* hybridization in staged mouse embryos to determine the expression patterns of *Cenpj* and *Cdk5rap2* during

cerebral cortical neurogenesis. *Cdk5rap2* and *Cenpj* were widely distributed in the developing embryo (**Supplementary Fig. 7** online) with highest expression in the brain and spinal cord (data not shown); primary expression was localized to the neuroepithelium of the frontal cortex early in neurogenesis. At embryonic day 15.5, *Cdk5rap2* had a subplate or neuronal localization and *Cenpj* had higher expression in the newly forming layers of the cortical plate. Therefore, like the other genes mutated in MCPH, *Cdk5rap2* and *Cenpj* are expressed in the neuroepithelium lining the lateral ventricles of the mouse forebrain, which contain the progenitor cells for cerebral cortical neurons[3,6]. Both *Cenpj* and *Cdk5rap2* have temporal and spatial expression patterns that are consistent with a role in regulating neurogenic mitosis. But expression of *Cenpj* and *Cdk5rap2* is not limited to the neuroepithelium, in contrast to *Calmbp1* (also called *Aspm*), which has solely neuroepithelial expression[3].

MCPH is hypothesized to affect neuronal precursor cell division; *D. melanogaster* Asp is crucially involved in neurogenic mitotic spindle integrity and cytokinesis[9]. We sought evidence of the involvement of CENPJ and CDK5RAP2 in mitosis. Immunohistochemistry and confocal microscopy of N-terminal antibodies in HeLa cells showed that CDK5RAP2 was centrosomal throughout mitosis and confirmed that CENPJ was similarly localized[13] (**Fig. 2**). We confirmed antibody specificity by peptide precompetition. During interphase, CDK5RAP2 and CENPJ were visible as two small dots external to the nucleus. As cells entered mitosis, CDK5RAP2 and CENPJ were concentrated at the mitotic spindle poles during prometaphase and metaphase. Signal intensity decreased during anaphase and remained at this level through telophase and cytokinesis.

We identified homozygous mutations in *CDK5RAP2* and *CENPJ* in families with MCPH linked to *MCPH3* and *MCPH6*, respectively. The mouse *MCPH3* and *MCPH6* homologs *Cdk5rap2* and *Cenpj* are expressed in the neuroepithelium at the time of prenatal neuron production. We showed that CDK5RAP2 localizes at the centrosome during mitosis and confirmed an identical profile for CENPJ. The finding that the MCPH proteins Asp, CDK5RAP2 and CENPJ are centrosomal components during mitosis further emphasizes the key role of the centrosome in each major stage of the development and function of the nervous system.

MCPH is characterized by a reduction in the size of the central nervous system present at birth[1–3]. The underlying defective neurodevelopmental process is unknown but is thought to be reduced neuron production during fetal life rather than defective neuron migration or increased apoptosis[1]. Central nervous system neurons are predominantly produced by asymmetric cell division of neural precursors residing in the neuroepithelium lining the brain. The data presented here support the hypothesis that MCPH is the consequence of a primary disorder of neurogenic mitosis[3,4,6]. Furthermore, our findings suggest that an unidentified centrosomal mechanism controls the number of neurons generated by neural precursor cells.

Ethical and licensing considerations. The study was approved by the Leeds East Research Ethic Committee. We obtained informed consent including consent to publish photographs from all subjects involved in the study and, for those less than 18 years of age, also from their parents. Mouse studies were approved by the Institutional Animal Care and Use Committee of the Harvard Medical School and the Beth Israel Deaconess Medical Center.

URLs. Spliceview is available at http://l25.itba.mi.cnr.it/~webgene/wwwspliceview.html. FlyBase is available at http://flybase.bio.indiana.edu/genes/. PIX is available at http://www.hgmp.mrc.ac.uk/Registered/Webapp/pix/. Tandem Repeat Finder is available at http://c3.biomath.mssm.edu/trf.html. Primer3 is available at http://www-genome.wi.mit.edu/cgi-bin/primer/primer3_www.cgi.

Accession numbers. GenBank: full-length human *CDK5RAP2* mRNA, NM_018249; full-length human *CENPJ* mRNA, NM_018451; full-length mouse *Cdk5rap2* mRNA, NM_145990; full-length mouse *Cenpj* mRNA, XM_127861. Assembly contig accession numbers for intron 26 of *CDK5RAP2*: chimp, AADA01048142; gorilla, AY917124; dog, AAEX01055387; mouse, AL929409; rat, AABR03041512. GenBank Protein: CENPJ protein: chimp, AY917123; mouse, XP_127861; rat, XP_224232; *D. melanogaster*, NP_649701; *Leishmania*, CAB89629.

Note: Supplementary information is available on the Nature Genetics website.

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COMPETING INTERESTS STATEMENT

The authors declare that they have no competing financial interests.

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