

What's the hype about CDK5RAP2?

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Abstract Cyclin dependent kinase 5 regulatory subunit-associated protein 2 (CDK5RAP2) has gained attention in the last years following the discovery, in 2005, that recessive mutations cause primary autosomal recessive microcephaly. This disease is seen as an isolated developmental defect of the brain, particularly of the cerebral cortex, and was thus historically also referred to as *microcephalia vera*. Unraveling the pathomechanisms leading to this human disease is fascinating scientists because it can convey insight into basic mechanisms of physiologic brain development (particularly of cortex formation). It also finds itself in the spotlight because of its implication in trends in

mammalian evolution with a massive increase in the size of the cerebral cortex in primates. Here, we provide a timely overview of the current knowledge on the function of CDK5RAP2 and mechanisms that might lead to disease in humans when the function of this protein is disturbed.

Keywords Microcephaly · MCPH · CDK5RAP2 · CDK5 · Centrosome · Spindle · Cell cycle · Mental retardation

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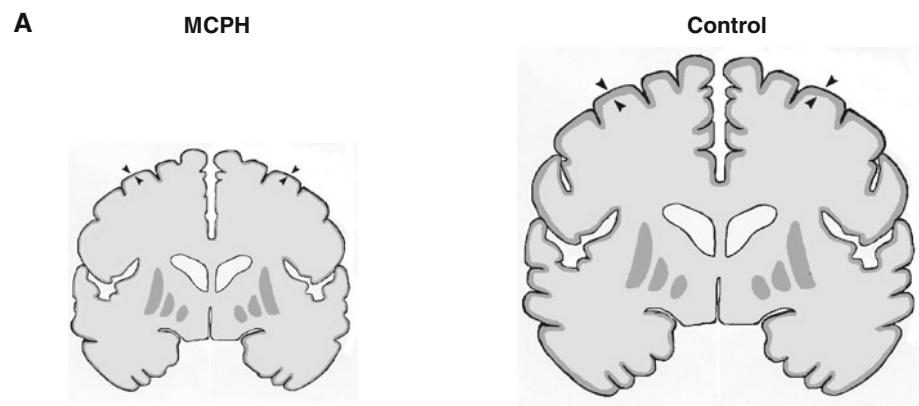
Introduction

Cyclin-dependent kinase 5, regulatory subunit-associated protein 2 (CDK5RAP2), also referred to as centrosome-associated protein 215 (CEP215), CDK5 activator binding protein C48 (C48; a fragment), KIAA1633 and DKFZp686D1070, has moved into the spotlight because of its central function in human brain development and evolution. Mutations in the *CDK5RAP2* gene cause the rare human disease primary autosomal recessive microcephaly (MCPH, for MicroCephalY Primary Hereditary) type 3 (Figs. 1, 2; Table 1). Here, we provide a timely overview of current knowledge on the function of CDK5RAP2 and mechanisms that might lead to disease in humans when the function of this protein is disturbed.

CDK5RAP2 gene and protein structure

Human *CDK5RAP2* was first cloned and designated as KIAA1633 by Nagase et al. [1] through sequencing clones obtained from a size-fractionated fetal brain cDNA library. In the same year, Ching et al. workers obtained a partial, 24 kDa CDK5RAP2 clone, designated as C48 through a

Fig. 1 Main features of patients with MCPH. **a** Illustration depicting typical coronal brain sections from a patient with MCPH and a control. Note the significant reduction in cerebral cortex volume (as shown by arrows) and the simplification of the gyral pattern in the MCPH patient in comparison to a control patient. **b** Main features seen in MCPH patients are listed



B Main features of MCPH

- Microcephaly at birth, further 'relative' reduction of OFC in the first years of life
- Reduction of cerebral cortex volume
- Simplification of gyral pattern
- Mild to severe mental retardation (normal IQ possible)

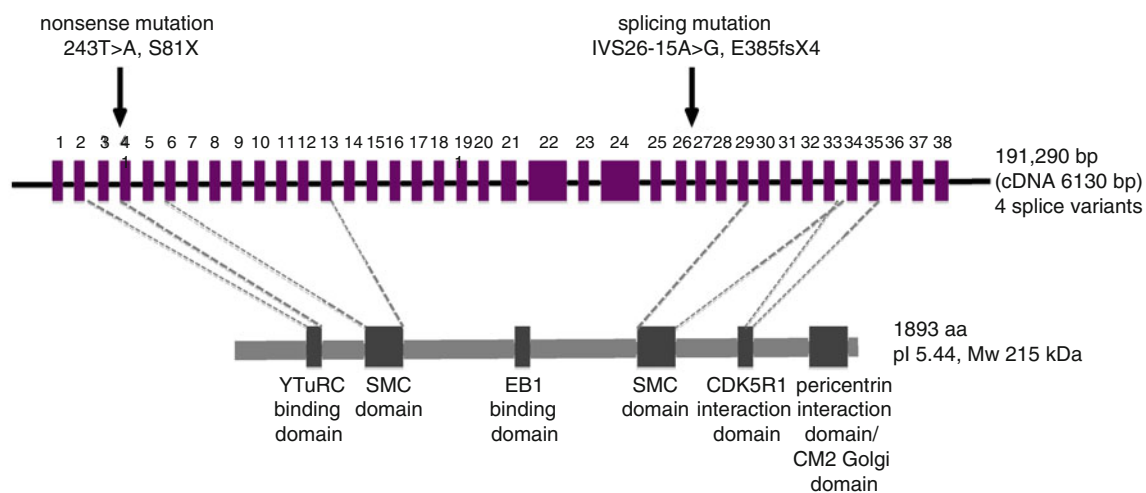


Fig. 2 Position of mutations within the *CDK5RAP2* gene and protein domains. The coding and non-coding regions of the *CDK5RAP2* gene are drawn to scale, and the exons are depicted as boxes (reference sequences according to NCBI genome viewer build 35.1 and Ensembl release 54); the localization and type of published mutations are given as well as the number of times a specific mutation has been reported. Known and predicted MCPH protein domains are presented, and the full-length protein is depicted even though four isoforms have been described (see Fig. 3). The exact positions of the two reported

structural maintenance-of-chromosome (SMC) domains [7] within the protein (SMC AA137–499, SMC_N AA793–1040; accession no. CAI16963) as well as that of the interaction site with CDK5R1 (AA1726–1768) are not clear and can only be predicted. The C-terminal Cnn Motif 2 (CM2) domain is known for Golgi complex interaction and binding to calmodulin. γ TuRC gamma tubulin ring complex, *EB1* plus-end binding protein 1, *CDK5R1* cyclin dependent kinase 5 regulatory kinase 1

yeast two-hybrid screen of two rat brain cDNA libraries using CDK5 regulatory kinase 1 CDK5R1 as bait (CDK5R1; syn. Cdk5 activator binding protein or p23; two CDK5R1 isoforms: p35 and truncated form p25). They isolated the full sequence using the novel cDNA sequence as a probe and demonstrated its interaction with CDK5R1 in an affinity pulldown assay [2]. CDK5RAP2 interacts with

the hydrophilic face of CDK5R1 [3], while the hydrophobic face of CDK5R1 is involved in CDK5 activation [4].

The human full length, 1893 aa CDK5RAP2 protein (NP_060719.4, Q96SN8) contains a predicted N-terminal interaction site with the gamma tubulin ring complex (γ TuRC, 59–133 aa; syn. CM1 [5]), two predicted structural maintenance-of-chromosomes (SMC) sites (137–499 aa,

Table 1 Overview of MCPH genes

MCPH	Protein	Gene	Chromosome	Ref.
MCPH1	Microcephalin	<i>MCPH1</i>	8p23	[28, 29]
MCPH2	WD-repeat containing protein 62	<i>WDR62</i>	19q13.12	[30–32]
MCPH3	Cyclin dependent kinase 5 regulatory subunit-associated protein 2	<i>CDK5RAP2</i>	9q33.3	[17, 27]
MCPH4	Centrosomal protein of 152 kDa	<i>CEP152</i>	15q21.1	[33, 34]
MCPH5	Abnormal spindle-like, microcephaly associated	<i>ASPM</i>	1q31	[35, 36]
MCPH6	Centromeric protein J	<i>CENPJ</i>	13q12.2	[17, 37]
MCPH7	SCL/TAL1 interrupting locus	<i>STIL</i>	1p32	[38]

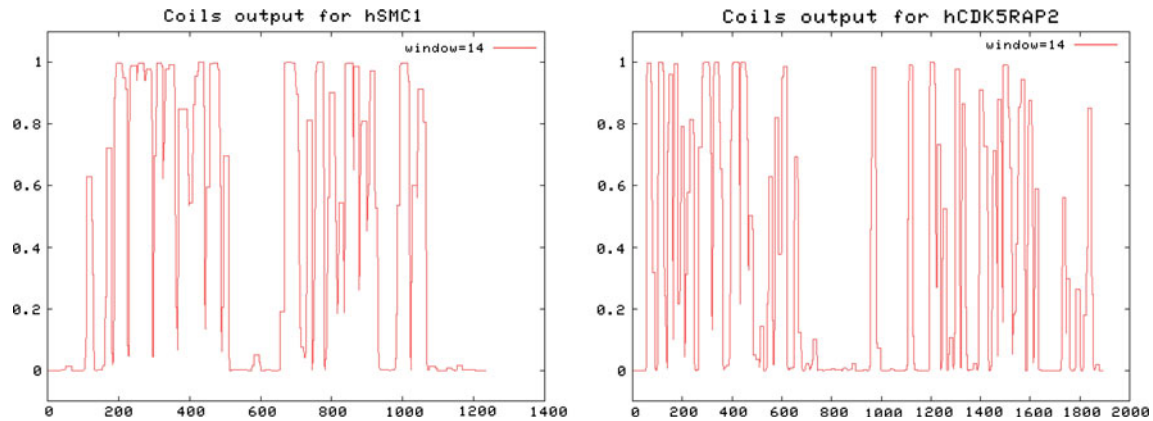


Fig. 3 Predicted coils in human SMC1 and CDK5RAP2. Coiled coil regions were predicted in (a) human SMC1 and (b) human CDK5RAP2 proteins using the corresponding EMBnet program (http://www.ch.embnet.org/software/COILS_form.html) according to Lupas et al. [11]. COILS is a program that compares a sequence to a database of known parallel two-stranded coiled-coils and derives a similarity score. By comparing this score to the distribution of scores in globular and coiled-coil proteins, the program then calculates the probability that the sequence (amino acid position represented in x-axis) will adopt a coiled-coil conformation (probability 0–1

represented in y-axis). A comparison of the predicted conformation of human SMC1 (structural maintenance of chromosomes protein 1A, NP_006297.2) and human CDK5RAP2 (NP_060719.4) reveals structural similarities between the two proteins. Both are composed of two long coiled-coil motifs connected by a rather unstructured domain that in the case of SMC proteins has been predicted to act as a flexible hinge that allows the proteins to gain a V-shaped structure necessary for the C- and N-terminal domains to come in close contact. The settings of the coils program were set to default, using a window width of 14, 21 and 28 amino acids

1395–1653 aa) known to be important for chromatid cohesion and DNA recombination during meiosis and mitosis [6–8], a Ser-rich motif for interaction with plus-end binding protein EB1 (926–1208 aa; [9]), an interaction site with pericentrin (within 1726–1893 aa; [10]), a C-terminal Cnn Motif 2 (CM2) domain for Golgi complex interaction and binding to calmodulin (within 1861–1870 aa; [10]) and a C-terminal interaction site with CDK5R1, an activator or modulator of CDK5 ([2]; Fig. 2).

Structure prediction using the method of Lupas et al. [11] revealed that CDK5RAP2 contains predominantly coiled-coil domains and may form a filamentous structure, which is a characteristic of pericentriolar matrix scaffold proteins [5]. Indeed, alignment of CDK5RAP2 to the NCBI GenBank conserved domains database reveals homologies to SMC proteins (COG1196, TIGR02168) involved in cell division and chromosome partitioning. These weak but significant homologies span approximately sequences aa

150–600 and aa 1300–1650 of CDK5RAP2. A direct alignment of full-length human CDK5RAP2 to human SMC1 (NP_006297) identifies stretches of homology that lie on both sides of the hinge region of SMC1 in the two long coiled-coil motifs connecting the highly conserved nucleotide binding N-terminal Walker A and C-terminal Walker B domains (for review [12]). Both CDK5RAP2 and SMC1 contain long coiled-coil motifs interrupted by a rather unstructured domain (Fig. 3). The latter has been discussed, in the case of SMC, to be a flexible hinge that allows the protein to gain its V-shaped structure necessary for the C- and the N-terminal domains to come in close contact. For this purpose, a hetero- or homodimer is generated using the coiled-coil domains to interact in an anti-parallel fashion. These structural similarities between CDK5RAP2 and SMC1 indicate potentially similar mechanism (dimer formation) for CDK5RAP2.

Four isoforms produced by alternative splicing have been reported so far: (1) isoform 1, Q96SN8-1, the full-length form; (2) isoform 2, Q96SN8-2 missing 702–733 aa (exon 19); (3) isoform 3, Q96SN8-3 missing 1009–1049 (exon 23); (4) isoform 4, Q96SN8-4 missing 1576–1654 (exon 32) (Fig. 4). The *in vivo* existence of these isoforms in humans is confirmed by GenBank EST data. None of the functional motifs identified so far are encoded by the exons alternatively spliced to generate the *CDK5RAP2* isoforms. Thus, the specific functions of the four isoforms remain to be elucidated. Similarly to the situation in humans, comparable isoforms also exist in other organisms such as mice and rats, and have been confirmed by GenBank *in silico* analysis. For mice and rats, the full-length isoform containing all three alternatively spliced exons is not represented in the “non-redundant nucleotide” or “non-redundant protein database” GenBank; however, a search of GenBank sequences shows the presence of the respective homologous exons on the genomic level. Further isoforms generated through co- and/or posttranslational modifications or degradation may exist as authors have described bands of 70, 90, 105 and 230 kDa in mice depending on the age and organ investigated as well as the anti-Cdk5rap2 antibody applied (see below and [13, 14]).

CDK5RAP2 in other organisms

CDK5RAP2 is a highly conserved protein, and ortholog genes with similar domain structures are also found in other organisms such as in apes, cows, dogs, rats, mice and chicken (Fig. 5; Supplementary Figure 1).

Other CDK5RAP proteins

In addition to CDK5RAP2, two further CDK5RAPs exist and are referred to as CDK5RAP1 and CDK5RAP3 or C42 and C53, respectively. While CDK5RAP1–3 compete for a common N-terminal region of (free or CDK5-bound) CDK5R1 (p35), no common CDK5RAP structural motif specific for CDK5R1 (p35) binding has been identified so far [3]. Various studies underline the importance of these CDK5RAPs in the regulation of the cell cycle, DNA damage checkpoints, and thus proliferation and apoptosis (e.g., [15, 16]), and they might thus have an interesting connection to developmental defects of the brain. Similar to CDK5RAP2, the other two CDK5RAPs are expressed in various organs.

Expression and localization of CDK5RAP2

CDK5RAP2 mRNA is widely expressed in human and in embryonal mouse tissue [2, 14, 17]. In murine embryos at

Fig. 4 Alignment of the four human *CDK5RAP2* isoforms. The human *CDK5RAP2* gene comprises 38 exons, and isoform Q96SN8-1 contains all of these 38 exons. Three further isoforms produced by alternative splicing have been reported so far: (1) isoform Q96SN8-2 missing exon 19 (aa 702–733); (2) isoform Q96SN8-3 missing exon 23 (aa 1009–1049); and (3) isoform Q96SN8-4 lacking exon 32 (aa 1576–1654). The *in vivo* existence of these isoforms in humans is confirmed by GenBank EST evidence. None of the functional motifs identified so far (see Fig. 2) are encoded by the alternatively spliced exons of the *CDK5RAP2* isoforms. Similarly to the situation in humans, comparable isoforms also exist in other organisms such as mice and rats, and have been confirmed by GenBank *in silico* analysis. In mice and rats, the isoform 1 is not represented in the GenBank; however, a search of GenBank sequences shows the presence of the respective homologous exon on the genomic level

E15.5, the highest levels were detected in the central nervous system (CNS; [17]). In humans, moderate to high expression was detected in all adult tissues and specific brain regions examined by quantitative RT-PCR ELISA with the highest levels in skeletal muscle, fetal liver, brain, kidney and ovary [1]. Within the brain, expression was particularly high within the thalamus, corpus callosum, substantia nigra, hippocampus and caudate nucleus [1]. In murine whole-brain protein extracts, Cdk5rap2 was detected at high levels between E10.5 and E13.5, remained robust at E15.5 and subsequently decreased with development [14]. At the same time, the authors detected a smaller band of about 90 kDa through Western blotting that first appeared at E13.5 and then increased in intensity, paralleling the decrease of the 200 kDa band. Similarly, Lizarraga et al. [13] detected a large, 230-kDa isoform in most tissues in addition to a smaller, approximately 105-kDa isoform in adult testis, thymus and brain as well as a 70-kDa testis-specific isoform through Western blots using adult tissues. In these organs, a 2.3-kb transcript was found to be expressed in most tissues, a 5.8-kb transcript in several tissues (particularly in testis and thymus) and a 1.4-kb transcript only in the testis by Northern blots. This indicates that there may be a change of gene expression and/or protein isoform synthesis throughout murine brain development and when comparing various organs. At E14.5, punctuate, centrosomal Cdk5rap2 signal was detected in young, Tuj1-positive neurons (Tuj1 = neuron-specific class III beta-tubulin) within the cortex. The most intense centrosomal signal was present at the luminal surface of the ventricular zone in neural stem cell marker nestin-positive progenitors.

CDK5RAP2 was localized to the centrosomes in the interphase and also to the spindle poles during mitosis in the human epithelial cervical cancer cell line HeLa [5, 17, 18]. Recently, the subcellular localization of Cdk5rap2 during the cell cycle was described in detail via immunocytochemistry in murine fibroblasts (antibody directed against the N-terminus, [19]). The authors found that Cdk5rap2 levels were relatively low at interphase centrosomes in a

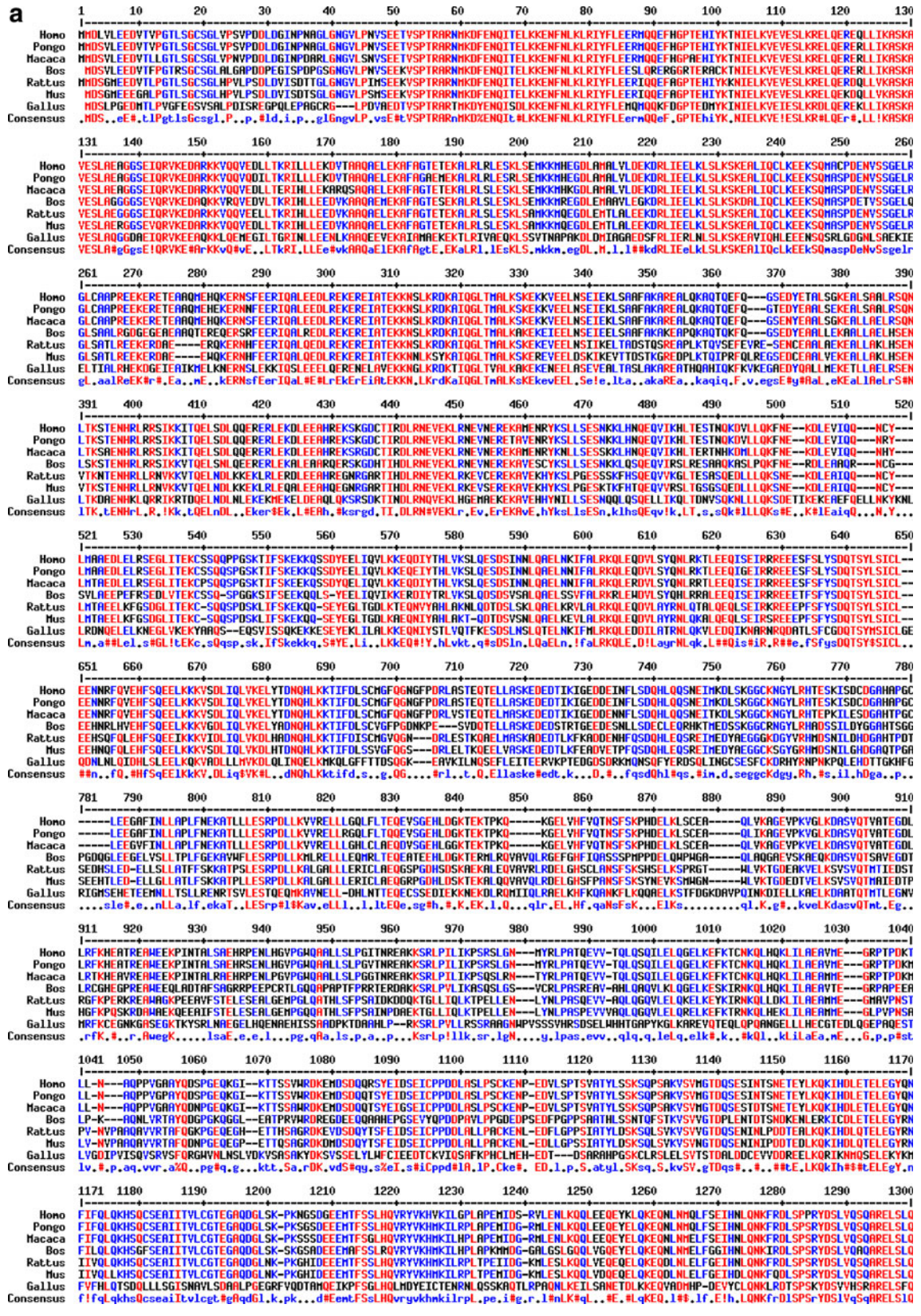


Fig. 5 CDK5RAP2 sequence in various organisms. Sequences of ortholog gene products are compared to that of human CDK5RAP2: orangutan (*Pongo abelii*), rhesus monkey (*Macaca mulatta*), cow (*Bos*

taurus), dog (*Canis lupus familiaris*), rat (*Rattus norvegicus*), mouse (*Mus musculus*) and chicken (*Gallus gallus*). Detailed information on sequences used for alignments can be found in the supplementary information box 1

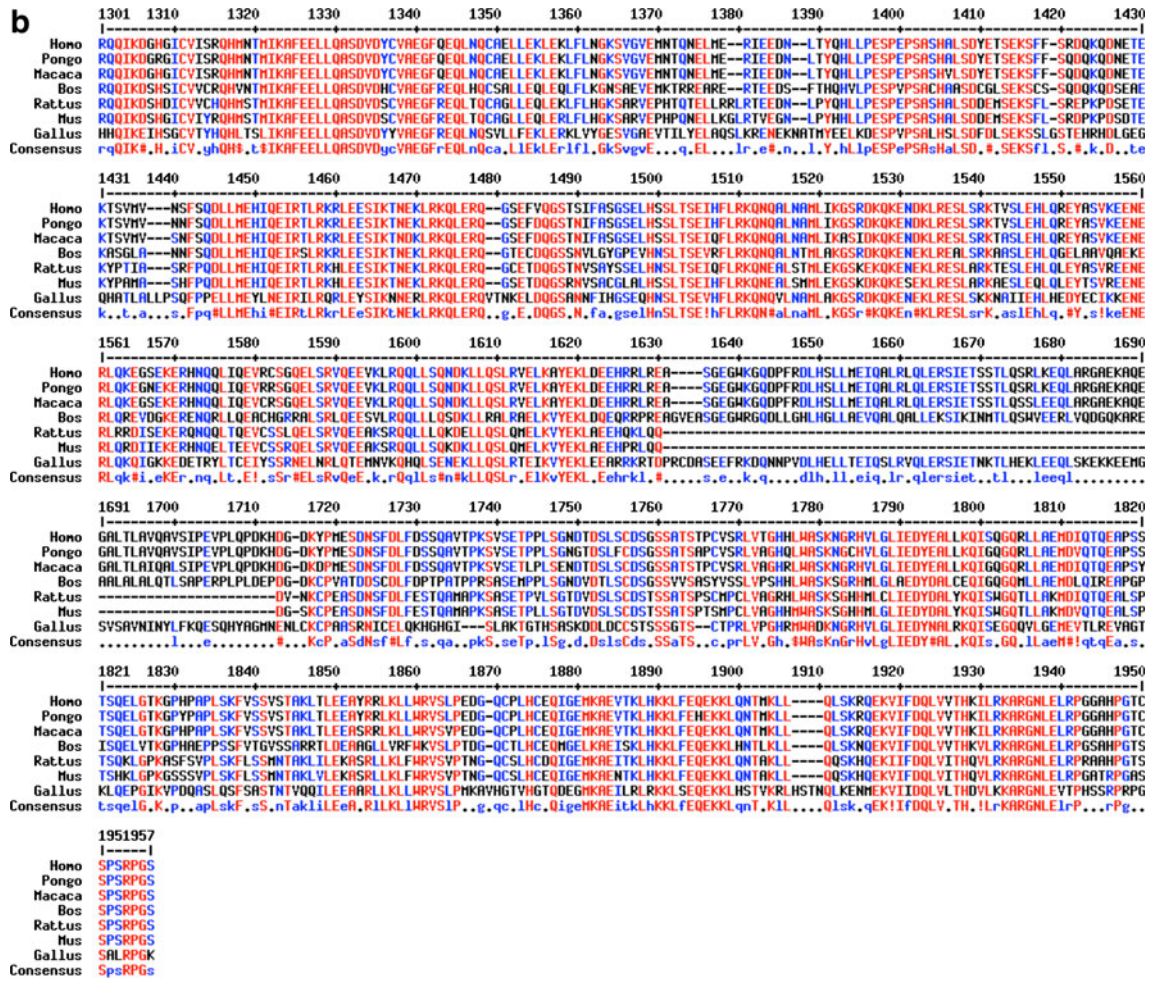


Fig. 5 continued

microtubule-independent way. The centrosomal Cdk5rap2 levels increased in the prophase and remained high throughout mitosis until the telophase, when the signal dropped again to interphase levels. These results underline that Cdk5rap2 is a centrosomal protein, and they illustrate that centrosomal Cdk5rap2 is regulated in a cell cycle-dependent manner. In addition to its centrosomal localization, CDK5RAP2 has been described to localize to the distal, growing tips of microtubules in human tumor cells (embryonic kidney cell line HEK293; HeLa and human osteosarcoma cell line U2OS) but not in rodent cells [9]. In these human tumor cells, CDK5RAP2 has been shown to regulate the plus-end dynamics via interaction with plus-end binding protein EB1, a prototypic member of the microtubule plus-end tracking proteins [9]. Only recently, the localization of CDK5RAP2 to the Golgi complex lasting throughout the interphase until mitosis has been demonstrated through colocalization with *trans*-Golgi marker mannosidase II in HeLa,

MCF7 and RPE1 cells (MCF7 = breast cancer cell line; RPE1 = human telomerase-immortalized retinal epithelial cell line [10]; Fig. 6). It has been further shown that the Golgi-localization of CDK5RAP2 is independent of microtubules, but dependent on centrosome function and energy supply [10]. It will be intriguing to identify further interaction partners of CDK5RAP2.

CDK5RAP2 as a regulator of CDK5R1 and CDK5

CDK5RAP2 has been suggested to inhibit or regulate CDK5 indirectly through *in vitro* studies. Ching et al. [2] demonstrated that high concentrations of C48 (resembling the C-terminal fragment of Cdk5rap2) were able to inhibit Cdk5 kinase activity up to about 20%, and their data also indicate that Cdk5rap2 can be phosphorylated by Cdk5. Apart from controlling Cdk5 kinase activity, a regulation of CDK5R1 isoform activity may also be important for the intracellular localization and substrate specificity of Cdk5

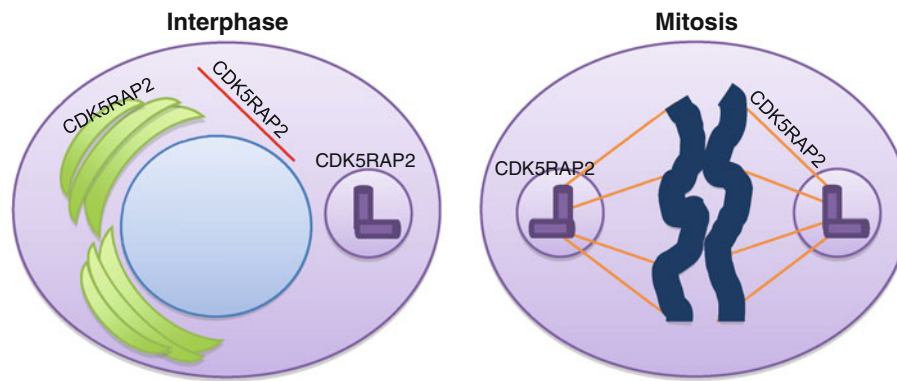


Fig. 6 Localization of CDK5RAP2 protein during the interphase and in mitosis. CDK5RAP2 protein is found at the centrosome and the Golgi complex during the interphase, and is localized at the centrosome in the mitotic cell (shown in tumor cells). Also, this protein has been localized to the plus end of microtubules. During the interphase cells are growing and duplicate their DNA (blue) in order

[20–26]. Still, the mechanism of the CDK5RAP2 inhibitory effect has not been completely elucidated, and convincing data illustrating especially an *in vivo* interaction with CDK5/p35 in the context of human brain development are lacking. While CDK5RAPs show a widespread tissue distribution, their target CDK5R1 is expressed mainly in the CNS. This indicates that CDK5R1-binding may not represent the primary function of these proteins in non-CNS tissues and might thus not explain the phenotype seen in patients with *CDK5RAP2* gene mutations.

Human disease caused by *CDK5RAP2* gene mutations

Homozygous mutations of the *CDK5RAP2* gene have been identified as a cause for MCPH3 in three families (MIM*608201): (1) a nonsense mutation in exon 4 (243T>A, S81X) and (2) an A to G transition in intron 26 (IVS26-15A>G, E385fsX4) introducing a new splice acceptor site, a frame shift and a premature stop codon [17, 27]. Both mutations are assumed to lead to non-functional, truncated protein products [5], but so far no study has clearly shown this. Other MCPH subtypes can be caused by homozygous mutations in the genes encoding microcephalin (MCPH1 [28, 29]), WD-repeat containing protein 62 WDR62 (MCPH2 [30–32]), centrosomal protein of 152 kDa CEP152 (MCPH4 [33, 34]), abnormal spindle-like, microcephaly associated ASPM (MCPH5 [35, 36]), centromeric protein J CENPJ (MCPH6 [17, 37]) and SCL/TAL1-interrupting locus STIL (MCPH7 [38]). Moreover, further genetic heterogeneity likely exists as in our experience about 20% of patients/families with a MCPH phenotype do not show linkage to any of the known loci (Table 1).

Patients with MCPH present typically at birth with microcephaly, a small cranium with significantly reduced

to later divide during mitosis through the mitotic spindle apparatus (orange). Growth and division of cells needs a constant supply of new proteins and lipids to the site of synthesis in the endoplasmic reticulum via the Golgi apparatus (green). During mitosis, the Golgi apparatus is fragmented into many small vesicles that are subsequently divided upon the later daughter cells



occipito-frontal head circumference (OFC), because of reduced brain volume (Fig. 1). Individual patients can have an OFC in the lower normal range at birth followed by the development of a ‘secondary microcephaly’ within the first year of life [39]. Many patients display mental retardation, and some have further neurological findings including speech delay, hyperactivity and attention deficit, aggressiveness, delay of developmental milestones, focal or generalized seizures, and pyramidal signs on clinical exam [39]. Moreover, abnormal height and weight [39, 40], and congenital hearing loss have been detected in some patients. We refer to Kaindl et al. [41] for further details.

Imaging studies reveal typically brains of ‘normal architecture’ but of reduced cerebral cortex (and white matter) volume [40]. The cerebral cortex structure has been described as simplified, and individual patients with MCPH provide evidence of periventricular neuronal heterotopias, infra-tentorial anomalies such as brainstem or cerebellar hypoplasia, dysmorphic and/or enlarged lateral ventricles, corpus callosum agenesis, and focal micropolygyria and/or dysplasia [39, 40]. Histological analysis, performed at a time when a genetic diagnosis was not possible, revealed a significantly reduced brain volume with an almost preserved convolution pattern [42, 43]. The manifestation of MCPH already at fetal age, its assumed non-progressiveness and the specific effect on the cerebral cortex have supported the hypothesis that the disease is caused by a deficiency in proliferation of cortical neural progenitor cells.

Mouse phenotype caused by mutation in *CDK5RAP2* homologous gene *Cdk5rap2* or shRNAi *in vivo*

The Hertwig’s anemia (an) mouse was identified recently as a model of MCPH3 by positional cloning [13] (Table 2).

Table 2 MCPH3 animal models overview

Animal	Gene	Animal model phenotype
 drosophila	<i>cnn</i> ^{-/-}	Viable flies Brains reportedly of normal size Centrosome dysfunction: Centrosome ‘rocketing’ within the cells due to inability to maintain connection with pericentriolar matrix Lack of centrosome cohesion Errors in centriole segregation Inability to dock γ TuRC to the centrosome Inability to maintain connection with astral microtubules Mitotic defects in syncytial embryos Only subtle defects of asymmetric cell divisions
 mouse	<i>Cdk5rap2</i> ^{-/-}	Premature death (rarely survive > P7) Males infertile, females cannot deliver pups Reduced brain and body size Thin cortex (esp. superficial layers), preserved layering Reduced size of ganglionic eminence, hippocampus, olfactory bulbs Reduced basal progenitors and neurogenesis Increase of M-phase cells, delayed mitotic progression Defect mitotic spindle orientation and abnormal mitotic figures Possible reduced symmetrical and increased asymmetric cell division Increase of premature cell cycle exit Increase of precursor and neuronal cell death Hematopoietic phenotype: Macrocytic, hypoproliferative anemia Leucopenia Predisposition to hematopoietic tumors

cnn Centrosomin

These mice have an inversion of exon 4 of the *Cdk5rap2* gene resulting in exon 4 skipping and have been well known for years for their hematopoietic phenotype of macrocytic, hypoproliferative anemia and leucopenia with an increase of severity in more mature hematopoietic progenitors as well as for their predisposition to hematopoietic tumors in homozygous mutants. Inbred *Cdk5rap2*^{an/an} B6.Cg mice rarely survive beyond 1 week of age, have reduced brain and body sizes already at E12.5 and defects in multiple organs including the brain, thymus and testis. While male *Cdk5rap2*^{an/an} B6.Cg mice are infertile because of a severe germ cell deficiency; females have less severe defects in their oocytes, but cannot deliver pups. In these mice, a high level of aneuploidy (abnormal number of chromosomes) in primary cell cultures of several organs derived from mutant animals has been reported [13].

In these mutant mice, Lizzaraga et al. detected small brains that display a thinning of the cerebral cortex, especially of the superficial, later-born Brn1/Cux1-positive neuronal layers (Brn1 = brain-specific homeobox/POU domain protein 1; Cux1 = homeobox protein cut-like 1),

and a reduced size of the ganglionic eminence, hippocampus and olfactory bulbs. The cortex layering was preserved, but there was a significant reduction of neurogenesis, a reduction in the number of Tbr2-immunopositive basal progenitors (Tbr2 = T-box brain protein 2), an increase of M-phase cells coupled with a delay in mitotic progression and defective mitotic spindle orientation and abnormal mitotic figures (aneupolar spindles) in cortical progenitors. At the ventricular surface, dividing cells in *Cdk5rap2*^{an/an} B6.Cg mice were found to have an increase of horizontal and a reduction of perpendicular cell division planes (thought to produce side-by-side daughter cells) compared to controls at E14.5 [13]. This finding was assumed to indicate a relative increase of asymmetric cell division [13].

Similarly, knockdown of *Cdk5rap2* through shRNAi plasmids introduced via in utero electroporation of mouse embryos led to (1) an altered cell distribution in the developing neocortex with significantly fewer cells remaining in the ventricular/subventricular zone and more entering the intermediate zone or cortical plate; (2)

significantly more postmitotic electroporated cells when compared to control cells; (3) a higher number of electroporated cells expressing layer VI marker *Foxp2* and (4) a trend towards fewer electroporated GFP-positive cells in the upper layers (layers II/III and IV; no layer-specific markers applied); (5) increased neuronal differentiation; (6) a significant decrease in cell proliferation; (7) an increase of neurogenesis; (8) an altered makeup of the progenitor pool with a decrease of Pax6-positive apical and an increase of Tbr2-positive basal progenitors (this is in contrast to the finding in KO mice, and the authors discuss a possible transient effect that might not be associated with an enduring phenotype); (9) an alteration of cell cycle parameters in the mouse neuroblastoma cell line Neuro-2A with a shift from S to G0/1 phase, a reduction of proliferation, an increase of cell-cycle length and an increase of cell-cycle exit, together leading to a depletion of the progenitor pool [14].

In comparison to the knockout mice mentioned above, Barrera et al. [19] recently published the phenotype of *Cdk5rap2* mutant mice that were viable, had no obvious behavioral deficits, and had normal brain size and morphology as well as normal body size and weight. These mice were derived from two Bay Genomics embryonic stem cell clones RRU0301 and RRF465, harboring transposon splice-trap insertions within introns 3 and 12, respectively, and resulting in translation of the first 64 and 435 amino acids of *Cdk5rap2*, respectively [19]. While males showed variable fertility (1/3 infertility), female mice were fertile. The difference in phenotype to the *Cdk5rap2^{an/an}* B6.Cg mice remains to be elucidated, but might be caused by a leaky mutation and/or a difference in genetic backgrounds of the mice.

Drosophila phenotype caused by loss of *CDK5RAP2* homologous gene *Centrosomin Cnn*

In *Drosophila*, a loss of the *CDK5RAP2* homologous gene *centrosomin cnn* causes centrosome malfunction in centrosomin mutant *Drosophila* embryos (*cnn^{-/-}*, [44, 45]; Table 2). In *cnn^{-/-}* flies, Lucas and Raff reported a failure of centrioles not to establish, but to maintain a stable connection to the pericentriolar matrix PCM (i.e., an attachment to the nuclear envelope in interphase and to the spindle poles in mitosis [44]). They further showed that this leads to microtubule-dependent rocketing of centrioles within the cells [44]. It has been suggested that the primary function of *cnn* may be to mechanically strengthen the PCM and that a weakened PCM in the absence of *cnn* initiates such centriole rocketing. Moreover, in *cnn^{-/-}* flies, centrosomes were able to organize astral microtubule arrays, but were unable to maintain a connection with

them. Thus, when the embryos entered mitosis, many nuclei were not associated with centrioles, and astral spindles assembled around the mitotic chromatin. Further, it has been demonstrated that *cnn* is required for centrosome cohesion (centrosome duplicates during the cell cycle but functions as a single microtubule-organizing center until shortly before mitosis [46]) and for docking of the γ TuRC to the centrosome via its N-terminal domain (but not for γ TuRC assembly) [44, 47]. While loss of *cnn* function results in ‘catastrophic’ failures in mitosis in syncytial embryos, somatic cells that lack *cnn* have few mitotic defects but have errors in centriole segregation [48]. Amazingly, only subtle defects of asymmetric divisions occurred in *cnn* mutant neuroblasts, there was no reduction of brain size, and *cnn* mutant flies were viable [44]. Flies with mutant *cnn* genes are able to compensate for the defect in a way that perhaps humans with homozygous *CDK5RAP2* mutations cannot. Recent studies have illustrated that mitotic spindle can form even in the absence of centrosomes and that individual cells can divide without this organelle [49]. However, it has been hypothesized that the ability of centrioles to recruit PCM has evolved to ensure equal partitioning of the centrioles during cell division rather than the efficient assembly of the mitotic spindle [50].

Further experimental data regarding the functions of *CDK5RAP2*

From the human phenotype and animal models it has become clear that *CDK5RAP2* has an influence on brain size regulation during fetal development. It is involved in cell cycle control and plays a role in regulating the shift in symmetric to asymmetric proliferation of progenitors and the proliferative potential of neuroblasts during neurogenesis. Moreover, *CDK5RAP2* may also be involved in brain enlargement during evolution. Several studies have focused on the functions of *CDK5RAP2* on a molecular level, and in the following we describe these functions in greater detail.

Centrosomal attachment of the γ -tubulin ring complex

CDK5RAP2 is a pericentriolar structural component that functions in gamma tubulin ring complex (γ TuRC) attachment and therefore in the microtubule organizing function of the centrosome [5]. Centrosomes consist of a pair of centrioles surrounded by amorphous pericentriolar material (PCM) including proteins such as gamma tubulin, the γ TuRC and pericentrin, and provide a docking site for the minus end of microtubules [51, 52]. *CDK5RAP2* is localized throughout the PCM in all stages of the cell cycle

in somatic cells, as demonstrated by Fong et al. [5]. As mentioned above, Cdk5rap2 levels at the centrosome have been found to be regulated in a cell cycle-dependent manner in murine fibroblasts (low levels in interphase, high levels in prophase until telophase; [19]). CDK5RAP2 is associated with the γ TuRC by a short conserved sequence present in several related proteins found in a broad range of organisms (Fig. 7). The binding of CDK5RAP2 is required for γ TuRC attachment to the centrosome but not for γ TuRC assembly [5]. The centrosome is the primary microtubule organizing center (MTOC), which plays a key role in the control of the temporal and spatial distribution of microtubule networks [46, 53, 54]. Hence, through its interaction with the γ TuRC, CDK5RAP2 plays a role in the microtubule organizing function of the centrosome. A disrupted CDK5RAP2 function inhibits centrosomal microtubule nucleation, which leads to disorganization of interphase microtubule arrays and formation of anastral mitotic spindles. It has been shown for other gamma tubulin binding proteins that a failure of γ tubulin to localize to the centrosome is associated with (1) a G2–M arrest of some cells followed by apoptosis or (2) highly defective mitoses with increased monopolar spindles for those cells bypassing the G2-M arrest [55]. It has been recently demonstrated that the γ tubulin depletion (along with the depletion of multiple other centrosomal constituents) results in intra-G1 arrest and cellular senescence via a p53-dependent mechanism [56].

Dynein-dependent accumulation of further pericentriolar matrix proteins for spindle pole formation

Cdk5rap2 has been implicated in dynein-dependent transport regulation (Fig. 8; [51]). Eukaryotic cells necessitate active, directional transport for the efficient and accurate localization of cellular components and accomplish this with the help of motor proteins such as dyneins, kinesins and myosins that ‘walk’ along cytoskeletal tracks. Dyneins

move along microtubules towards the microtubule minus ends, which in most cells are collected into the microtubule-organizing center (MTOC) near the nucleus. Dynactin is an essential dynein adaptor that targets dynein to specific cellular locations and activates dynein. A knockdown of *Cdk5rap2* by siRNA has been shown to lead to an increase of cell proliferation of HeLa cells (increase of phospho H3 levels) accompanied by abnormal spindle formations such as unfocused spindle poles and centrosomes that were detached from the spindle poles [51]. Such a depletion of Cdk5rap2 further led to a reduction of dynein at the centrosome, but did not affect its total protein levels or its levels at microtubule [51]. On the other hand, the centrosomal localization of Cdk5rap2 was also shown to depend on the function of the dynein-dynactin complex. In view of these findings, Lee and Rhee thus hypothesized that Cdk5rap2 regulates a dynein-dependent transport of the pericentriolar matrix proteins during centrosome maturation.

Spindle checkpoint function

CDK5RAP2 is essential for spindle formation [17] and proper chromosome segregation during mitosis, and has been suggested to be required for the spindle checkpoint-signaling pathway [57]. The mitotic spindle checkpoint prevents chromosome missegregation by delaying sister chromatid separation and thus entry of a mitotic cell into anaphase until all chromosomes are bipolarly attached via their kinetochores to spindle microtubules [58, 59]. This pattern of attachment ensures that each daughter cell receives one copy of each chromosome. Inhibition of *Cdk5rap2* via siRNA led to spindle checkpoint failure with chromosome missegregation, anastral mitotic spindles and reduced expression of the spindle checkpoint proteins BUBR1 (budding uninhibited by benzimidazoles 1 homolog beta) and MAD2 (mitotic arrest-deficient 2) via interaction with their promoters in HeLa cells (Fig. 9; [57]).

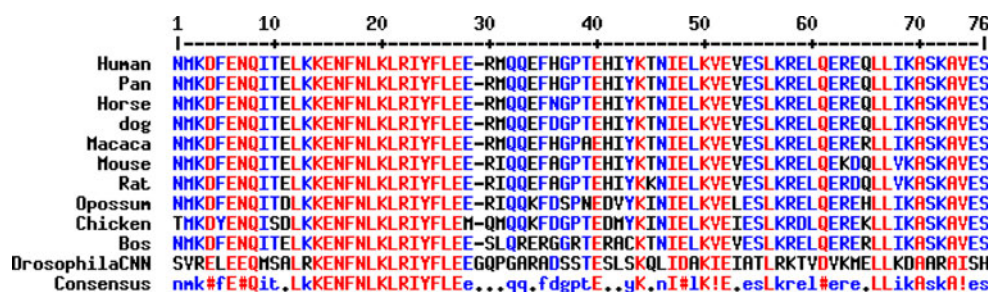
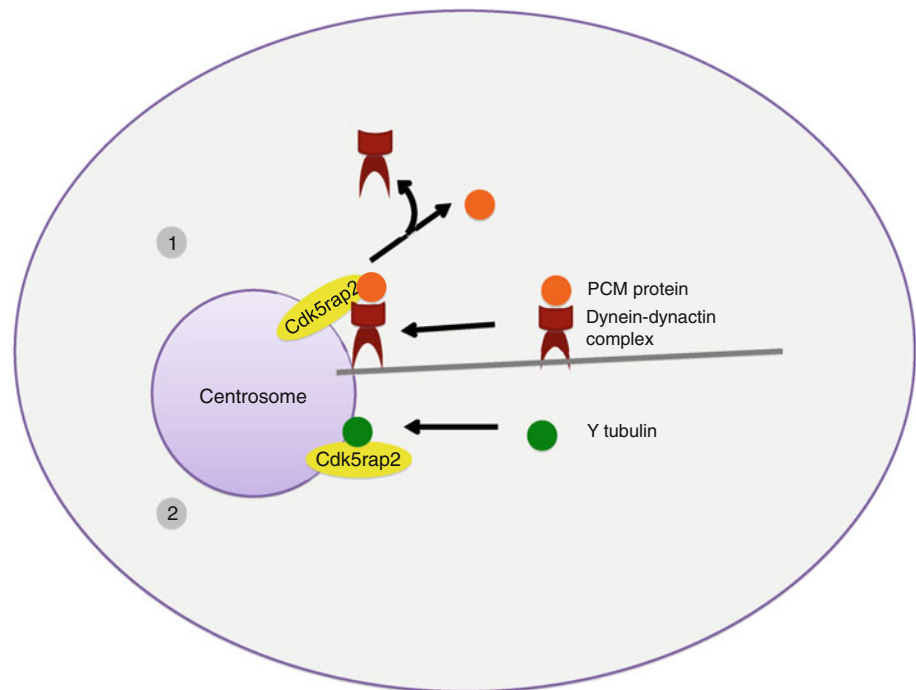


Fig. 7 Alignment of the gamma tubulin ring complex (γ TuRC) site sequence in various organisms. Sequences of the γ TuRC in ortholog gene products of various organisms are compared to that of human CDK5RAP2: chimpanzee (*Pan troglodytes*), horse (*Equus caballus*), dog (*Canis lupus familiaris*), rhesus monkey (*Macaca mulatta*),

mouse (*Mus musculus*), rat (*Rattus norvegicus*), opossum (*Monodelphis domestica*), chicken (*Gallus gallus*), cow (*Bos taurus*), fruit fly (*Drosophila melanogaster*). Detailed information on sequences used for alignments can be found in the supplementary info box 1

Fig. 8 Cdk5rap2 and dynein-dependent transport regulation: a hypothesis. Dynein and its activating adaptor dynactin move along microtubules towards the microtubule minus ends. It has been suggested that (1) Cdk5rap2 may be transported to the centrosome by the dynein-dynactin complex and that, on the other hand, Cdk5rap2 may regulate the dynein-dependent transport of other pericentriolar matrix (PCM) proteins during centrosome maturation. (2) In addition, Cdk5rap2 can attract PCM proteins such as gamma tubulin in a dynein-dynactin-independent fashion



CDK5RAP2 has been reported to reside on the *BUBR1* and *MAD2* promoters and to function as a positive transcriptional regulator [57]. Moreover, loss of CDK5RAP2 function was associated with an increase in chromatin-associated CDC20 [57]. Chromosome segregation is initiated by activation of the spindle checkpoint target APC (anaphase-promoting complex), which functions as an E3 ligase when bound to its activator CDC20 and drives cells from metaphase into anaphase by inducing degradation of securin and mitotic cyclins [57, 59, 60]. The spindle checkpoint ensures that the CDC20-dependent activation of APC does not occur until all chromosomes have achieved bipolar kinetochore-microtubule attachment [57]. The spindle checkpoint proteins BUBR1 and MAD2 are part of an inhibitory complex for APC by sequestering CDC20 through directly binding and thus inhibiting APC activity [57]. Zhang et al. suggested a model in which downregulation of CDK5RAP2 reduces its binding to CDC20 and inhibits transcription of BUBR1 and MAD2, consequently decreases BUBR1 and MAD2 protein levels, and releases BUBR1- and MAD2-sequestered CDC20. Thereby, more free CDC20 would be available to bind chromatin, to activate the APC and thus to allow the transition from metaphase to anaphase in the presence of unattached kinetochores and/or kinetochores that lack tension in CDK5RAP2-inhibited cells. Additionally, cytosolic CDK5RAP2 sequesters CDC20 from chromatin. These intriguing results regarding the role of Cdk5rap2 remain to be repeated independently and to be complemented by further detailed studies.

Microtubule dynamics

CDK5RAP2 localizes to microtubules and concentrates at their distal tips in addition to its centrosomal localization in human tumor cell lines HeLa, HEK293T and U2OS, but not in rodent cells [9]. CDK5RAP2 interacts with EB1, a prototypic member of microtubule plus-end tracking proteins (+TIPs), and contains the basic and Ser-rich motif responsible for EB1 binding [61, 62]. The CDK5RAP2-EB1 complex regulates microtubule dynamics and stability. Fong et al. [9] also showed that the CDK5RAP2-EB1 complex induces microtubule bundling and acetylation when expressed in cell cultures, and stimulates microtubule polymerization, assembly and bundle formation, but does not induce nucleation in vitro. Therefore, CDK5RAP2 may participate in microtubule reorganization through the selective stabilization of microtubules and thus play a role in microtubule-dependent processes such as cell polarization and directional movement.

The EB-1 binding motif, spanning aa 926–956 in the human sequence, is well conserved in higher mammals such as apes, cattle and dogs. However, it shows increasing divergence in lower mammals such as mice and rats as well as in non-mammals such as chickens (Fig. 10). Fong et al. [9] recently suggested a gain of function of the EB1 site during mammalian evolution. This hypothesis was based on their finding that mouse Cdk5rap2 does not show EB1-binding and plus-end tracking properties in a respective assay and the fact that two residues including the proline of a critical Ile/Leu-Pro motif (position 14 in Fig. 10) are

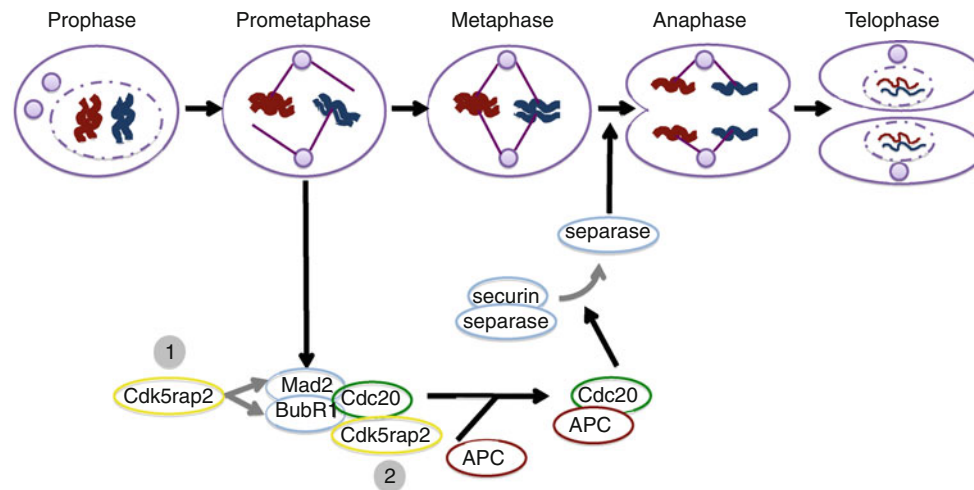


Fig. 9 Cdk5rap2 and spindle checkpoint function: a hypothesis. The mitotic spindle checkpoint prevents chromosome missegregation by delaying sister chromatid separation and thus entry of a mitotic cell into anaphase until all chromosomes are bipolarly attached via their kinetochores to spindle microtubules. Chromosome segregation is initiated by spindle checkpoint target APC, which is activated by binding to its activator CDC20 and drives cells from metaphase into anaphase by inducing degradation of securin and mitotic cyclins. The spindle checkpoint proteins BUBR1 and MAD2 are part of an

inhibitory complex for APC by sequestering CDC20 through directly binding and thus inhibiting APC activity. It has been suggested that Cdk5rap2 downregulation reduces its binding to CDC20 and inhibits transcription of BUBR1 and MAD2, consequently decreases BUBR1 and MAD2 protein levels, and releases BUBR1- and MAD2-sequestered CDC20. Thereby, more free CDC20 would be available to bind chromatin, to activate the APC and thus to allow transition from metaphase to anaphase in the presence of unattached kinetochores and/or kinetochores that lack tension in CDK5RAP2-inhibited cells

substituted in the rat and mouse sequences. Moreover, they showed that a mutation of these two residues to Ala in the human sequence resulted in a loss of activity in an EB1-binding assay. However, as shown in Fig. 10, the chicken sequence has a conserved Ile/Leu-Pro motif. This indicates that other residues may also need to be conserved for EB1 binding if one assumes that the chicken motif is not capable of binding EB1. This will need to be analyzed through further experiments. The basic and Ser-rich motif was not found in the sequences of Cnn, Mto1p and Pcp1p, implying that the tip-tracking function may not be conserved in *Drosophila* and *S. pombe*.

Centriole engagement and cohesion

Only recently it has been demonstrated by Barrera et al. [19] that Cdk5rap2 is required for the maintenance of centriole engagement and cohesion in mice. Centrosomes contain two structurally distinct centrioles, a mature ‘mother’ centriole and a ‘daughter’ centriole, that are amplified in a tightly regulated centriole duplication cycle to ensure that it occurs only once per cell cycle (brief overview in [19]): (1) G1 phase: mother centriole initiates formation of the primary cilium; (2) S phase: each centriole templates the assembly of a single daughter centriole, which grows from its proximal base and remains tightly bound, or “engaged,” until disengagement occurs at mitosis; (3) G2 phase: engaged centriole pairs remain tethered by cohesion. Centriole cohesion refers to the

	1	10	20	3032																									
Human	P	G	I	T	N	R	E	A	K	S	R	L	P	I	L	I	K	P	S	R	S	L	G	N	M	Y	R	L	P
Pan	P	G	Y	T	N	R	E	A	K	S	R	L	P	I	L	I	K	P	S	R	S	L	G	N	M	Y	R	L	P
Orang	P	G	Y	T	N	R	E	A	K	S	R	L	P	I	L	I	K	P	S	R	S	L	G	N	M	Y	R	L	P
Horse	P	S	G	T	H	K	D	A	K	S	R	L	P	V	L	L	R	P	S	R	S	L	G	S	L	Y	R	L	P
Dog	P	S	G	A	D	K	A	K	S	R	L	P	I	L	I	K	P	S	R	S	L	G	N	Y	R	L	P		
Bos	P	R	R	T	E	R	D	A	K	S	R	L	P	V	L	I	K	A	S	Q	S	L	G	S	V	C	R	L	P
Opossum	S	S	I	V	D	K	S	T	K	S	R	L	P	I	L	L	K	P	S	R	S	L	G	S	I	N	L	N	S
Rat	P	S	A	I	D	K	D	D	K	T	G	L	L	I	Q	L	K	T	P	E	L	L	N	L	N	L	P		
Mouse	P	S	A	I	N	P	D	A	E	K	T	G	L	L	I	Q	L	K	T	P	E	L	L	N	L	N	L	P	
Chicken	P	K	T	D	A	A	H	L	P	R	K	S	R	L	P	V	L	L	R	S	S	R	A	G	N	A	P	V	S
Consensus	.ps	...	da	.K	s	r	L	p	!l	k	.s	r	s	L	g	n	.y	.l	p	a									

Fig. 10 Alignment of the EB1 binding site sequence in various organisms. Sequences of the EB1 binding site in ortholog gene products of various organisms are compared to that of human CDK5RAP2: chimpanzee (*Pan troglodytes*), orang (*Pongo abelii*), horse (*Equus caballus*), dog (*Canis lupus familiaris*), cow (*Bos taurus*), opossum (*Monodelphis domestica*), rat (*Rattus norvegicus*), mouse (*Mus musculus*) and chicken (*Gallus gallus*). Detailed information on sequences used for alignments can be found in the supplementary info box 1

tethering of centriole pairs by cohesion fibers during interphase, and it is physiologically lost at mitotic onset to allow centrosome separation in preparation for mitotic spindle assembly. After cell division, a cell inherits a pair of disengaged but cohered centrioles. Barrera et al. reported that loss of Cdk5rap2 function in murine embryonic fibroblasts (MEFs) is concomitant with centriole amplification; however, centrioles are frequently single, unpaired and showed an increase in daughter-daughter centriole pairs. Centrioles were disengaged in mutant MEFs, and early in mitosis, amplified centrosomes assembled multipolar spindles in *Cdk5rap2* mutant cells. With their results,

the authors indicate that *Cdk5rap2* is required to maintain centriole engagement and cohesion, thereby restricting centriole replication.

Switch from symmetric to asymmetric cell division and neuronal differentiation

During neurogenesis, the length of the cell cycle and the proportion of symmetric and cell fate-determining asymmetric divisions of neural progenitors crucially affect the brain size. In the MCPH3 *Cdk5rap2^{an/an}* B6.Cg mouse model, Lizarraga et al. [13] reported a change in cleavage plane orientation with an increase of horizontal cleavage plane positioning in knockout cells. The authors argue that this is likely associated with an increase of asymmetric and a decrease of symmetric cell proliferation [13]. In other mouse models, such a shift from symmetric to asymmetric proliferation has been shown to lead to a depletion of the progenitor pool, a reduction of brain size, and an increase of precursor and neuronal cell death [13, 63–65].

Some of the CDK5RAP2 effects on neuronal differentiation may be elicited via its interaction with CDK5R1 and indirectly CDK5, though it needs to be pointed out that binding of CDK5RAP2 to human CDK5R1 and regulation of CDK5 has not been seriously studied in human tissues. For CDK5, a pivotal role in neuronal differentiation, cell polarity and synaptic function has been reported [22, 66–68]. Moreover, the direct *Cdk5rap2* interaction partner *Cdk5r1* (p35) not only interacts with *Cdk5*, but also with Notch signaling pathway regulators [69]. It would be intriguing to analyze the role of *Cdk5rap2* in these pathways. *Cdk5* is otherwise an unusual member of the Cdk protein family since, unlike other Cdks, it is not believed to regulate a ‘typical cell cycle.’

Does CDK5RAP2 play a role in migration?

While migration defects are not mainstream features of patients with MCPH3 and also not a striking feature in the *Cdk5rap2^{an/an}* B6.Cg mice or *cnn^{-/-}* flies, brain malformations that indicate migration defects, such as heterotopias, have been described in individual patients with other MCPH subtypes (only three families with MCPH3 have been described so far). It thus remains to be elucidated whether *Cdk5rap2* plays a subtle role in migration in humans. *Cdk5rap2* is believed to inhibit (or regulate) *Cdk5/Cdk5r1*(p35), a complex required for the proper migration of neurons to the cortical plate during corticogenesis [22]. *Cdk5* and its regulatory subunits *Cdk5r1* (p35) and *Cdk5r2* (p39) are essential for one of the two pathways for radial migration identified in mice (reviewed in [22, 70]). This *Cdk5* pathway is of considerable importance in radial migration as *Cdk5*, *p35* and *p39* knockout mice reveal

cortical inversion to varying degrees (but apparently no change of brain size; [71–74]). It remains to be elucidated whether *Cdk5rap2* interacts with other regulators of p35 such as Brain-1 (*Brn1*) and Brain-2 (*Brn2*) [75], and Heat Shock Factor 2 (*Hsf2*) [76].

Does CDK5RAP2 play a role in cerebellar development?

Developmentally controlled proliferation, differentiation, migration and apoptosis of cells through processes such as centrosome function, cell cycle control, spindle assembly and division plane orientation are not unique for the cerebral cortex. They also occur throughout the development of the cerebellar cortex. Subtle cerebellar defects have been described in individual patients with MCPH such as cerebellar hypoplasia. However, why a loss of CDK5RAP2 protein function does not lead to a gross reduction of the cortex in the cerebellum as it does in the cerebrum is unknown and raises questions regarding the difference in cortex development within the brain. Moreover, the role of CDK5RAP2 has not been studied in cerebellar development, and it is thus not known whether the *CDK5RAP2* gene is expressed in this context.

It thus remains to be elucidated what role *Cdk5rap2* plays in cerebellar development, especially in light of the crystallizing respective role of *Cdk5*. In the perinatally lethal *Cdk5* knockout mice, cerebellar development was disrupted with regard to cell migration, especially of the Purkinje cells (the Purkinje layer could not be distinguished at E18.5) [77]. *Cdk5* chimeric knockout mice, containing both *Cdk5* wild type and *Cdk5* knockout cells, have hypoplastic cerebella with aberrantly localized, *Cdk5* mutant Purkinje cells in the white matter. Moreover, some of the *Cdk5* mutant granule cells in chimeric mice displayed a migration defect, as they did not migrate to the internal granule layer (IGL). On the other hand, *Cdk5* mutant cells of the deep cerebellar nuclei (DCN) had normal configuration, indicating that the cells of the nuclei migrate by a *Cdk5* independent pathway, even though they are generated at a similar time and in the same location as the Purkinje cells [77]. The *Cdk5* mutation divides neuronal migration into different types: an early *Cdk5*-independent and a late *Cdk5*-dependent form. Since the granule cells can migrate from the rhombic lip to the cerebellum even in the absence of *Cdk5*, it seems the same cell can make use of different mechanisms of migration at different times [77].

CDK5RAP2 and evolution

The rapid increase in brain size over the past 3–4 million years together with the associated increase in cognitive

skills is one of the most striking features of hominid evolution. MCPH genes are obvious candidates for this strong evolutionary movement and several, sometimes controversial, studies have attended to this point [7, 78–96]. From an evolutionary perspective, primary microcephaly can be viewed as an example of ‘evolutionary regression’ whereby the brain dimensions of the affected individuals revert back to the level resembling that of very early hominids [85, 97], a probably simplistic interpretation as hominids had small brains.

Evans et al. (2006) compared the evolutionary rates of CDK5RAP2 among primates, rodents and carnivores to verify the hypothesis that genes regulating brain size during development might also play a role in brain evolution in primates and especially humans [98]. They showed that the protein evolutionary rate of CDK5RAP2 is significantly higher in primates than in rodents or carnivores, especially in functional domains that are implicated in chromosome cohesion and condensation, such as the SMC domains [7]. Moreover, the plus-end binding protein EB1 motif detected in CDK5RAP2 that is important for microtubule dynamics and stability [9] is conserved in the gene sequences of chimpanzees, bovines and dogs, but not in those of rats and mice (see discussion above). Fong et al. [9] argue that CDK5RAP2 is possibly engaged in evolutionary brain development in part by association with microtubule distal tips. Rimol et al. [99] described sex-specific associations between SNPs and cortical surface area; such SNPs may have effects upon specific groups of neuronal progenitor populations. Still, the functional role of the adaptive evolution of MCPH genes remains speculative, and no clear answer has emerged from genetic epidemiology.

Conclusion

Brain size at birth is largely determined by the relative rates of proliferation, differentiation and cell death. CDK5RAP2 holds a role in cell cycle regulation, cell cycle checkpoint control and DNA repair, chromosome condensation, centrosome function, spindle formation and dynamics, kinetochore attachment to spindles, cellular abscission and apoptosis. It thereby contributes to the human phenotype. The phenotype of humans with MCPH and of animal models indicates that pathomechanisms underlying MCPH include a decrease or abnormal cell proliferation/differentiation, increased rates of cell death and possibly neuronal migration defects. All MCPH genes encode centrosomal proteins such as Cdk5rap2, implicating a critical role for the centrosome in brain development/growth. Further centrosomal proteins such as pericentrin have been associated with diseases that involve microcephaly, in the latter case Seckel syndrome (MIM*210600)

and microcephalic osteodysplastic primordial dwarfism type II (MIM*210720). Future work will need to address the molecular function of Cdk5rap2 and its interaction with other proteins in greater detail.

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