Kinetochore KMN network gene CASC5 mutated in primary microcephaly

Anne Genin1, Julie Desir1,2, Nelle Lambert1,2, Martine Biervliet3, Nathalie Van Der Aa3, Genevieve Pierquin4, Audrey Killian5, Mario Tosi5, Montse Urbina2, Anne Lefort1, Frederick Libert1, Isabelle Pirson1,* and Marc Abramowicz1,2

1Institute of Interdisciplinary Research IRIBHM and 2Medical Genetics, Hôpital Erasme, Université Libre de Bruxelles, Anderlecht 1070, Belgium, 3Department of Medical Genetics, University Hospital Antwerp, Antwerp 2000, Belgium, 4Human Genetics, University of Liège, Liège 4000, Belgium and 5Faculty of Medicine, Inserm U1079, IRIB, Rouen 76000, France,

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Several genes expressed at the centrosome or spindle pole have been reported to underlie autosomal recessive primary microcephaly (MCPH), a neurodevelopmental disorder consisting of an important brain size reduction present since birth, associated with mild-to-moderate mental handicap and no other neurological feature nor associated malformation. Here, we report a mutation of CASC5 (aka Blinkin, or KNL1, or hSPC105) in MCPH patients from three consanguineous families, in one of which we initially reported the MCPH4 locus. The combined logarithm of odds score of the three families was >6. All patients shared a very rare homozygous mutation of CASC5. The mutation induced skipping of exon 18 with subsequent frameshift and truncation of the predicted protein. CASC5 is part of the KMN network of the kinetochore and is required for proper microtubule attachment to the chromosome centromere and for spindle-assembly checkpoint (SAC) activation during mitosis. Like MCPH gene ASPM, CASC5 is upregulated in the ventricular zone (VZ) of the human fetal brain. CASC5 binds BUB1, BUBR1, ZWINT-1 and interestingly it binds to MIS12 through a protein domain which is truncated by the mutation. CASC5 localized at the equatorial plate like ZWINT-1 and BUBR1, while ASPM, CEP152 and PCTN localized at the spindle poles in our patients and in controls. Comparison of primate and rodent lineages indicates accelerated evolution of CASC5 in the human lineage. Our data provide strong evidence for CASC5 as a novel MCPH gene, and underscore the role of kinetochore integrity in proper volumetric development of the human brain.

INTRODUCTION

A large, convoluted brain is a hallmark of the Homo sapiens lineage, and is associated with human-specific skills, including the ability to orchestrate symbolic thought, language, tool use and emotional adaptation to the social environment. Conversely, a small brain, and particularly a small cerebral cortex, is a major feature of many developmental brain disorders (1).

The brain size correlates closely with the head circumference (HC). Microcephaly refers to a small HC in terms of standard deviations (SD) below the mean for age and gender. Primary microcephaly refers to a small brain size present since birth, with no structural malformation of the brain, with a HC < −2SD at birth and < −3SD after 1 year of age. From a theoretical standpoint, primary microcephaly may result from decreased cell proliferation during development, increased cell death or migration defects, these three groups of causes not excluding one another. Microcephaly, Primary Hereditary (MCPH) [MIM 251200] is an autosomal recessive trait with primary microcephaly, associated with mild-to-moderate mental handicap and absence of additional neurological or somatic disease. The brain may be very small, with an estimated volume hardly more than a third of the normal volume in some patients (2). MCPH is a very rare disorder in outbred populations, affecting <1/100 000 newborns (3), but is more frequent in some populations with a high rate of consanguinity (4,5).

*To whom correspondence should be addressed at: 808, Route de Lennik, Anderlecht 1070, Belgium. Tel: +32 25554137; Email: ilpirson@ulb.ac.be

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Because MCPH is Mendelian (single gene defect) and because microcephaly in general is a feature of many developmental brain disorders (1), MCPH is an illuminating model to dissect molecular mechanisms of brain development. MCPH is very heterogeneous, with ten loci and nine genes reported to date: Microcephalin (MCPH1, or BRIT1) (5), WDR62 (MCPH2) (6), CDK5RAP2 (MCPH3) (7), CEP152 (MCPH4) (8), ASPM (MCPH5) (2), CENPJ (MCPH6) (7), STIL (MCPH7) (9), CEP63 (10) and CEP135 (11). In addition, an orphan locus has recently been reported at 10q (12). More genetic heterogeneity is to be expected considering the complexity of the process of human brain development. All nine genes reported to date encode proteins that localize to the centrosome (7,13,14) suggesting that a centrosomal mechanism is responsible for controlling the neural cell number in the developing human brain (7), with data supporting a role in regulating progenitors proliferation and cell cycle exit leading to neuronal differentiation (15,16). Another mechanism is, however, not excluded. MCPH1 has been involved in DNA repair (17).

In addition to genetic heterogeneity, some MCPH defects are allelic to complex syndromes that share microcephaly as a feature, e.g. lissencephaly with additional brain malformations (WDR62) (6), and Seckel syndrome (CEP152 (8), CENPJ (7)).

We initially reported a relatively large (25 cM) MCPH linkage region on chromosome 15, MCPH4, after homozygosity mapping in a Moroccan family (18). In 2010, Guernsey et al. reported mutations of CEP152 in unrelated MCPH patients, CEP152 being located in our initial MCPH4 linkage region (8). Here, we report the mutation of a novel MCPH gene, CASC5, and the exclusion of CEP152, in our initial MCPH4 family and two previously unreported families.

RESULTS

Mapping and identification of a CASC5 homozygous mutation

The ASPM gene (the most frequently mutated gene in MCPH) was sequenced in each of the three families and no mutation was found. Genotyping the original MCPH4 family E, and families S and Y using 250 K GeneChip® microarrays showed a common region of homozygosity spanning 3.7 Mb at 15q14–15.1 (Fig. 1C, open box). This region was the only region in the whole genome that showed homozygosity.
in the three families over a significant length consistent with
autozygosity (>2 cm in each family). This 3.7 Mb segment
clearly excluded the CEP152 gene transcription unit, which
is located 7 Mb outside of the linkage region. Furthermore,
direct sequencing of all coding exons and intronic junctions
of CEP152 showed no mutation.

The three families originated from neighboring small villages
in rural Morocco. Within the 3.7 Mb homozygous region, we
observed a 2.7 Mb haplotype that was common to the three
families, strongly suggesting linkage disequilibrium around an
ancestral mutation (Fig. 1C).

This genomic region is rich in protein-coding genes, and the
2.7 Mb segment contains 38 genes. Two in silico methods to
prioritize the best candidates were used: the Endeavour
program and $K_s/K_a$ analysis. The Endeavour program (www.
esat.kuleuven.be/endeavour) aims at ranking a defined set
of target genes (here, all genes of our 2.7 Mb segment) according
to their expression, cellular function and other publicly available
data after comparison with a defined set of educating
genes. For the latter set, we used the known MCPH genes,
as well as all genes known to be involved in centrosomal or
spindle organization and function, and/or in the regulation of
the mitotic cell cycle (Fig. 2A).

We computed the human/macaque and rat/mouse $K_s/K_a$ for
the range of our candidate genes in order to prioritize those
with high ratios (Fig. 2B) (18). We analyzed the patient’s lymphoblasts transcriptome by the
Affymetrix® gene expression array (Fig. 2C). We reasoned that
a sizable, probably large fraction of mutations causing highly
penetrant recessive phenotypes are complete loss-of-function
mutations where premature stop codons cause nonsense-
mediated mRNA decay. We focused on the genes contained
in the critical interval, as well as on CEP152. CASC5,
EIF2AK4, FAM82C, CHAC1 and THBS1 showed very low
expression when compared with control in both probands for at
least one of the probes, contrasting with CEP152 which
yielded a higher signal in the patients.

RAD51, BUB1B, EIF2AK4, NUSAP1, PAK6, VPS18,
ZFVE19 and CASC5 were sequenced by the Sanger method
(Fig. 3A). Only one novel variant was discovered, a missense
mutation in exon 18 of the CASC5 gene. This homozygous-
coding variant, c.6125 G>A, changed a methionine into an iso-
leucine residue at position 2041 of the protein (p. M2041I).
Met2041 is highly conserved across species (Fig. 3B) and the
substitution was scored as “probably damaging” by software
PolyPhen2 (score of 0.996). Fifty-eight unrelated healthy con-
trols of the same (Moroccan) ethnic origin and a 100 unrelated
controls with mixed ethnicities were sequenced for this variant
and all of them were negative (0 occurrence of the mutation in
316 alleles).

Under the assumption of a mutation frequency equal to
0.003, the combined logarithm of odds (LOD) score over the
three families was >6.
The CASC5 gene has 27 exons and codes for a protein
which localizes at the kinetochore. The CASC5 protein per-
forms two crucial functions during mitosis: it is required for
correct attachment of chromosome centromeres to the micro-
tubule apparatus, and is essential for spindle-assembly check-
point (SAC) signaling (19). CASC5 directly links BUB1 and
BUB1B to kinetochores (19). CASC5 also directly binds
MIS12 which is essential for kinetochore formation and
proper chromosome segregation during mitosis (20), and the
interactions between NDC80, MIS12 and KNL1 (CASC5)
are referred to as the KMN network. The C-terminal portion
of CASC5 (AA 1981–2108), containing the mutation, interacts
with ZWINT-1, a kinetochore protein, required for kineto-
chores assembly and for proper SAC silencing at metaphase
(Fig. 5A).

In order to rule out a disease-causing mutation in another
gene, the full genomic 2.7 Mb critical region was captured
on a customized microarray and sequenced using the 454
Roche FLX Genome Sequencer. Using a customized Nimble-
Gen array with 60–90 nucleotide-long probes covering 74.3%
target bases, including 93.3% of protein-coding sequences
and untranslated regions, we enriched DNA for the 2.7 Mb
locus and sequenced the captured library. A total of
1 389 296 reads (445 659 360 bp) with an average length of
317 bp were sequenced. The length of the hybridized frag-
ments being much larger than the capture probes, we were
able to cover 97.5% of the target region. The depth of sequen-
cing was >20-fold for 78% of the bases, >10-fold for 88% and
>4-fold for 97%. After filtering the raw data for changes affect-
ing less than two reads or <10% of the reads, we identified
1982 variants in the region, all homozygous as expected.
One thousand seven hundred and nineteen were
reported in single-nucleotide polymorphism (SNP) databases
dbSNP128 and 263 were unknown. Variants were further fil-
tered to remove polymorphisms present in dbSNP135. Two
hundred and eight homozygous variants remained after filtering,
consisting of 113 noncoding indels, and 94 noncoding single
nucleotide variants (SNVs), in locations unlikely to have a func-
tional effect (e.g. not located in first or last intronic dinucleo-
tide). We identified a single coding, rare, unreported SNV,
the c.6125 G>A mutation in CASC5. Of note, FLX sequencing
also identified an unknown coding SNV in RTF1, but the latter
was not confirmed by Sanger Sequencing (Table 1). A virtual
de novo assembly of the FLX sequencing reads failed to
detect a chimeric segment that would indicate a chromosomal
rearrangement. In short, the only candidate mutation found in
the 2.7 Mb fragment was the CASC5 mutation (Fig. 3C).

Functional analysis of the mutation

The CASC5 mutation was predicted to produce a semi-
conservative amino acid change, transforming a methionine
into an isoleucine residue, with a likely functional effect.
More importantly, the mutation was predicted to inactivate
an exonic splicing enhancer (ESE), as indicated by both the
ESEfinder and RESCUE-ESE algorithms, leading to an abnor-
mal transcript with an absent exon 18. Besides the important
role of the exon 18-encoded protein domain (see discussion),
skipping this exon produces a frameshift, with a premature
stop codon in exon 19.

To demonstrate the abnormal splicing of the mutated CASC5
in patients, RNA was extracted from lymphoblastoid cell lines,
reverse transcribed into complementary DNA (cDNA) and
amplified using primers targeting a 316 bp fragment encom-
passing exon 17 to exon 19 of CASC5 (Fig. 4A). Two bands
were observed after acrylamide gel electrophoresis. One band
of normal size (316 bp) was present in all subjects. A smaller
band of expected size (228 bp) for exon 18 deletion was observed only in the homozygous patient and heterozygous parents. This 228 bp smaller band seemed more intense in the patient’s sample. Both the bands were extracted from the gel and sequenced, which confirmed that they consisted of exons 17-18-19, containing the variant c.6125 G>A and 17–19, respectively.

The impact of the mutation in abnormal CASC5 splicing was further studied by a minigene experiment. The wild-type and mutant exon 18, flanked by 150 bp of intron sequences and inserted into the pCAS-1 minigene, was transfected into HeLa cells. Splicing products were visualized in agarose gels (Fig. 4B). An abnormal band of the expected size corresponding to the deletion of exon 18 was only observed from the mutated construct, confirming that the mutation was able to cause exon skipping in vitro.

CASC5 is a large protein of 265 KDa. The skipping of exon 18 is thus predicted to cause a frameshift and a premature stop codon in exon 19, which would result in a truncated, or absent protein if subject to nonsense-mediated mRNA decay. We

![Figure 2. Prioritization of candidate genes. (A) Global ranking of our candidate genes using the Endeavor program, higher ranking indicating more likely candidate. (B) Computation of the $K_a/K_s$ ratios in primates (human versus macaque, first column) and in rodents (mouse versus rat, second column). Higher ratios reflect more rapid evolution. The third column is the ratio between $K_a/K_s$ in primates and rodents. A ratio of >1 indicates accelerated evolution of the gene in the Homo Sapiens lineage. MCPH1, CDK5RAP2, CENP3 and ASPM are known MCPH genes. (C) Transcriptome study. Heatmap generated using the GenePattern software, comparing expressions of all genes in the 2.7 Mb linkage interval, as well as CEP152, in control lymphoblasts and in lymphoblasts from patients E3 and S1. Red indicates the highest gene expression; dark blue indicates the lowest expression. Some transcripts are targeted by several probes indicated as a, b, c, d.
used antibodies to detect a truncated or absent CASC5 protein in our patients affected with primary microcephaly. A CASC5 commercial antibody (Bethyl, catA300-805A) was first tested against human CASC5 from cell extracts (HEK293T, HELA and HTB10 human cell lines) and the endogenous protein was detectable at 265 kDa with higher expression in human embryonic kidney (Fig. 4C).

Western blots using whole cell lysates from patient’s lymphoblastoid cell lines were performed. The endogenous protein was present in all subjects, including affected patients (Fig. 4D). In spite of intensive efforts using two commercials and non-commercial antibodies, including an antibody generously given by Kiyomitsu et al. (19), we failed to observe a clear and reproducible alteration in CASC5 protein expression in lymphoblastoid cell lines of affected subjects.

**Cellular analyses**

The CASC5 knockdown in HeLa cells by siRNA causes a misalignment of chromosomes and premature entry into mitosis (19). We measured the growth rate of our patient’s lymphoblast cells to determine whether the mitoses were more...
frequent and/or abnormal, and searched for aneuploidies, which in turn can lead to apoptosis. Cell counting was performed every 48 h during 1 week and we compared lines from patients, unaffected family members and controls. However, the results showed no significant growth rate alteration (data not shown).

We also studied the cellular phenotype of our patients’ lymphoblasts. Knocking down CASC5 has been shown to produce micronuclei, a consequence of the misalignment of chromosomes during metaphase (19). No micronuclei were observed in our patients’ fibroblasts when compared with controls (data not shown).

We used immunofluorescence (IF) to study the intracellular localization of CASC5, ZWINT-1, BUBR1, ASPM, CEP152 and PCTN during different phases of mitosis. Antibodies against these proteins were merged with an antibody recognizing alpha-tubulin, specific to the microtubules of the mitotic spindle (Fig. 5B). No specific defects were observed in fibroblasts of patients when compared with controls. Indeed, all proteins were correctly expressed and the mitotic spindles were normal, in our experimental setting.

**DISCUSSION**

We initially reported a linkage region (MCPH4) on chromosome 15 in a consanguineous family with primary microcephaly (18). Here, we re-analyzed this original family, and two other consanguineous families, using a genome-wide 250K GeneChip® SNP microarray. None of the families showed homozygosity at any known MCPH locus. One and only one region was found where the three families were homozygous over a significant length consistent with autozygosity (>2 cM in each family), a 3.7 Mb interval at 15q14–15.1. The three families originated from three small villages in rural Morocco before they immigrated to our country, consistent with a relatively recent common ancestor. The 3.7 Mb interval contained a 2.7 Mb-long haplotype that was shared with the three families (Fig. 1C), suggesting linkage disequilibrium around a common mutation. In the 2.7 Mb segment, we identified one rare coding mutation in CASC5, also known as KNL1, Blinkin, AF15q14, or D40. The mutation, c.6125 G>A, was absent from 316 unrelated control chromosomes (116 ethnically matched and 200 of various ethnicities), and was not observed among the 9500 alleles compiled by the Exome Variant Server (http://evs.gs.washington.edu/EVS/). The mutation was present in the three families, and segregated with primary microcephaly. Under the assumption of a mutation frequency of 0.003, the combined LOD score in the three families was >6. The genome-wide exclusion of other linkage regions, and the high LOD score, indicate that the MCPH-causing mutation lies within the 2.7 Mb interval.

CEP152 mutations have been reported as a cause of primary microcephaly within the 25 Mb MCPH4 locus that we initially reported (8). We excluded CEP152 in the present families by three lines of evidence. First, no mutation was found by Sanger sequencing of the CEP152 exons and flanking intronic sequences. Second, CEP152 is located 7 Mb outside of the 3.7 Mb linkage region (Fig. 1C). Such a large distance is hardly compatible with the hypothesis of a mutation in the linkage interval acting as a distant regulator of CEP152 gene expression (21), enhancing more distant than 1 Mb being exceptionally reported (22). Third, the CEP152 protein was clearly present and normally expressed at the spindle poles in our patients’ fibroblasts (Fig. 5B).

Sequencing CASC5 in a cohort of patients with sporadic or familial primary microcephaly did not reveal any additional mutation, including three probes from consanguineous

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Table 1. Table showing all coding variants found in our 2.7 Mb MCPH4 locus

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Only one remains after ruling out a 454 FLX sequencing artifact not confirmed by Sanger sequencing (nc) (depth > 20, number of reads >90%).
families with homozygosity at the MCPH4 locus. We did not identify CEP152 mutations in our cohort either, suggesting that both genes might be very rare causes of MCPH. Only four null mutations of CASC5 are reported among 9500 alleles in the Exome Variant Server, suggesting a mutated allele frequency, 1/2000 and a homozygote frequency, 1/4 000 000 in an outbred population.

To further validate the CASC5 mutation as the cause of MCPH in our patients, we sequenced the whole 2.7 Mb common homozygous haplotype by next generation sequencing. We used a customized hybridization array to capture the interval from our patients’ DNA and sequenced the target-enriched DNA using a GS FLX 454 pyrosequencer. The De novo assembly of the sequencing reads showed no junction fragment that would have indicated an intra- or interchromosomal rearrangement. While this analysis produced 208 novel variants, only one unknown rare variant was observed in a protein-coding exon, c.6125 G>A. None of the noncoding variants affected a conserved noncoding element (23), and the critical interval did not contain an ultra-conserved element (24). Although this analysis did not formally rule out a noncoding mutation with a functional effect, it supported the CASC5 c.6125 mutation as the only coding and potentially highly penetrant mutation in our critical interval.

The transcriptome study focused on genes from the critical interval in lymphoblasts from two patients showed low signals for CASC5 and normal signals for CEP152. Although these experiments are sensitive to cell culture conditions and we only had two patients’ cell lines available for the study, these data provide some evidence for CASC5 loss of function and for CEP152 integrity.

Recently Fietz et al. analyzed the transcriptome of the germinial zone of the human fetal neocortex at 13–16 gestational weeks by RNA sequencing (25). They showed that the expression levels of ASPM, CEP135 and CASC5 were significantly upregulated in the human ventricular zone (VZ) compared with the subventricular zones (ISVZ and OSVZ) and cortical plate (CP) (hVZ > hISVZ + hOSVZ + hCP) and that the expression levels of WDR62, CEP152, CEPNJ, ASPM and CASC5 were down regulated in the CP compared with VZ and SVZs (hCP < hVZ + hISVZ + hOSVZ). These human fetal brain expression data identify CASC5 as a gene which is upregulated in the VZ and downregulated in the CP, like ASPM, and are fully consistent with a CASC5 mutation affecting cell proliferation in the VZ, hence causing microcephaly.

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**Figure 4. Functional effect of the mutation.** (A) RT-PCR of random-primed RNA extracted from our patient’s lymphoblastoid cell lines. Amplified cDNAs using primers surrounding CASC5 exon 18 were loaded on 6% acrylamide gel. F: Unaffected father (family E); P: MCPH patient E3; C1-C4: unrelated normal controls; RT ±: positive and negative control of retrotranscription. Arrows indicate primers. A smaller band is observed in the homozygous patient and faintly in heterozygous father. Direct sequencing of the GC band extracted from the gel showed read-through from exon 17 into exon 19. Sequencing of the larger band at the expected size showed inclusion of exon 18 containing the mutation. (B) Ex vivo splicing assay for CASC5 variant. The cells were transfected with vectors containing two exons surrounding our exon of interest. One of the vectors contained the mutated exon 18 (EM), the other contained the wild-type exon 18 (EWT). Expressed mRNAs were detected on agarose gels. (C) Indicated amounts of whole cell lysates from HEK293T, HELA and HTB10 cells were separated on SDS-PAGE. CASC5 was detected by western blot using a commercial anti-CASC5 antibody. (D) Electrophoresis 100 μg of whole cell lysates from our patient's lymphoblastoid cell lines, and western blotting detection of CASC5 using a polyclonal antibody against customized CASC5 peptides (C-term: left; N-term: right). F: Unaffected father (family E); P: MCPH patient E3.
CASC5 is a member of the conserved KMN (KNL1/Mis12 complex/Ndc80 complex) network of proteins which allows the docking of chromosome kinetochores to the microtubule apparatus. Correct docking is essential for adequate segregation of sister chromatids at anaphase, and is required for the spindle checkpoint of the mitotic cycle (19).

**Figure 5.** Mitotic morphology of our patients spindle in mitosis compared with control. (A) The longest isoform of protein CASC5 is made of 2342 amino acids encompassing 27 coding exons. The mutation found in our patients is indicated by the arrow. *Stop* codon in exon 19 induced by exon 18 skipping and subsequent frameshift. The areas of known interactions with partners are shown. (B) Immunofluorescence staining of fibroblasts carrying the M2041I mutation and control fibroblasts with antibodies against six proteins of interest (red, left column), α-tubulin (green, central column) and Hoechst (blue, central column) and the merged images (right column). The CASC5 antibody used in this experiment was the anti-Blinkin mouse monoclonal antibody 31F2. No specific defects observed.
localization to the kinetochore is constant from G2 until late anaphase (26). Aurora Kinase B (AURKB) is a serine–threonine kinase that functions in the attachment of the mitotic spindle to the centromere during cell division for accurate chromosome segregation (27). A conserved motif in CASC5 directly interacts with and targets protein phosphatase 1 (PP1) to the outer kinetochore where it dephosphorylates AURKB substrates and stabilizes microtubule attachment. CASC5 thus opposes AURKB activity (28). Interestingly, CASC5 itself is a substrate for AURKB and phosphorylation of CASC5 by AURKB disrupts the CASC5-PP1 interaction (29). Moreover, the association of PP1 with the Spc7/Spc105/KNL1 family of kinetochore proteins is necessary to stabilize microtubule–kinetochore attachments and silence the SAC and the phospho-dependent recruitment of Bub1 and Bub3 to Spc7/KNL1 by Mph1 kinase maintains the spindle checkpoint (30).

The primate/rodent \( K_\alpha/K_\beta \) analysis of CASC5 shows a ratio of \( >1 \) (ratio = 1.63). This is very similar to other MCPH proteins MCPH1, CDK5RAP2, ASPM and CENPJ, strongly suggesting positive selection of CASC5 in primate evolution leading to \( Homo sapiens \) and providing further evidence for CASC5 as a MCPH gene. Of note, CASC5 is part of the ASPM co-expression network in an exon-level study of the spatio-temporal transcriptome of the human brain (31).

CASC5 was recently identified as a protein presenting a human-specific phosphorylation site at serine 1076 that originated after the human–chimpanzee divergence (32). This might have a role in regulating CASC5 protein interaction networks in human cell division during brain development.

Analysis of the CASC5 transcript in our patients’ lymphoblasts, and of a mutated CASC5 minigene in vitro, clearly showed that the exon 18 mutation causes exon skipping, which in turn produces a frameshift and a premature stop codon in exon 19 (Fig. 4A and B). The c.6125 G>A mutation is located in a portion of CASC5 ranging from amino acid 1981 to 2108 known to specifically interact with ZWINT-1 (33). Downstream from the mutation, the C-terminus of CASC5 is essential for interacting with NSL1 and the MIS12 complex (34). Hence, the skipping of exon 18, frameshift and truncation induced by the mutation are expected to cause severe loss of CASC5 function.

CASC5 binds to BUBR1 (also known as BUB1B) via an N-terminal domain, aa 151–250 (Fig. 5A) (35,36). A 2.2 Å resolution crystal structure of the complex has allowed detailed analysis of the binding interface between CASC5 and BUBR1, defining the stoichiometry and the affinity of the interaction (35). Kiyomitsu et al. described distinct and essential binding domains for BUB1 and BUBR1 at the N-terminus of CASC5 and for ZWINT-1 and hMis14/hNsl1 at the C-terminus of the protein (33). BUB1 loss of function in man causes Mosaic Variegated Aneuploidy (MVA) Syndrome 1 (MIM 257300), an autosomal recessive microcephaly phenotype linked to a mitotic chromosomal segregation defect leading to aneuploidy for various chromosomes in fractions of cells in many tissues (37). It is hence reasonable to speculate that primary microcephaly in both CASC5 and BUBR1 defects results from a common mechanism involving chromosome missegregation.

CASC5 protein was present in lymphoblasts of our patients with a homozygous mutation. Furthermore, in contrast with MVA or other syndromes with microcephaly like the PCNT defect (38), our lymphoblasts morphological studies did not detect aneuploidy, abnormal mitoses or other nuclear anomalies. We speculate that only some tissues, including neural cell progenitors, fully express the defect and would show a truncated, or absent, CASC5 protein. The normal phenotype in the non-neurological cells we studied is consistent with the absence of leukemia or tumors in our patients, which contrasts with cancer-proneness in MVA.

The CASC5 knockdown in HeLa cells by siRNA causes a misalignment of chromosomes and premature entry into mitosis (19). Premature entry into mitosis is also observed in MCPH caused by the microcephalin (MCPHI) defect, where it is thought to cause microcephaly by its critical role in neural progenitors. In the latter cells, coupling of the mitosis and centrosome cycles is required for proper symmetric versus asymmetric division and hence for appropriate proliferation, which in turn will result in an appropriate number of neurons and a normal-sized brain (16). MCPH1 patients’ cells show premature chromosome condensation (39) and abnormal chromosome alignment is observed in a mouse MCPH1 model (16). Our CASC5-mutated patients’ lymphoblasts did not display premature chromosome condensation or nuclear features associated with the CASC5 knockdown, e.g. micronuclei. Our results may reflect normality of the CASC5 function in the cell types we tested, i.e. fibroblasts and lymphoblasts from our patients, and/or functional redundancy in these cells, as opposed to our patients’ neural progenitor cells. Indeed, mRNA and protein analyses in lymphoblasts showed the presence of some normally spliced transcript (Fig. 4A) and the presence of a normal-sized protein. Inducing pluripotent stem cells from our patients’ fibroblasts and deriving them in vitro into the brain cortex (40) may eventually answer this question.

In conclusion, we identified a rare mutation of the kinetochore KMN network gene CASC5 in three families with autosomal recessive primary microcephaly (MCPH). We provide evidence that the mutation disrupts CASC5 function, and show features of accelerated evolution of CASC5 in the Homo sapiens lineage. CASC5 is upregulated in the VZ of the human fetal brain, it is required for the spindle checkpoint of the mitotic cycle and its knockdown causes premature entry into mitosis. Our data provide strong evidence for CASC5 as a novel microcephaly gene, underscoring the role of the kinetochore in proper volumetric development of the human brain.

MATERIALS AND METHODS

Patients and families

Family E has been reported previously (18). Two consanguineous families S and Y from Morocco with three and one affected children, respectively, were later referred to us for primary microcephaly. The three families were unaware of common ancestry but originate from three villages <50 km apart in rural Morocco. All patients have HC –4 to –7 SD relative to the mean for age and gender, and all have
congenital microcephaly except S2, whose HC at birth was reported at fifth centile (Fig. 1A–C). All patients have a normal stature and reached motor milestones within the normal time limits. None had epilepsy or neurological deficits. No additional malformation was reported and medical histories are unremarkable. The oldest patients are now older than 30 years with no history of leukemia or tumors. All have mild-to-moderate mental retardation.

**Homozygosity mapping and Sanger sequencing**
DNAs from index patients from the three families were genotyped using 250K SNP GeneChip® microarrays (Affymetrix™, Inc.) following manufacturer’s instructions. Homozygous stretches were delineated using the HomozygosityMapper software (41).

**Prioritization of candidate genes**

\[
\frac{K_s}{K_u} = \text{standardized ratio of nonsynonymous mutations in the protein, } K_u = \text{standardized ratio of synonymous mutations, occurring in the degenerate positions of codon.}
\]

A ratio \(\frac{K_s}{K_u} \geq 1\) is a sign of positive selection (42). Protein sequences were compared between human and macaque and between mouse and rat. Homozygous mutations, occurring in the degenerate positions of codon.

**Transcriptome study**
Five micrograms of RNA were purified using the RNeasy mini kit (Qiagen) from E3, S1 and one unrelated control. They were hybridized to an Affymetrix kit (Qiagen) from E3, S1 and one unrelated control. They were then purified on RNeasy columns (Qiagen) according to the manufacturer’s instructions. Once all the samples were hybridized to an Affymetrix GeneChip Human Genome U133 Plus 2.0 Array. Experiments and analyses were performed following manufacturer’s instructions.

**Sanger sequencing**
PCR primers for all candidate genes were designed using the Exonprimer software. ‘Touchdown’ PCR products were loaded on agarose gels. The coding regions and exon–intron junctions of the candidate genes were sequenced using the Big Dye Terminator cycle sequencing kit v2 (Applied Biosystems, Foster City, CA, USA), and analyzed on a 3130 Genetic Analyzer sequencing machine (Applied Biosystems). Sequences were inspected in silico for mutations using the SeqScape software V.2.0. (Applied Biosystems).

**Next generation sequencing of the critical region**
A 2.7 Mb genomic segment corresponding to the critical linkage region of the MCPH4 locus was captured from a sonicated sample of 21 µg purified genomic DNA of E1, E2, E3, S1 and S3 on the solid phase using a customized Sequence Capture 385K Human Array, designed and manufactured by Roche NimbleGen. A total of 385 000 unique, non-tiling probes 60–90 nucleotides in length were designed to encompass the whole critical region (chromosome 15: 36,948,439-39,645,979; NCBI build 36, hg18), excluding repetitive DNA. The target region-enriched DNA was then sequenced using a GS-FLX 454 pyrosequencer. Emulsion PCR and pyrosequencing were performed in a PicoTiter Plate as described in the GS-FLX Titanium sequencing manual. Sequencing data were filtered for changes present in <10% of reads or in fewer than two reads. The filtered data were analyzed using the following software: GS De Novo Assembler (which allows for the detection of junction fragments in chromosomal translocations or inversions), GS Reference Mapper (which compares the sequence of interest with the reference sequence and identifies small insertions or deletions, and SNPs) and GS Amplicon Variant Analyzer (which identifies unknown variants and their respective frequencies in large data pool).

**Cell culture**
Lymphoblastoid cell lines were cultured in Roswell Park Memorial Institute medium 1640 (Invitrogen) supplemented with 10% fetal bovine serum (Invitrogen), 100 U/ml penicillin (Invitrogen), 100 µg/ml streptomycin (Invitrogen), 2.5 µg/ml fungizone (Invitrogen) and 2 mM l-glutamine (Invitrogen). Fibroblasts were cultured in Dulbecco’s modified Eagle’s medium (Invitrogen) supplemented with 10% fetal bovine serum (Invitrogen), 100 U/ml penicillin (Invitrogen), 100 µg/ml streptomycin (Invitrogen), 2.5 µg/ml fungizone (Invitrogen) and 2 mM Na pyruvate (Invitrogen). Cells were grown at 37°C in a 5% CO2-humidified atmosphere.

**RNA extraction and RT-PCR**
Lymphoblastoid cell lines were lysed in Tri-reagent (Ambion) and centrifuged for 10 min at 13 000 rpm at 4°C. Two hundred microliters of RNase-free chloroform (VWR Prolabo) were added to the supernatant. Each sample was then centrifuged at 15 000g for 15 min at 4°C. One volume of ice cold 70% ethanol was then added to the supernatant. Each sample was then purified on RNeasy columns (Qiagen) according to the manufacturer’s instructions. Once purified, 2.5 µl of the RNA samples was subjected to reverse transcription using the Super-Script II Reverse Transcriptase kit (Invitrogen) following the manual instructions. Once all the samples were reverse transcribed, 50 ng of template cDNA was engaged in a ‘touchdown’ PCR: initial denaturation at 94°C for 2.5 min, 20 cycles including denaturation at 94°C, hybridization at 62°C–52°C (−0.5°C each cycle) and elongation at 72°C, followed by 20 other cycles at a constant 52°C melting temperature. The primers used for CASC5 cDNA amplification were: 5’-TCCTGACAAAGAGCTGAAGGC-3’ and 5’-CTTAACCTTGTCGACTTTTGA-3’. PCR products were then loaded on 6% tris, borate, EDTA gel (Invitrogen) and 2 mM Na pyruvate (Invitrogen). Cells were grown at 37°C in a 5% CO2-humidified atmosphere.

**Minigene construction and expression**
CASC5 exon 18 was amplified by PCR, with 150 bp of 5’ and 3’ flanking intron sequences, using forward and reverse primers carrying 5’ tails containing appropriate restriction sites, and inserted into the BamHI and MluI restriction sites of the pCAS-1 vector (43). Minigenes carrying the wild-type or the variant allele were then identified by direct sequencing and were transfected separately into HeLa cells in the same
experiment. RNA was extracted from transfected cells and the splicing patterns corresponding to the wild-type and mutant alleles were then compared by RT-PCR analysis, using universal primers in the exons of the pCAS minigene and by sequencing all RT-PCR products.

**Antibodies**

Anti-CASC5 rabbit polyclonal antibody (Bethyl, catA300-805A; Abcam, ab70537), Anti-Blakin mouse monoclonal antibody 31F2 (19), anti-ZWINT-1 rabbit polyclonal antibody (Abcam, ab84367), anti-BUBR1 mouse monoclonal antibody (Abcam, ab54894), anti-ASPM rabbit polyclonal antibody (Bethyl, catIHC-00058), anti-CEP152 rabbit polyclonal antibody (Bethyl, catA302-480A), anti-PTEN rabbit polyclonal antibody (Covance, pRb-432C) and anti-\(\alpha\)-TUBULIN mouse polyclonal (Sigma, T6793) were obtained commercially. Polyclonal Antibodies against costumed peptides of CASC5 protein were produced by Eurogentec (https://secure.eurogentec.com/product/research-double-peptide-a-nti-peptide-polyclonal-packages.html). N-terminal peptide: (AA 1–15) MDGVSSEANEDNDI and C-terminal peptide: (AA 2036-2050) IKIDEMDKILKIDN.

**Immunoblotting**

Lymphoblastoid cell lines were washed twice with ice cold phosphate-buffered saline (PBS) and harvested with lysis buffer [50 mM Tris HCl pH 8, 150 mM NaCl, 1% NP-40, 0.5% sodium deoxycholate 0.1% sodium dodecyl sulfate (SDS), 4 mM sodium orthovanadate, 5 mM Na4P2O7, 1 mM okadaic acid and a mix of protease inhibitors Complete (Roche Diagnostics)] for 60 min at 4°C. The cell lysates were clarified by centrifugation at 15 000 \(\times g\) for 10 min at 4°C and the protein concentration was measured using the Bradford assay. Different quantities of the whole cell lysate were separated by sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE), transferred onto nitrocellulose membranes and immunoblotted using appropriate antibodies. Proteins of interest were then revealed using ECL (enhanced chemiluminescence) kits (PerkinElmer) or using an Odyssey Infrared Imaging System (LI-COR Biosciences).

**Immunofluorescence**

Fibroblasts were seeded on coverslips in 12-well plates at 5 \(\times 10^4\) cells per well in the supplemented Dulbecco’s modified Eagle’s medium as described above. Forty-eight hours after seeding, the cells were washed twice with PBS, fixed with 4% paraformaldehyde (PFA 4%) for 30 min at room temperature or methanol for 10 min at \(-20^\circ\)C and washed again twice with PBS. The cells were then incubated with blocking/permeabilization solution for 30 min, with the primary antibody solution for 2 h, washed three times with PBS and incubated with the secondary antibody for an hour preserved from light. Coverslips were then washed three times with PBS, incubated with Hoechst 33342 (Invitrogen), washed three times with PBS and fixed with Fluorsafe (Calbiochem). Finally, IF was analyzed using a Zeiss Axiosplan 2 Imaging microscope.

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