Aus der Klinik für Pädiatrie mit Schwerpunkt Neurologie
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DISSERTATION

The Role of CDK5RAP2 in
Autosomal Recessive Primary Microcephaly (MCPH)

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"Nothing in life is to be feared, it is only to be understood. Now is the time to understand more, so that we may fear less."

Marie Curie

To my husband

and

to my family

Table of contents

Figures

Abstract

Autosomal recessive primary microcephaly (MCPH) is a rare, genetically heterogeneous disease characterized by a reduced head circumference at birth and intellectual deficit. Biallelic mutations in the Cyclin-dependent kinase 5, regulatory subunit-associated protein 2 gene *CDK5RAP2* cause MCPH type 3 (MCPH3). CDK5RAP2 function contributes to cellular processes such as centrosome function, cell cycle checkpoint control, DNA repair, chromosome condensation, and kinetochore attachment to spindles. One current model for the microcephaly phenotype in MCPH3 invokes a premature shift from symmetric to asymmetric cell divisions and thus premature neurogenesis with a subsequent depletion of the progenitor pool. In addition, other mechanisms may also play a role.

The aim of my PhD thesis work was to better understand the role of CDK5RAP2 in physiological brain development and in disease. Specific aims were (i) to characterize the spatiotemporal CDK5RAP2 expression during normal murine and human brain development, (ii) to characterize the phenotype of a *Cdk5rap2* mutant mouse generated in our research group, and (iii) to identify further MCPH3 patients with novel *CDK5RAP2* mutations and thereby better characterize the phenotype spectrum of MCPH3. We found that in the murine brain *Cdk5rap2* expression boosts during early embryonic stages, when proliferation rates are high and neocortical development is initiated. In human and murine brain, CDK5RAP2 is present in brain structures with high proliferative rates and, colocalizes with progenitor cells, glial cells, and early neurons. As the brain matures, CDK5RAP2 is refined to specific substructures within regions, which correspond to preserved proliferation zones. Moreover, we found concordance between regions of high CDK5RAP2 expression in the mouse and sites of pathology suggested by neuroimaging studies in humans and mouse. Our findings in human and mouse tissue confirm the function of CDK5RAP2 in cell proliferation. In addition, we described for the first time in detail the clinical, radiological, and also the cellular phenotype of MCPH3 patients with a novel homozygous nonsense mutation in the *CDK5RAP2* gene. Cells from the patients showed mitotic spindle defects and disrupted γ-tubulin localization to the centrosome, which underlines the importance of CDK5RAP2 in mitotic spindle pole organization and centrosome integrity. These findings suggest that MCPH3 pathomechanism is partially due to mitotic spindle and centrosomal defects. To further mimic the human mutation and study the function of CDK5RAP2 *in*

vivo, we generated a transgenic *Cdk5rap2* mouse. These mice did not display the expected microcephaly phenotype. Our further analysis indicated that a previously unknown splice variant of the *Cdk5rap2* gene exists, which allows translation of Cdk5rap2, even in the mutant mice.

With our studies, we provide for the first time a systematic description of the spatiotemporal expression of CDK5RAP2 in murine and human developing brain. Moreover, the cellular phenotype findings we collected from the new MCPH3 patients provide a glimpse into processes that could lead to the microcephaly phenotype in MCPH.

Zusammenfassung

Die Autosomal-rezessive primäre Mikrozephalie (MCPH) ist eine seltene und genetisch heterogene Erkrankung, die sich durch einen kongenital reduzierten Kopfumfang und eine mentale Retardierung auszeichnet. Biallelische Mutationen im Cyclin-dependent-kinase-5-regulatory-subunit-associated-Protein 2 Gen *CDK5RAP2* verursachen die MCPH vom Typ 3 (MCPH3). Das CDK5RAP2 spielt eine Rolle in zellulären Prozessen wie der Funktion von Zentrosomen, der Kontrolle der Zellzyklus-Checkpoints, DNA-Reparatur, Chromosomenkondensation und Kinetochor Befestigung an Spindeln. Eine der Haupthypothesen für die Entstehung des Mikrozephalie-Phänotyps bei MCPH3 beinhaltet einen vorzeitigen Wechsel von symmetrischer zu asymmetrischer Zellteilungen und damit eine vorzeitige Neurogenese sowie eine Depletion des Vorläuferpools. Darüber hinaus können auch andere Mechanismen eine Rolle spielen.

Das Ziel der vorliegenden Doktorarbeit war es, die Rolle des CDK5RAP2-Proteins in der physiologischen Entwicklung des Gehirns und in der Entstehung von MCPH besser zu verstehen. Spezifische Ziele waren (i) die zeitliche und örtliche Expression von CDK5RAP2 während der normalen Entwicklung des Gehirns von Menschen und Mäusen zu veranschaulichen, (ii) den Phänotyp einer in unserer Forschungsgruppe erzeugten *Cdk5rap2*-Mausmutanten zu charakterisieren, und (iii) weitere MCPH3 Patienten mit bisher nicht beschriebenen Mutationen im *CDK5RAP2-Gen* zu identifizieren und damit das Phänotypspektrum der MCPH3 besser zu erforschen. Wir fanden, dass Cdk5rap2 während der frühen embryonalen Stadien im murinen Gehirn besonders hoch exprimiert wird, d.h. während einer Zeit in der hohe Proliferationsraten auftreten und die Neokortex-Entwicklung bei Mensch und Maus beginnt. In humanen und murinen Gehirnen, ist CDK5RAP2 in Hirnstrukturen mit hoher Proliferationsrate vorhanden und kolokalisiert mit Vorläuferzellen, Gliazellen und unreifen Neuronen. Mit der Entwicklung des Gehirns reduziert sich das Vorhandensein von CDK5RAP2 auf spezifische Unterstrukturen, in denen die Proliferation anhält. Darüber hinaus fanden wir Übereinstimmungen zwischen Regionen mit hoher CDK5RAP2 Expression in der Maus und der durch bildgebende Verfahren beschriebenen Pathologie bei Mensch und Maus. Unsere Ergebnisse in Human- und Mausgewebe bestätigen die Rolle von CDK5RAP2 in der Zellproliferation. Zusätzlich beschrieben wir zum ersten Mal detailliert den klinischen, radiologischen und auch den zellulären Phänotyp von Patienten mit MCPH3 mit einer bisher nicht beschriebenen homozygoten Nonsense-Mutation im

CDK5RAP2-Gen. Wir fanden, dass Zellen von Patienten eine abnorme Morphe ihrer mitotischen Spindelapparate sowie eine gestörte Lokalisation des γ-Tubulins am Zentrosom aufwiesen. Diese Ergebnisse unterstreichen die Bedeutung von CDK5RAP2 in der Organisation der mitotischen Spindel und der Zentrosomintegrität. Diese Ergebnisse legen nahe, dass der MCPH3-Pathomechanismus teilweise auf Defekte der mitotischen Spindel und der Zentrosomen zurückzuführen ist. Um die Auswirkungen einer *CDK5RAP2*-Mutation im Menschen weiter zu imitieren und die Funktion des CDK5RAP2-Proteins *in vivo* zu untersuchen, generierten wir eine transgene *Cdk5rap2*-Mausmutante. Diese Mäuse bildeten aber nicht den erwarteten Mikrozephalie-Phänotyp aus. Weitere Analysen zeigten, dass es eine bisher unbekannte Spleißvariante des *Cdk5rap2*-Gens gibt, die selbst in den Mausmutanten die Translation von *Cdk5rap2* ermöglicht.

Diesen Studien bieten zum ersten Mal eine systematische Beschreibung der CDK5RAP2-Expression während der Entwicklung muriner und humaner Gehirne. Darüber hinaus geben unsere am Patienten erhobenen Ergebnisse des zellulären Phänotyps einen Einblick in Prozesse, die zum Mikrozephalie-Phänotyp bei MCPH führen könnten.

1. Introduction

1.1 Microcephaly definition and epidemiology

Microcephaly is defined by a reduction of the occipito-frontal head circumference (OFC) of more than two standard deviations (SD) below the mean for age, gender, and ethnicity (severe microcephaly is defined by an OFC below -3 SD). Microcephaly can be caused by genetic and/or environmental factors and classified as primary (congenital) or secondary (postnatal). About 2-3% of the general population are microcephalic, depending on the population and the applied SD threshold to define microcephaly. Moreover, primary, nonsyndromal microcephaly has an incidence of 1:30,000 to 1:250,000 live-births (reviewed in (1-3)).

1.2 Autosomal recessive primary microcephaly (MCPH)

Autosomal recessive primary microcephaly or MCPH for **M**icro**C**ephaly **P**rimary **H**ereditary is a rare and genetically heterogeneous disease reported in about 200 families world-wide. MCPH is considered as a model disorder for understanding the mammalian evolutionary brain size expansion, especially the expansion of the cerebral cortex (reviewed in (1, 3, 4)).

1.2.1 Phenotype

MCPH patients display a pronounced reduction in brain volume at birth and simplified gyration of otherwise architectonical normal brains on magnetic resonance imaging (MRI) studies (Figure 1; reviewed in (1, 2, 5)). In addition, patients suffer from mental retardation of various degrees. Individual patients with periventricular neuronal heterotopias as an indication of a neuronal migration defect have been reported (2, 5, 6). Further cerebral abnormalities are now known (7). Moreover, a short statue can occur in MCPH patients, particularly in those with subtypes MCPH1 and MCPH5 (reviwed in (1)).

Figure 1. MCPH patient phenotype.

(A) MCPH patient with microcephaly and typical sloping of the forehead. **(B)** Typical reduction of the brain volume, particularly of the cerebral cortex, as well as simplified gyration in an MCPH patient compared to a healthy control on magnetic resonance (MRI) studies. Adapted from (1).

1.2.2 Genotype

MCPH subtypes 1-11 are caused by mutations in genes encoding microcephalin (MCPH1; MIM#251200 (8, 9)), WD repeat-containing protein 62 WDR62 (MCPH2; MIM#604317 (10, 11)), cyclin-dependent kinase 5 regulatory associated protein 2 CDK5RAP2 (MCPH3; MIM#604804 (12, 13)), cancer susceptibility candidate 5 CASC5 (MCPH4; MIM#604321 (14)), abnormal spindle-like microcephaly associated ASPM (MCPH5; MIM#608716 (15-17)), centrosomic protein J CENPJ (MCPH6; MIM#608393 (13, 18)), SCL/TAL1 interrupting locus STIL (MCPH7; MIM#612703 (19)),135 kDa centrosomal protein CEP135 (MCPH8; MIM#614673 (20)), centrosomal protein 152 kDa CEP152 (MCPH9; MIM#604852 (21, 22)), and polyhomeotic-like protein 1 ZNF335 (MCPH11; MIM#615414 (23)). In addition, linkage to chromosome 10q11.23 has been reported (24). MCPH genes are highly conserved among species and have been suggested to be involved in the evolutionary enlargement of the human brain ((2, 25, 26). Mutations in *CENPJ* and *CEP152* have not only been identified in patients with

MCPH, but also in those with Seckel syndrome (other Seckel syndrome genes include *ATR* and *RBBP8*) (27, 28). There is not only an overlap of the genotype, but also of the MCPH and Seckel syndrome phenotype such as short stature, severe microcephaly, and mental retardation (27). Moreover, there is an overlap of the phenotype of Majewski osteodysplastic primordial dwarfism type II (MOPDII) caused by mutations in the pericentrin gene *PCNT* with that of MCPH and of Seckel syndrome (29). The gene products associated with these diseases also share common functions (30).

1.2.3 MCPH protein function

MCPH genes encode proteins that are involved 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 (reviewed in (1, 30)). MCPH proteins localize predominantly to the centrosome and the pericentriolar matrix. In animal cells, the centrosome, as the major microtubule organizing center (MTOC), organizes and orientates the mitotic spindle poles to ensure proper cell division (31). MCPH proteins further play a role in mitotic cleavage plane orientation and thereby regulate the balance between symmetric and asymmetric progenitor cell division during neurogenesis (reviewed in (1)). Abnormal orientation of the mitotic cleavage plane is believed to partially contribute to the microcephaly phenotype (1, 32). In addition, CDK5RAP2, CENPJ, CEP152, and STIL are involved in various centrosomal processes such as centriole duplication, engagement, and cohesion, centrosome attachment to the spindle pole, recruitment of important factors to the centrosome, and establishment of proper mitotic spindles (33-42).

1.2.4 Pathomechanism of MCPH

Neurons in the mammalian CNS are all generated from neuroepithelial (NE) cells, which are pluripotent stem cells. NE cells undergo three types of cell division: (i) symmetric, proliferative division giving rise to two NE cells, (ii) asymmetric division yielding one NE cell and one neuron, (iii) symmetric division yielding two neurons (43). The mammalian cortex is highly organized and has a laminated structure, consisting of six layers (44). It develops in an inside-out sequence, where early postmitotic cells generated from the neuroepithelium build up the layers nearest to the ventricular zone (deep layers) and later generated postmitotic cells migrate beyond the first generated deeper layers giving

rise to the next layer and so on (45). In that fashion the six cortical layers evolve (45). The size of the cortex depends on the relative rate of cell proliferation, the balance of symmetric versus asymmetric progenitor cell proliferation, cell death and cell differentiation/maturation (46). One current model for the microcephaly phenotype in MCPH invokes a premature shift from symmetric to asymmetric cell divisions and thus premature neurogenesis with a subsequent depletion of the progenitor pool (Figure 2; (47-49)). In addition, increased levels of cell death and premature cell cycle exit have also been suggested to contribute to the pathomechanism of MCPH (48, 49). However, other mechanisms might also play a role, as a progressive cellular defect during stem cell differentiation has been reported (50-52).
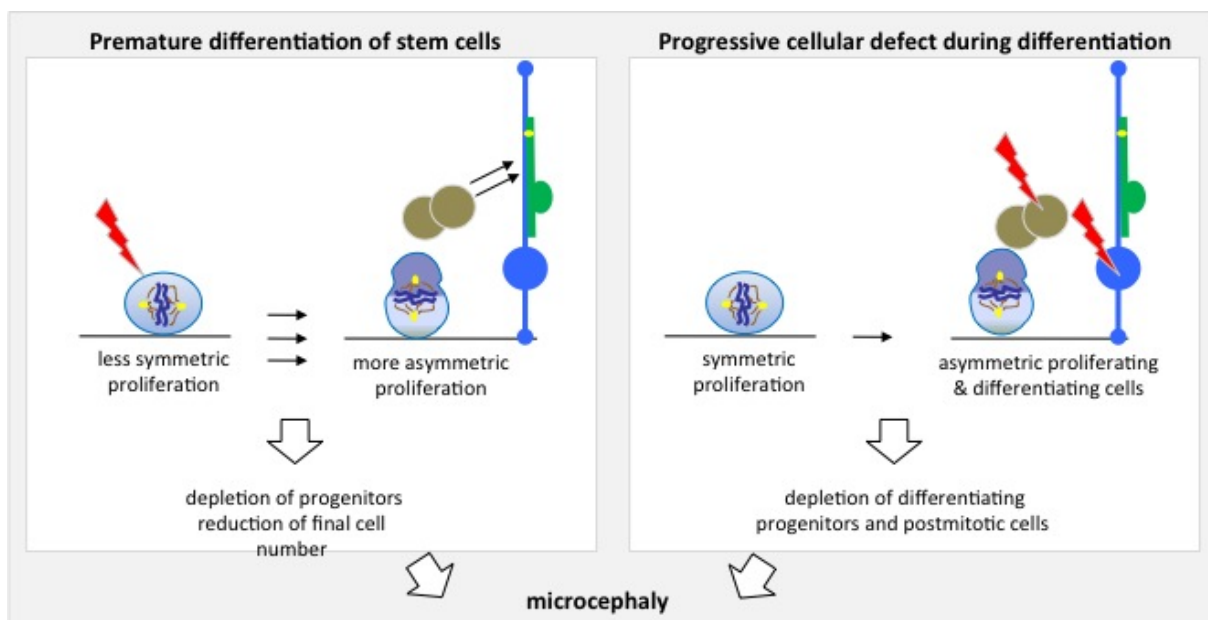
Figure 2. Working hypothesis for MCPH3 pathomechanism.

A current model suggests a premature shift from symmetric to asymmetric cell division, leading to a depletion of the progenitor pool and a reduction of the final neuron number. Adapted from (53).

1.3 MCPH type 3 (MCPH3)

Homozygous mutations in the Cyclin-dependent kinase 5, regulatory-subunit associated protein 2 gene *CDK5RAP2* were identified as a cause of MCPH type 3 (MCPH3) in 2005 (13). Currently there are three identified *CDK5RAP2* mutations in three Pakistani families and, one Somali child: (i) a nonsense mutation in exon 4 (c.246T>A, p.Y82X) introducing a new splice acceptor site, (ii) a nonsense mutation in exon 8 (c.700G>T,

p.E234X) introducing a frame shift, and (iii) a mutation in intron 26 (IVS26-15A>G, R1334SfsX5) resulting in a premature stop codon and finally (iv) a heterozygous mutation introducing a frame shift and a splicing respectively (c.524_528del and c.4005-1 G>A) (Figure 3; (13, 54-56)). All mutations are proposed to lead to truncated proteins and a loss of CDK5RAP2 function.
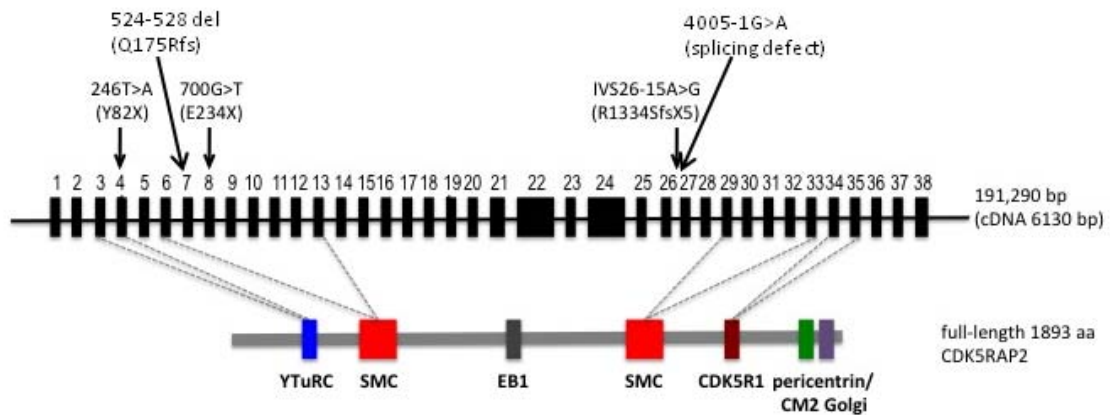

Figure 3. Position of *CDK5RAP2* mutations within gene and protein domain.

Known and predicted MCPH protein domains: Structural maintenance of chromosome (SMC), C-terminal Cnn Motif 2 that might mediate Golgi complex interaction and binding to calmodulin (CM2), Gamma tubulin ring complex (yTuRC), EB1 plus-end binding protein 1 (EB1), Cyclin-dependent kinase-5 regulatory kinase 1 (CDK5R1). Figure adapted from (57).

1.3.1 CDK5RAP2

CDK5RAP2, also referred to as centrosome-associated protein 215 (CEP215), or CDK5 activator binding protein C48, is a highly conserved protein, and ortholog genes are found in other organisms such as apes, cows, dogs, rats, mice, and chicken (57). The protein was discovered by two groups independently in 2000 and associated with the disease MCPH five years later (12, 13, 58, 59). Studies performed in somatic cells revealed the importance of CDK5RAP2 in centrosomal microtubule organization; it recruits the y-tubulin ring complex (y-TuRC) to the centrosome, which is key for microtubule nucleation (60). Cdk5rap2 localization to the pericentriolar matrix (PCM) is evident throughout all stages of the cell cycle, and its centrosomal level is regulated in a cell cycle dependent manner in mouse embryonic fibroblasts (36, 60). Centrosomal

microtubule organization and PCM component recruitment regulated through CDK5RAP2 are important for proper mitotic progression (61). In human tumor cells, but not in rodents, CDK5RAP2 has been found to localize at the distal, growing tips of microtubules where it indirectly regulates the microtubule plus-end dynamics (62). Proper spindle formation, chromosomal segregation, spindle checkpoint signaling pathway, indirect regulation of CDK5, and regulation of centriole cohesion and duplication cycle are additional functions of CDK5RAP2 (13, 36, 58, 63). Apart from the centrosomal localization of CDK5RAP2, the protein has also been shown to localize to the Golgi network during interphase in a centrosomal and energy dependent manner; however, the importance of this localization is not known so far (64).
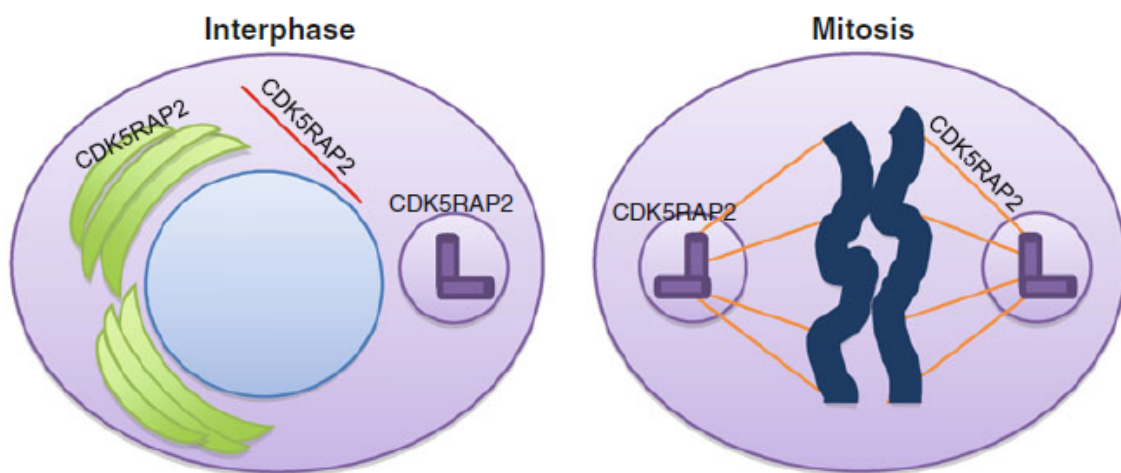
Figure 4. CDK5RAP2 intracellular localization.

Localization of CDK5RAP2 protein during interphase and in mitosis. CDK5RAP2 protein is found at the centrosome and the Golgi complex (green) during the interphase, and is localized at the centrosome in the mitotic cell (shown in tumor cells). Also, this protein has been described to localize to the plus end of microtubules (orange). During interphase cells are growing and duplicate their DNA (blue) in order 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 endoplasmatic 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. Figure adaptebd from (57).

2. Aims

When I started my PhD thesis in 2009, CDK5RAP2 was known to be a centrosome-associated protein, involved in various centrosome related functions such as the recruitment of the y-tubulin ring complex to the centrosome, maintenance of connection between centrioles and pericentriolar matrix, centrosome cohesion, and spindle checkpoint regulation. In addition, the protein was described to be an indirect regulator of the highly conserved serine threonine kinase CDK5 (reviewed in (1)). The latter result has not been reproduced. Further mechanisms were - at that time point - not described. In particular, no systematic description of the spatiotemporal expression of CDK5RAP2 in the developing mouse and human brain as well as no mouse model had been reported. Moreover, only two *CDK5RAP2* mutations with sparse clinical data had been published. Thus, the main aim of my PhD thesis project was to characterize the role of CDK5RAP2 in physiological brain development and in the occurrence of acquired and hereditary brain malformation. The specific aims were:

(i) Characterization of the spatiotemporal CDK5RAP2 expression during normal murine and human brain development.

(ii) Characterization of a *Cdk5rap2* mutant mouse generated in our research group.

(iii) Identification of further MCPH3 patients with novel *CDK5RAP2* mutations and characterization of their phenotype.

3. Methods

3.1 Animal and human samples

Embryonal (E), postnatal (P) and adult *C57Bl6* mice (E10, E12, E16, P0, P5, P10, P56) were obtained from the animal facility FEM of the Charité - Universitätsmedizin Berlin, Germany. In addition, we generated transgenic *Cdk5rap2* mice (see below for details). All experiments were carried out in accordance to the national ethic principles (registration no. T0309/09 and G0113/08).

Informed consent was obtained from the parents of the patients for the molecular genetic analysis, the publication of clinical data, photos, magnetic resonance images (MRI) and studies on immortalized lymphocytes (LCLs). DNA was extracted from EDTA blood samples using the Illustra BACC2 DNA extraction kit (GE Healthcare, Munich, Germany). Samples from microcephaly patients and controls were used in this study with approval from the local ethics committees of the Charité and the Freiburg University (approval nos. EA1/212/08 and 494/11, respectively).

3.2 Cell culture

Ebstein-Barr virus transformed lymphocytes (LCLs) were established according to the protocol published by (65). Non-adherent LCLs were cultured in RPMI 1640 with L-Glutamine (Invitrogen, Darmstadt, Germany) supplemented with 20% v/v fetal bovine serum (Invitrogen) and 1% v/v penicillin-streptomycin (Sigma-Aldrich, Taufkirchen, Germany).

3.3 RNA extraction and quantitative real time PCR

Total RNA was extracted using standard techniques from *C57Bl6* mouse cerebral cortex at the age of E10, E12, E14, E16, P0, P5, P10, P20, and P56 (n=6 per group) and organs from P0 pups as kidney, thymus, lung, heart, bladder, liver, and placenta (n=3-6 per group). In addition, RNA was extracted from transgenic *Cdk5rap2* mouse and control specimen. Total RNA was extracted using TRI-Reagent® (Sigma-Aldrich) according to the manufacturer's recommendations from tissue samples, and cDNA was prepared by reverse transcription using the ThermoScript® RT-PCR System (Invitrogen), using a combination of oligo(dT)$_{20}$ and random hexamer primers. cDNA synthesis was performed with 1 µg of RNA. For quantitative real-time PCR, 1 µl of 1:10 diluted cDNA was used as template. For primer and probe sequences, please refer to

(66, 67). Experiments (n=5-6 per group) were run in triplicate, using an Applied Biosystems 7500 Fast Real-time PCR System (Applied Biosystems Inc., Norwalk, CT, USA) in 96-well microtiter plates. Threshold cycle (Ct) values were calculated using the 7500 Fast System SDS Software (Applied Biosystems Inc.), and further statistical calculations were performed using Microsoft Excel (Microsoft Corporation, Bellevue, WA, USA) and GraphPad Prism 5 software (GraphPad Software Inc., La Jolla, CA, USA). The $2^{-\Delta\Delta Ct}$ method was applied for the quantification of the relative expression of the *Cdk5rap2* mRNA using the reference gene hypoxanthine phophoribosyltransferase (*Hprt)* as the endogenous control for normalization.

3.4 Protein extraction and Western blot
Proteins were extracted from murine tissues and human LCLs using standard techniques. Protein concentrations were determined using a bicinchoninic acid (BCA) based assay, according to the instructions of the manufacturer (BCA Protein Assay Kit; Pierce Biotechnology, Rockford IL, USA). Protein extracts were denaturated in Laemmli sample loading buffer at 95 °C for 5 min, separated by sodium dodecyl sulphate polyacrylamide gel electrophoresis (SDS-PAGE), and electrophoretically transferred in transfer buffer in a semi-dry fashion using Trans-Blot SD Semi-Dry transfer cell (BioRad, Munich, Germany) onto nitrocellulose membrane (BioRad). The membranes were incubated for 1 h at room temperature (RT) in blocking buffer (TBS-T 1x with 5% bovine serum albumin (BSA)), rinsed three times with TBS-T 1x for 8 min each at RT on a shaker followed by incubation with primary and secondary antibodies. The immunoreactive proteins were visualized using a technique based on a chemiluminescent reaction. The gel pictures were obtained with the Molecular images ChemiDoc XRS+ (BioRad). Western blot experiments were run in triplicates.

3.5 Immunohistology and -cytology
Cryostat sections of embryonic, postnatal, and adult murine brain tissue and organs from P0 pups (kidney, thymus, lung, heart, liver, and intestines) were briefly air-dried prior to rising in phosphate buffer saline (PBS 1x) for 10 min and in staining buffer (0,2% gelatine, 0,25% Triton X-100 in PBS 1x) for 20 min. In a 30 min blocking step, sections were incubated in 10% donkey or goat normal serum (DNS, GNS) in staining buffer at RT. Sections were incubated overnight at RT with primary antibodies in the staining buffer containing 10% DNS or GNS followed by an incubation with the corresponding secondary antibodies for 2 h at RT, and 30 min rinsing with PBS 1x prior to mounting

with Immu-mount (Thermo scientific, Schwerte, Germany). Nuclei were labeled with 4',6-diamidino-2-phenylindole (DAPI, 1:1000, Sigma-Aldrich).

Human paraffin sections were deparaffinized, rinsed in staining buffer, incubated 30 min in 10% GNS at RT, incubated overnight at RT with the primary antibody followed by an incubation with streptavidin goat anti-rabbit biotinylated antibody (Invitrogen) 1:400 for 2 h at RT. Endogenous peroxidase was quenched through subsequent incubation in 0.3% H2O2 for 10 min at RT, and signal amplification was performed using the Vectastain ABC elite system® (Vector laboratories, Orton Southgate, UK). Color development was achieved by adding 17 µl of H_2O_2 30% to sections incubated in a solution containing 200 mg/L DAB, 0.05 M Tris and 0.6% $NiNH_4SO_4$. Color development was stopped through rinsing sections in 0.05 M Tris solution, and sections were dehydrated and mounted with Entelan® (Merck, Darmstadt, Germany). Human sections were also stained with Hematoxylin and Eosin staining (H&E) and anti MIB1 immunostaining (Dako, Hamburg, Germany); these immunostainings were done using standard procedures. For immunocytology human LCLs were plated on Poly-L-lysine (Sigma-Aldrich) coated coverslips, cultured for 30 min at standard conditions, and incubated in 37 °C PFA 4% for 10 min prior to rinsing with phosphate buffered saline (PBS 1x). Coverslips were further incubated at RT in staining buffer (0,2% gelatin, 0,25% Triton X-100 in PBS 1x) for 20 min and subsequently in 10% donkey normal serum (DNS) in staining buffer for 30 min for blocking. Coverslips were incubated overnight at 4 °C with primary antibodies in the staining buffer containing 10% DNS followed by an incubation with the corresponding secondary antibodies for 2 h at RT. Nuclei were labeled with DAPI (1:1000).

Fluorescently labeled tissue and cells were analyzed and imaged by a fluorescent Olympus BX51 microscope with the software Magnafire 2.1B (2001; Olympus, Hamburg, Germany) and with the Zeiss Lsm5 exciter confocal microscope with the software Zen 2009 (Zeiss, Berlin, Germany). All images were processed using Adobe Photoshop. For antibody information, please refer to the original publications (67, 68).

3.6 Haplotype analysis using microsatellite markers
For linkage analysis with respect to the loci of *MCPH1-7* and *PNKP* in patients with primary microcephaly, we selected microsatellite markers for each locus in such a way that three markers were located on each side of each gene. The markers flanking the *CDK5RAP2* gene were: CHLC.GGAA23B10, D9S258, D9S2152, D9S103, D9S116,

and D9S1823. PCR fragments were resolved by capillary electrophoresis on an ABI 3100 sequencer (Applied Biosystems, Darmstadt, Germany), and fragment analysis was performed using the GeneScan software (Applied Biosystems). Haplotypes were constructed in the family by inspection of the microsatellite fragment length.

3.7 *CDK5RAP2* gene sequencing

Thirty-eight coding exons of the *CDK5RPAP2* gene and at least 50 bp of the intronic, exon-flanking sequence were analyzed through PCR, and cycle sequencing using the ABI Prism BigDye Terminator Cycle Sequencing Ready Reaction Kit Version 1.1 (Applied Biosystems). Capillary electrophoresis was performed using an ABI 3100 sequencer (Applied Biosystems), and sequence data were analyzed using SeqPilot DNA sequence analysis software (JSI, Kippenheim, Germany). The database sequence NM_018249 for the *CDK5RAP2* gene was used as reference sequence.

3.8 Generation and characterization of *Cdk5rap2* mutant mice

The conditional gene-targeting vector for the *Cdk5rap2 LoxP*$^{+/+}$ mice was produced from a mouse genomic library clone (*C57Bl6*) by my supervisor and colleagues (Figure 5). The targeting strategy was to conditionally delete exon 3 of *Cdk5rap2* and generate a subsequent stop codon at the beginning of exon 4 by using a Cre-LoxP strategy. Exon 3 and 4 encode the γ-tubulin ring complex (γTuRC)-binding domain of Cdk5rap2 (Figure 3). The targeting vector was constructed by successive cloning of PCR products and contained 3.4 kb 5′ and 3.5 kb 3′ homology arms and a neomycin selection cassette. A *LoxP* sequence was introduced into intronic regions between exon 2 and 3 as well as between exon 3 and 4, i.e., with positions about 320 bp before and about 2.2 kp at the end of exon 3. An *FRT-Neo* selection cassette with two flanking *FRT* sites for later removal by FLP recombinase was inserted about 300 bp into intron 3 (first *FRT* site) and 16 bp (second *FRT* site) before the 3' *LoxP* site The linearized targeting construct was electroporated into *C57BL/6N* mouse embryonic stem cells (ESC) and selected using neomycin. For the resultant clones, the correct insertion of the targeting construct into the genome was subsequently confirmed by PCR over the homologous recombination arm using external primers and further confirmed by Southern blot with Neo internal probe and with 5′ and 3′ external probes.

The *Cdk5rap2 LoxP*$^{+/-}$ mouse line was established at the Institut Clinique de la Souris (ICS; Ilkirch, France) in accordance to the French law. One verified stem cell clone was

selected for *C57Bl6* blastocysts injection, and ESC-derived chimeras gave germline transmission. The resulting chimeric line was verified by PCR using external primers, further confirmed by Southern blot with Neo internal probe and crossed with a Flip *C57Bl6* deleter mouse to excise the FRT site-flanked Neo cassette on F1 progenies. The F1 animals were crossed with *C57BL6* mice to generate F2 animals. Conditional *Cdk5rap2 LoxP+/+ hCMV Cre+* mice (cKO) were generated to obtain complete excision of the Cdk5rap2 exon 3 and introduce a stop codon in exon 4. Breeding of *Cdk5rap2 LoxP+/+* mice with *hCMV Cre+* mice resulted in heterozygous *Cdk5rap2 LoxP+/- hCMV Cre+* mice that were then crossed with *Cdk5rap2 LoxP+/+* mice. The latter mice were further breed among each other. *Cdk5rap2 LoxP+/+ hCMV Cre-* and *Cdk5rap2 LoxP+/- hCMV Cre-* were used as controls. Breeding was performed at the animal facility FEM of the Charité - Universitätsmedizin Berlin, Germany, and all experiments were carried out in accordance to the national ethic principles (registration no. T0309/09 and G0113/08).

Genomic DNA was isolated from tail cuts by proteinase K digestion (Invisorb spin tissue mini kit, Stratec-molecular, Berlin, Germany) using standard methods, and genotyping was performed by PCR (primer sequences are available on request).

Characterization of our conditional *Cdk5rap2 LoxP$^{+/+}$ / hCMV Cre* mice (cKO) was achieved through various strategies including quantitative real-time PCR (qPCR) of *Cdk5rap2* mRNA products, sequencing of cDNA, Western blot, histology (hematoxylin and eosin staining), immunohistology, inspection of the animals, and cranial MRI analysis.
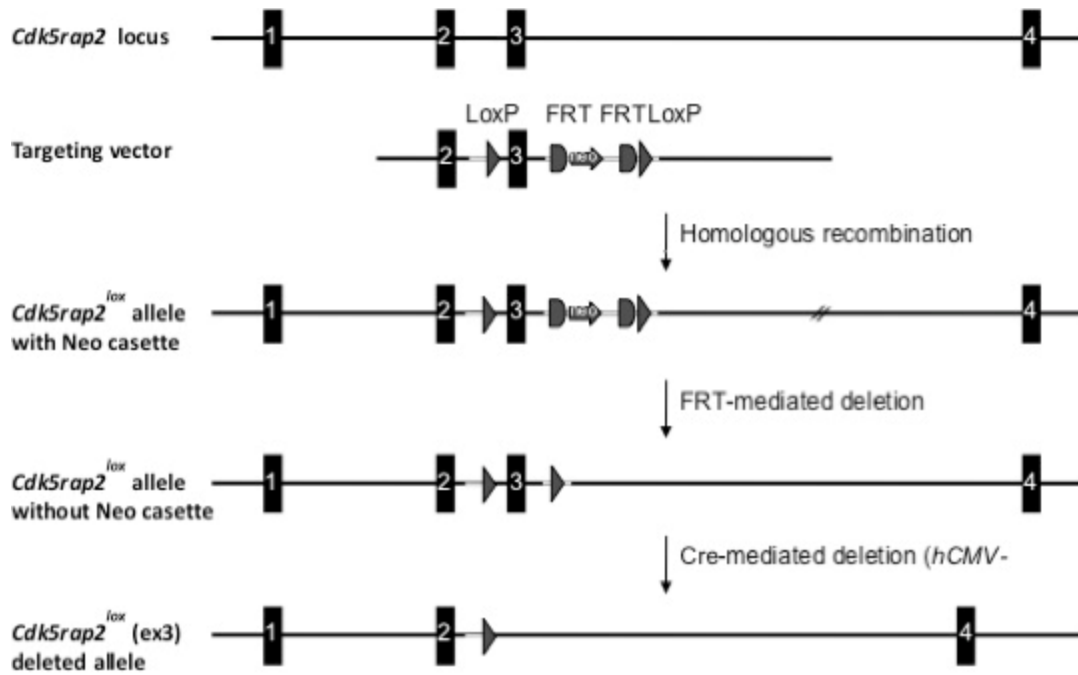
Figure 5. Transgenic *Cdk5rap2 LoxP / hCMV Cre* mouse generation.

Schematic representation of the targeting vector. Homologous recombination into the *Cdk5rap2* wild-type allele of mESC resulted in the *Cdk5rap2 LoxP* genotype. A correctly targeted ESC clone was injected into blastocystes to generate chimeric mice. Chimeras were bred with FLIP expressing transgenic animals to generate *Cdk5rap2 LoxP* mice lacking the Neo cassette, and the latter were bred with *hCMV Cre* mice to delete exon 3 and generate a stop codon downstream.

4. Results

4.1 Reference genes in the developing murine brain and in differentiating embryonic stem cells

To chose the most appropriate reference gene for normalization purpose of the qPCR results, we analyzed, *in vivo* and *in vitro*, five candidate genes throughout mouse embryonic stem cell differentiation and the developing murine brain neocortex: *Actb*, *18s*, *Gapdh*, *Hprt*, and *RPII*. Thereby, we identified *Gapdh* and *Hprt* as reference genes most suitable for *Cdk5rap2* gene expression analysis in the developing murine brain neocortex *in vivo* (66).

4.2 Spatiotemporal expression of CDK5RAP2

We started to study CDK5RAP2 function by characterizing its spatiotemporal expression in pre- and postnatal murine and human brain and in other organs (Figure 6). CDK5RAP2 was detected in various murine and human organs with the highest protein and mRNA levels in murine brain, kidney, and thymus. CDK5RAP2 immunopositivity was present particularly within regions of high proliferation in murine and human fetal organs. The intracellular enrichment of CDK5RAP2 at the centrosomes and the Golgi apparatus was confirmed by immunohistology. We found CDK5RAP2 spatio-temporal localization to be strictly regulated during murine and human brain development. During early neurogenesis in murine brain (E10.5-E12.5), Cdk5rap2 localizes to symmetrically and asymmetrically dividing neural progenitors at the ventricular and subventricular zone (VZ, SVZ). Later in development, the protein is present in glial cells and early neurons but is rarely detected in mature neurons. Cdk5rap2 is strongly downregulated as the brain matures; however, it remains to be expressed in preserved proliferation zones. At the age of P0, Cdk5rap2 is present throughout the cerebral cortex, particularly in Cux1-positive upper layer neurons. Later, in the adult brain, the distribution of the protein within the cerebral cortex is uniform. CDK5RAP2 also localizes to other brain regions such as the hippocampus and the cerebellum, where again we found its immunoreactivity in proliferative zones. Our results obtained in human brain are consistent with those of the murine brain and suggest that CDK5RAP2 may be important for neural progenitor proliferation (67).
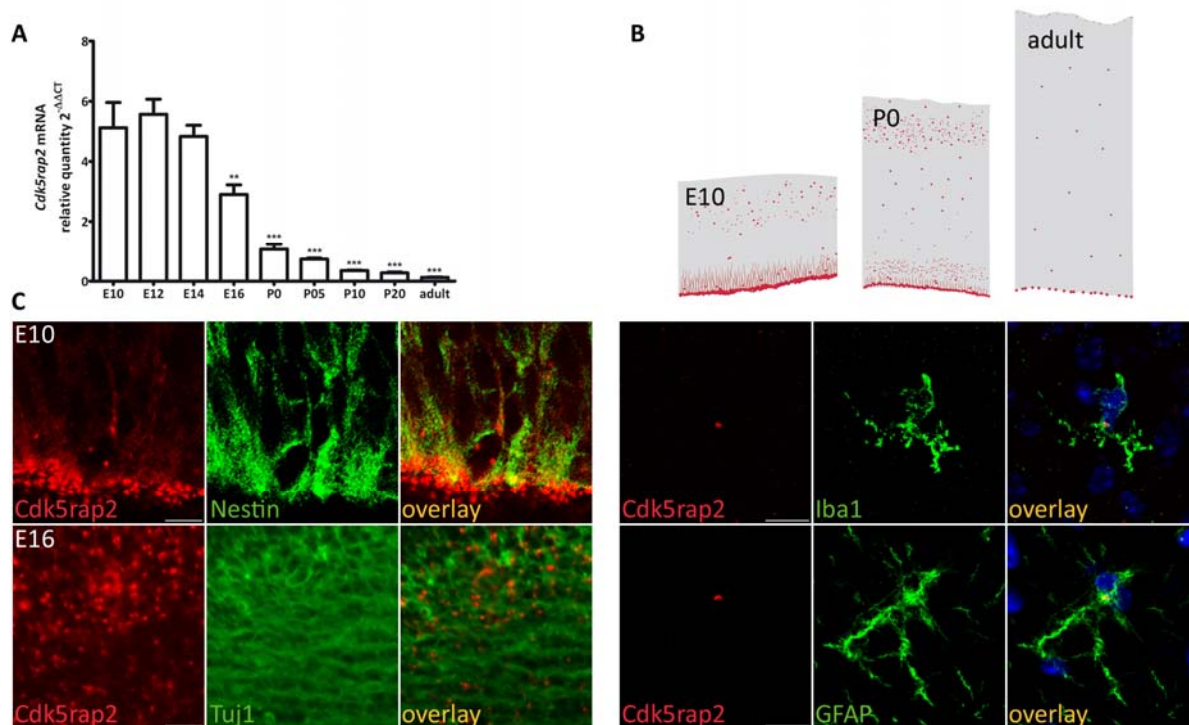
Figure 6. Cdk5rap2 in the developing murine cortex.

(A) *Cdk5rap2* mRNA expression in the murine cortex at various embryonic and postnatal ages. **(B)** A scheme representing the spatiotemporal protein localization in the murine neocortex. **(C)** Cdk5rap2 colocalizes with nestin-positive progenitor cells, and Tuj1-positive early neurons. Also, it is found in glial cells (Iba1-positive microglia, GFAP-positive astrocytes).

4.3 Clinical and cellular phenotype of MCPH3 patients with novel *CDK5RAP2* mutation

We identified, in collaboration, a novel homozygous nonsense mutation in the *CDK5RAP2* gene in two affected boys of Italian descent (c.4441C>T, p.R1481X; Figure 7), and described for the first time in detail the clinical, radiological, and also the cellular phenotype of MCPH3 patients (68).

Both patients were microcephalic at birth and were later diagnosed to suffer from intellectual disability, developmental speech delay, and behavioral problems. Cranial MRI revealed microcephaly, simplified gyration (frontal pachygyria in one patient), and hypogenesis of the corpus callosum. We specifically detected neither multiple organ defects nor a hematopoietic phenotype recently reported in a mouse model of MCPH3 ('*Hertwig's* anemia mouse'; (48, 68)).
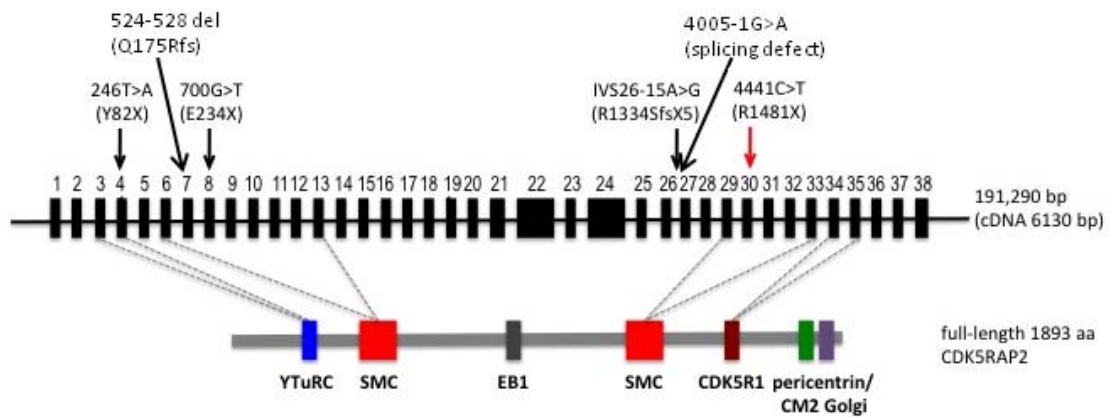
Figure 7. Novel mutation within the CDK5RAP2 gene and protein.

Exons are depicted as boxes. The coding and non-coding regions are drawn to scale. The localization and type of the published mutations are shown in black. The novel mutation is shown in red. Figure adapted from (68).

We investigated the pathogenicity of the identified mutation in LCLs from the two patients with MCPH3 and from controls (Figure 8). Consistent to results of studies performed on murine cells, CDK5RAP2 localized to the centrosome of control LCLs throughout mitosis. Centrosomal CDK5RAP2 levels in interphase cells were weaker in comparison to those at prophase and telophase. CDK5RAP2 was below detection level in mutant LCLs, as detected by Western blot and immunocytology. The Golgi domain of the CDK5RAP2 protein located at the c-terminus is predicted to be lost in our patients. Therefore, we analyzed the integrity of the Golgi apparatus in the patient cells by immunocytology using an antibody against the cis-Golgi matrix protein GM130. The Golgi-apparatus is known to be fragmented during the course of mitosis to ensure proper and equal distribution between the two daughter cells (69). Our experiments indicate that the Golgi fragmentation in the patient cells occurs earlier during mitosis compared to that in control cells. Next, we examined the integrity of centrosomes and mitotic spindle apparatus in patient and control cells through immunocytology. The centrosomal protein y-tubulin was dispersed around the centrosomes in patient cells (normal total y-tubulin protein levels on Western blots), while the centrosomal protein pericentrin displayed a normal centrosomal localization. Moreover, we detected mitotic spindle defects as abnormal spindles with unfocused and broad arrangement of the microtubule poles in patient LCLs. Patient cells also showed a trend towards an

16

increase in multipolar spindles in combination to a decrease of spindle pole distance. Lagging chromosomes were significantly increased in one of the patients but merely showed a trend towards an increase in the other patient. While it has been previously shown that the level of Chk1 protein is downregulated in mouse *Cdk5rap2* mutant cells (37), we did not detect a significant change of CHK1 protein in patient cells.
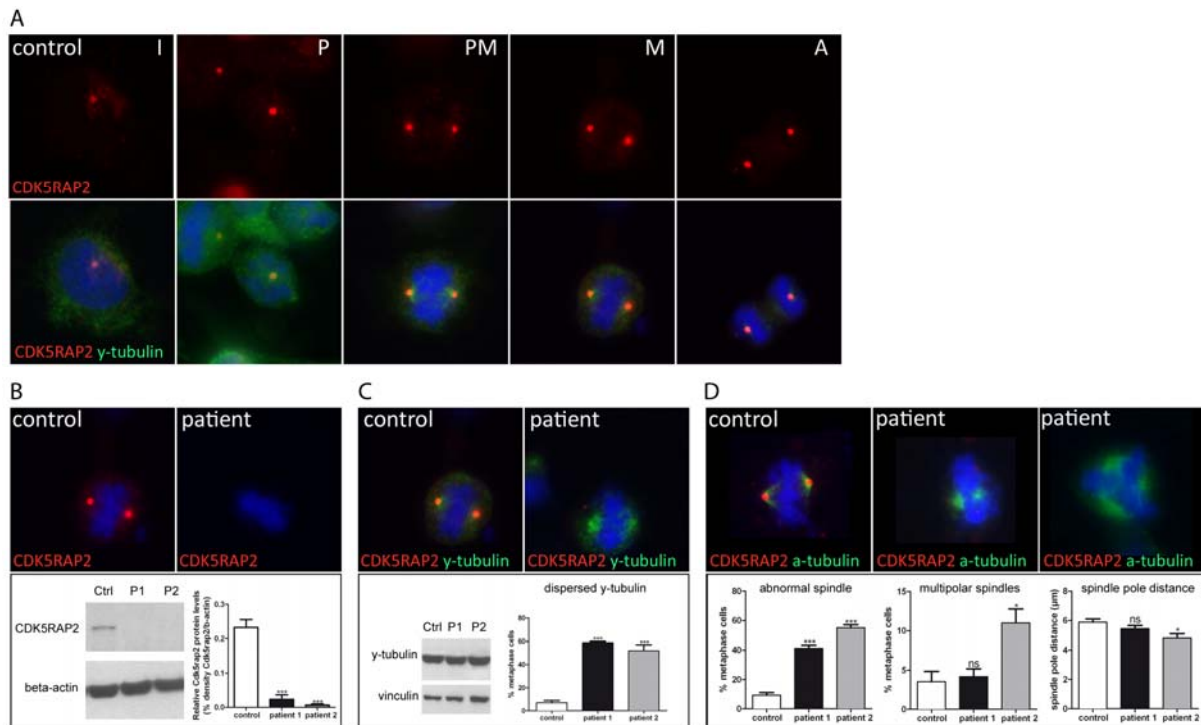

Figure 8. Cellular phenotype in *CDK5RAP2* mutant LCLs.

(A) CDK5RAP2 is enriched at the y-tubulin-positive centrosome throughout the cell cycle in control LCLs. In mutant LCLs; **(B)** CDK5RAP2 is below detection level; **(C)** y-tubulin is more dispersed around the centrosome despite normal total-y-tubulin levels; **(D)** abnormal spindles with broad and unfocused poles and decreased spindle pole distance appear; multi-polar spindles were observed in one patient ($*p<0.05$, $**p>0.01$, $***p<0.001$, One-way ANOVA; (68)). Interphase (I), prophase (P), prometaphase (PM), metaphase (M) and anaphase (A).

4.4 *Cdk5rap2* mutant mouse

To mimic the human mutation and further study the function of CDK5RAP2 *in vivo*, we generated a transgenic *Cdk5rap2* mouse. Through a Cre-LoxP strategy, a part of the y-tubulin ring complex (yTuRC)-binding domain was conditionally deleted, resulting in a frameshift and subsequently a stop codon at the beginning of exon 4 (see materials and methods; *Cdk5rap2 LoxP / hCMV Cre* (cKO)). Sequencing genomic DNA and cDNA as well as Southern blot analysis confirmed the generation of the planned cKO allele, the

successful Cre-mediated deletion of exon 3, and the presence of the translational stop codon. Still, we detected Cdk5rap2 protein using an antibody that recognizes the C-terminus, and the cKO mice did not display the expected microcephaly phenotype. Our further analysis indicated that a previously unknown splice variant of the *Cdk5rap2* gene exists, which allows translation of Cdk5rap2, even in the absence of exon 3. This has been confirmed by cDNA analysis. Two further splice trap mutation mice were also not microcephalic (*Cdk5rap2^{RRF465}*, *Cdk5rap2^{RRU031}*; (36)). While protein could be detected in low amounts in one of these mice, the mechanism responsible for the lack of phenotype in the other mouse line is unknown. These results are currently prepared for publication.

5. Discussion

The current hypothesis for the microcephaly phenotype in MCPH describes a premature shift from symmetric to asymmetric cell divisions, resulting to premature neurogenesis with a subsequent depletion of the progenitor cell pool (30, 47-49, 57). The underlying mechanisms for the suggested hypothesis include a deregulation of the CDK5RAP2 function at the centrosomes, a lack of proper spindle assembly and orientation, a lack of proper cleavage plane and/or a deregulation of the response to DNA damage (reviewed in (57)). However, these have not been demonstrated in humans, and there might be other unknown mechanisms involved since microcephaly occurs in an MCPH mouse model despite normal cleavage plane (70).

With the isolated brain phenotype of MCPH patients in mind, we began to study CDK5RAP2 function by characterizing the temporal and spatial expression pattern in the pre- and postnatal developing mouse and human brains including other organs (67). Our results provide the first systematic description of the temporal and spatial expression pattern of CDK5RAP2 in murine and human brain, and we detected a high degree of similarity between these two species with respect to CDK5RAP2 expression. We confirmed the high enrichment of CDK5RAP2 at the centrosomes and its association to the Golgi apparatus *in vivo*. We observed large numbers of CDK5RAP2-positive cells and high levels of CDK5RAP2 protein and mRNA expression in the germinal matrix and the neocortex of murine embryos and human fetuses. Here, CDK5AP2 is present in both symmetrically and asymmetrically dividing neural progenitors. Cdk5rap2 is strongly downregulated as the brain matures, but it remains present in preserved proliferation zones of the mature mouse and human brain. These finding are in line with results of neuroimaging studies demonstrating a reduced brain volume especially of the neocortex in MCPH patients (2, 5) as well as with experimental data demonstrating the role of CDK5RAP2 in neural progenitor proliferation (reviewed in Megraw et al. 2011).

In the murine and human neocortex, we found CDK5RAP2 immunopositivity particularly in the superficial cerebral cortex layers (67). These results are supported by the specific thinning of the superficial cerebral cortex layers in the MCPH3 mouse model *Hertwig´s* anemia mouse (48). In addition, superficial neocortical layers have been reported to be especially thinned in postmortem brains of non-genotyped *microcephaly*

vera patients (71, 72). Given the localization of CDK5RAP2 outside of the germinal matrix in murine and human neocortices, we analyzed its cell-type specific localization. Cdk5rap2 was highly present in early, but not in mature neurons. To our surprise, as the white matter is only slightly affected in MCPH patients, we detected high levels of Cdk5rap2 immunopositivity in glial cells (astrocytes, microglia, oligodendocytes). CDK5RAP2 is also present in other brain regions than the neocortex, especially in the hippocampus and in the cerebellum. Moreover, we detected Cdk5rap2 in migratory pathways within the mouse brain such as the rostral migratory stream, where progenitors and early differentiating neurons migrate toward the olfactory bulb, and the migration stream from the ganglionic eminences towards the cerebral cortex. It remains to be elucidated why the white matter as well as other brain regions are not more severely affected in patients, even given recent reports on a broader MCPH phenotype (e.g., cerebellar hypoplasia, corpus callosum agenesis, periventricular heterotopias). The brain phenotype of MCPH3 mouse models apart from the obvious differences of the neocortex has not been reported (48), and we will therefore address this point in future projects.

CDK5RAP2 is a centrosomal protein, and it is astonishing therefore that patients with homozygous mutations in the corresponding gene suffer only from an isolated brain phenotype instead of a multi-organ disorder. In one of the MCPH3 mouse models, "multiple organ defects" were noted but not further specified (48). In addition, one MCPH3 patient with acute leukemia as well as two MCPH3 patients with sensineural hearing loss have been reported (55). We detected high levels of CDK5RAP2 in various murine and human organs including thymus, kidney, heart, lung, liver, spleen, placenta, testes and intestines (67). To address this point, we analyzed in detail the phenotype of patients with a homozygous *CDK5RAP2* mutation (68). We specifically detected neither multiple organ defects nor a hematopoietic phenotype.

Three homozygous *CDK5RAP2* mutations had been described in three Pakistani families and in one patient from Somalia at the beginning of my PhD thesis project. All of these mutations had been proposed (but not shown) to lead to truncated proteins and loss of CDK5RAP2 function. Therefore, in parallel to our investigation of the spatiotemporal expression pattern of CDK5RAP2, we searched for further patients with MCPH3. We were able to identify a novel homozygous nonsense mutation in the *CDK5RAP2* gene (c.4441C>T, p.R1481X) in the first two European patients (68). These

patients show classical MCPH phenotypes and also previously not acknowledged severe behavioral problems. We investigated the pathogenicity of the identified mutation in immortalized patient and control lymphocytes. In patient cells, CDK5RAP2 protein levels were below detection level, which further supports the proposed loss of CDK5RAP2 function in MCPH3. Moreover, mitotic spindle defects and disrupted γ-tubulin localization to the centrosome were apparent. We were thereby able to attribute the microcephaly phenotype in MCPH3 at least partially to a loss of centrosome integrity and to a mitotic spindle defect. We identified further patients with *CDK5RAP2* mutations, and we are planning *in vitro* studies on human primary cells in order to study specifically proliferation.

To study the role of Cdk5rap2 and to mimic the human mutation *in vivo*, we generated a transgenic Cdk5rap2 mouse model (cKO) in which exon 3 encoding a part of the γ-tubulin ring complex (γTuRC)-binding domain can be conditionally deleted (Kraemer et al., in preparation). The deletion should result in a frameshift and a subsequent stop codon at the beginning of exon 4. We confirmed the successful Cre-mediated deletion of exon 3, and the presence of the translational stop codon that should be caused in the mRNA containing the frameshift by sequencing genomic DNA and cDNA as well as by Southern blot analysis. Despite the generation of the planned cKO allele, Cdk5rap2 protein could still be detected in the transgenic mice. In addition, the cKO mice did not show the expected microcephaly phenotype. Further analysis revealed the existence of a previously unknown splice variant of the *Cdk5rap2* gene, allowing translation of *Cdk5rap2* in the absence of exon 3. This has been confirmed by cDNA analysis. It remains to be elucidated whether this variant is also present in the human.

Brain size at birth is largely determined by the relative rates of proliferation and cell death. By highlighting regions of physiological CDK5RAP2 expression in human fetuses and infants, we offer a further glimpse into how a disruption of the CDK5RAP2 gene may impact on the development of particular brain systems in humans. CDK5RAP2 localizes to the germinal zones of the cortex in mice and humans, and its colocalization with markers of proliferating/progenitor cells underlines its proposed role in symmetric and asymmetric progenitor cell divisions and subsequent neocortical expansion during brain development. MCPH is considered as a predominant "neuronal disorder". However, our results indicate a further function of Cdk5rap2 in glia cells, where

Cdk5rap2 is also expressed. Future studies will need to address the molecular function of Cdk5rap2 in the white matter as well as in other brain regions and other organs in animal models and patients (by neuroimaging and on the basis of postmortem samples). Moreover, it needs to be addressed how various MCPH-associated proteins cause the same human phenotype when dysfunctional and whether these proteins interact directly or indirectly. In addition, an identification and in-depth characterization of further patients with biallelic *CDK5RAP2* mutations may provide a means to investigate processes that cause MCPH and to verify mechanisms described in other model systems and in settings where animal models are neither sufficient nor satisfactory.

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68. Issa L, Mueller K, Seufert K, Kraemer N, Rosenkotter H, Ninnemann O*, et al.* Clinical and cellular features in patients with primary autosomal recessive microcephaly and a novel CDK5RAP2 mutation. *Orphanet J Rare Dis* 2013; 8: 59.

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7. Affirmation in lieu of an oath (Eidesstattliche Versicherung)

„Ich, Lina Jahns, versichere an Eides statt durch meine eigenhändige Unterschrift, dass ich die vorgelegte Dissertation mit dem Thema: „The Role of CDK5RAP2 in Autosomal Recessive Primary Microcephaly (MCPH)" selbstständig und ohne nicht offengelegte Hilfe Dritter verfasst und keine anderen als die angegebenen Quellen und Hilfsmittel genutzt habe.

Alle Stellen, die wörtlich oder dem Sinne nach auf Publikationen oder Vorträgen anderer Autoren beruhen, sind als solche in korrekter Zitierung (siehe „Uniform Requirements for Manuscripts (URM)" des ICMJE -*www.icmje.org*) kenntlich gemacht. Die Abschnitte zu Methodik (insbesondere praktische Arbeiten, Laborbestimmungen, statistische Aufarbeitung) und Resultaten (insbesondere Abbildungen, Graphiken und Tabellen) entsprechen den URM (s.o) und werden von mir verantwortet.

Meine Anteile an den ausgewählten Publikationen entsprechen denen, die in der untenstehenden gemeinsamen Erklärung mit dem/der Betreuer/in, angegeben sind. Sämtliche Publikationen, die aus dieser Dissertation hervorgegangen sind und bei denen ich Autor bin, entsprechen den URM (s.o) und werden von mir verantwortet.

Die Bedeutung dieser eidesstattlichen Versicherung und die strafrechtlichen Folgen einer unwahren eidesstattlichen Versicherung (§156,161 des Strafgesetzbuches) sind mir bekannt und bewusst."

_____

Datum / Date   Unterschrift / Signature

8. Authors contributions (Anteilserklärung an den erfolgten Publikationen)

Lina Jahns (geb. Issa) hatte folgenden Anteil an den folgenden Publikationen:

Publications selected for PhD:

Publication 1: Housekeeping genes and development

Kraemer N, Neubert G, **Issa L**, Ninnemann O, Seiler AE, Kaindl AM. Reference genes in the developing murine brain and in differentiating embryonic stem cells. Neurol Res. 2012;34(7):664-8.

Contribution:

LI isolated murine brain samples, participated in the qPCR process, and proofread the manuscript.

Publication 2: Cdk5rap2 in brain development

Issa L, Kraemer N, Rickert CH, Sifringer M, Ninnemann O, Stoltenburg-Didinger G, Kaindl AM. CDK5RAP2 Expression During Murine and Human Brain Development Correlates with Pathology in Primary Autosomal Recessive Microcephaly. Cereb Cortex. 2013;23(9):2245-60.

Contribution:

LI performed the human and murine brain analysis, generated figures, participated in writing of the manuscript and proofread it.

Publication 3: Clinical and cellular *CDK5RAP2* phenotype:

Issa L, Mueller K, Seufert K, Kraemer N, Rosenkotter H, Ninnemann O, Buob M, Kaindl AM, Morris-Rosendahl DJ. Clinical and cellular features in patients with primary autosomal recessive microcephaly and a novel CDK5RAP2 mutation. Orphanet J Rare Dis. 2013;8:59.

Contribution:

LI performed the lymphocyte analysis, generated figures, participated in writing of the manuscript and proofread it.

Additional publications:

Publikation 4: Many roads lead to primary autosomal recessive microcephaly

Kaindl AM, Passemard S, Kumar P, Kraemer N, **Issa L**, Zwirner A, Gerard B, Verloes A, Mani S, Gressens P. Many roads lead to primary autosomal recessive microcephaly. Prog Neurobiol. 2010; 90(3):363-83.

Contribution:
LI participated in writing of the manuscript, and proofread it.

Publikation 5: What's the hype about CDK5RAP2?

Kraemer N, **Issa L**, Hauck SC, Mani S, Ninnemann O, Kaindl AM. What's the hype about CDK5RAP2? Cell Mol Life Sci. 2011;68(10):1719-36

Contribution:
LI participated in writing of the manuscript, and proofread it.

Unterschrift, Datum und Stempel des betreuenden Hochschullehrers/der betreuenden Hochschullehrerin

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8.1 Housekeeping genes and development

Kraemer N, Neubert G, **Issa L**, Ninnemann O, Seiler AE, Kaindl AM. Reference genes in the developing murine brain and in differentiating embryonic stem cells. Neurol Res. 2012;34(7):664-8.

Quantitive real-time PCR (qPCR) is a powerful method to analyze gene expression. For obtaining successful and reliable results by qPCR, a reference gene, which is used as an internal standard must be chosen. The aim of the present study was to identify suitable reference gene for *Cdk5rap2* gene expression analysis the developing murine neocortex *in vivo* and in undifferentiated, as well as differentiating mouse embryonic stem cells (mESC) *in vitro.* We found *RNA polymerase II* (*RpII*) to be a good reference gene to use when *Cdk5rap2* gene expression is studied in mESC. Furthermore our results show that glycerinaldehyde-3- phosphate dehydrogenase (*Gapdh*) and hypoxanthine phophoribosyltransferase (*Hprt*) are good reference gene candidates for *Cdk5rap2* expression analysis in murine neocortex.

http://dx.doi.org/10.1179/1743132812Y.0000000060

8.2 Cdk5rap2 in brain development

Issa L, Kraemer N, Rickert CH, Sifringer M, Ninnemann O, Stoltenburg-Didinger G, Kaindl AM. CDK5RAP2 Expression During Murine and Human Brain Development Correlates with Pathology in Primary Autosomal Recessive Microcephaly. Cereb Cortex. 2013;23(9):2245-60.

Primary autosomal recessive microcephaly (MCPH) is a rare neurodevelopmental disorder, characterized by a pronounced reduction of brain volume, particularly of the cerebral cortex. MCPH genes are proposed to have a role in the mammalian brain expansion and stem-cell biology. Homozygous mutations in the cyclin-dependent kinase-5 regulatory subunit-associated protein 2 gene *CDK5RAP2* cause MCPH type 3. In this study, we show, for the first time, the spatiotemporal expression of CDK5RAP2 in the developing brain of mouse and human. From our studies, we found intriguing concordance between regions of high CDK5RAP2 expression in the mouse and sites of pathology suggested by neuroimaging studies in humans and mouse. We show that CDK5RAP2 is highly expressed in proliferative regions of the developing murine and human brain, underlining the function of CDK5RAP2 in cell proliferation and arguing for its role in the expansion of the mammalian cerebral cortex.

8.3 Clinical and cellular *CDK5RAP2* phenotype

Issa L, Mueller K, Seufert K, Kraemer N, Rosenkotter H, Ninnemann O, Buob M, Kaindl AM, Morris-Rosendahl DJ. Clinical and cellular features in patients with primary autosomal recessive microcephaly and a novel CDK5RAP2 mutation. Orphanet J Rare Dis. 2013;8:59.

In 2005, homozygous mutations in *CDK5RAP2* gene were first reported to cause MCPH3. At the time of this research work, three different *CDK5RAP2* mutations had been identified; however, no detailed radiological descriptions of patients or functional analyses in patient samples had been reported. In this study, we report a novel *CDK5RAP2* gene mutation and, for the first time, provide a detailed clinical, radiological, and cellular description. From the cellular phenotype detected in our patients, we could show that the microcephaly phenotype in MCPH3 is at least partially caused by mitotic spindle defect and centrosome disorganization.

8.4 Many roads lead to primary autosomal recessive microcephaly

Kaindl AM, Passemard S, Kumar P, Kraemer N, **Issa L**, Zwirner A, Gerard B, Verloes A, Mani S, Gressens P. Many roads lead to primary autosomal recessive microcephaly. Prog Neurobiol. 2010; 90(3):363-83.

Primary autosomal recessive microcephaly (MCPH) is a heterogeneous, rare neurodevelopmental disease. Understanding MCPH might give us an insight into the physiologic brain development and especially that of the cerebral cortex. *MCPH* genes have moved into the spotlight, since they might have contributed to the evolutionary expansion of the mammalian cerebral cortex. Here, we give a summary of MCPH phenotype and genotype in addition to the pathomechanisms suggested to contribute to this disease.

8.5 What's the hype about CDK5RAP2?

Kraemer N, **Issa L**, Hauck SC, Mani S, Ninnemann O, Kaindl AM. What's the hype about CDK5RAP2? Cell Mol Life Sci. 2011;68(10):1719-36.

Biallelic mutations in the *CDK5RAP2* gene cause the neurodevelopmental disease MCPH3. Over the years, researchers have been investigating the pathogenesis of MCPH3 and the role of CDK5RAP2 in the pathomechanism of MCPH3. Here, we give a timely overview of what has been described so far regarding CDK5RAP2.

Mein Lebenslauf wird aus datenschutzrechtlichen Gründen in der elektronischen Version meiner Arbeit nicht veröffentlicht.

My curriculum vitae is not published for privacy reasons in the electronic version of my thesis.

10. List of publications

Original publications

1   Kaindl AM, Degos V, Peineau S, Gouadon E, Chhor V, Loron G, Le Charpentier T, Josserand J, Ali C, Vivien D, Collingridge GL, Lombet A, **Issa L**, Rene F, Loeffler JP, Kavelaars A, Verney C, Mantz J, Gressens P. Activation of microglial N-methyl-D-aspartate receptors triggers inflammation and neuronal cell death in the developing and mature brain. Ann Neurol. 2012;72(4):536-49.

2   Kraemer N, Neubert G, **Issa L**, Ninnemann O, Seiler AE, Kaindl AM. Reference genes in the developing murine brain and in differentiating embryonic stem cells. Neurol Res. 2012;34(7):664-8.

3   **Issa L**, Kraemer N, Rickert CH, Sifringer M, Ninnemann O, Stoltenburg-Didinger G, Kaindl AM. CDK5RAP2 Expression During Murine and Human Brain Development Correlates with Pathology in Primary Autosomal Recessive Microcephaly. Cereb Cortex. 2013;23(9):2245-60.

4   **Issa L**, Mueller K, Seufert K, Kraemer N, Rosenkotter H, Ninnemann O, Buob M, Kaindl AM, Morris-Rosendahl DJ. Clinical and cellular features in patients with primary autosomal recessive microcephaly and a novel CDK5RAP2 mutation. Orphanet J Rare Dis. 2013;8:59.

Reviews

1   Kaindl AM, Passemard S, Kumar P, Kraemer N, **Issa L**, Zwirner A, Gerard B, Verloes A, Mani S, Gressens P. Many roads lead to primary autosomal recessive microcephaly. Prog Neurobiol. 2010; 90(3):363-83.

2   Kraemer N, **Issa L**, Hauck SC, Mani S, Ninnemann O, Kaindl AM. What's the hype about CDK5RAP2? Cell Mol Life Sci. 2011;68(10):1719-36.

Abstracts for congress presentations

1   Kraemer N, **Issa L**, Zwirner A, Kaindl AM. Expression Pattern of Cdk5rap2 in Murine Brain Development, Berlin Neuroscience Forum, Berlin, Germany, 10-11.6.2010 (Poster).

2   **Issa L**, Kraemer N, Zwirner A, Ninnemann O, Stoltenburg-Didinger G, Kaindl AM. Cdk5rap2 In Murine Cortical Development, 5th congress of Federation of Asia and Oceanian Neuroscience, Lucknow, India, 25-28.11.2010 (Poster).

3   Krämer N, **Issa L**, Zwirner A, Kaindl AM.   Primäre autosomal rezessive Mikrozephalie (MCPH) und Cdk5rap2, 1. Otto Heubner Wissenschaftsretreat 2011, Berlin, Germany, 08.01.2011 (Lecture).

4   **Issa L**, Kraemer N, Zwirner A, Stoltenburg-Didinger G, Kaindl AM. Cdk5rap2 in The developing murine cortex, Cortical Development Meeting, Chania, Greece, 19-22.5.2011 (Poster).

5   Krämer N, **Issa L**, Zwirner A, König J, Neubert G , Kaindl AM. Primäre autosomal rezessive Mikrozephalie (MCPH) und Cdk5rap2, 107. DGKJ Jahrestagung, Bielefeld, Germany, 22.-25.09.2011 (Poster).

6   **Issa L**, Kraemer N, Zwirner A, Rickert C, Ninnemann O, Stoltenburg-Didinger G, Kaindl AM. Cdk5rap2 in murine and human brain development, Forschungsseminar Neuropädiatrie, Berlin, Germany, 20.10.2011 (Poster).

7   Krämer N, **Issa L**, Neubert G, Seiler A, Ninnemann O, Kaindl AM. Cdk5rap2 in murinen embryonalen Stammzellen (mESC): Etablierung eines *in vitro* Mikrozephalie Modells, Forschungsseminar Neuropädiatrie, Berlin, Germany, 20.10.2011 (Lecture).

8   **Issa L**, Kraemer N, Zwirner A, Rickert C, Ninnemann O, Stoltenburg-Didinger G, Kaindl AM. Cdk5rap2 in Murine and Human Brain Development, SFB665 meeting, Berlin, Germany, 27-29.10.2011 (Poster).

9   Krämer N, **Issa L**, Neubert G, Zwirner A, König J, Ninnemann O, Kaindl AM. Cdk5rap2 in Murine Embryonic Stem Cells: Establishment of an *In vitro* Model for Microcephaly. SFB665 meeting, Berlin, Germany, 27-29.10.2011 (Poster).

10   **Issa L**, Kraemer N, Zwirner A, Rickert C, Ninnemann O, Stoltenburg-Didinger G, Kaindl AM. Cdk5rap2 in the developing murine and human brain, Centrosomen Workshop, Berlin, Germany, 30.11.2011 (Lecture).

11   **Issa L**, Kraemer N, Zwirner A, Rickert C, Ninnemann O, Stoltenburg-Didinger G, Kaindl AM. Cdk5rap2 in Murine and Human Brain Development, Berlin Brain Days, Berlin, Germany, 7-9.12.2011 (Poster).

12   Krämer N, **Issa L**, Neubert G, Seiler A, Ninnemann O, Kaindl AM. Microcephaly and Effects of Cdk5rap2 Downregulation in Murine Embryonic Stem Cells. 38. Jahrestagung GNP, Münster, Germany, 19.-22.04.2012 (Lecture; abstract, published in: Neuropediatrics 2012;43(2):105-106).

13   Krämer N, **Issa L**, Neubert G, Seiler A, Ninnemann O, Kaindl AM. Mikrozephalie und Effekte einer Cdk5rap2 Runterregulierung in murinen embryonalen Stammzellen. 38. Jahrestagung GNP, Münster, Germany, 19.-22.04.2012 (Lecture; abstract, published in: Neuropädiatrie in Klinik und Praxis 2012;2:64).

14   **Issa L**, Mueller K, Kraemer N, Morris-Rosendahl D, Kaindl AM. Spindle Defects and Disruption of Centrosome Integrity in Primary Microcephaly Patients With CDK5RAP2 Mutation, Berlin Neuroscience Forum, Berlin, Germany, 31.5-1.6.2012 (Poster).

15   **Issa L**, Kraemer N, Zwirner A, Rickert C, Ninnemann O, Stoltenburg-Didinger G, Kaindl AM. CDK5RAP2 expression during murine and human brain development correlates with pathology in primary autosomal recessive microcephaly, 57th Annual Meeting of the German Society for Neuropathology and Neuroanatomy, Erlangen, Germany, 12-15.9.2012 (Poster).

16   **Issa L**, Kraemer N, Zwirner A, Rickert C, Ninnemann O, Stoltenburg-Didinger G, Morris-Rosendahl D, Kaindl AM. CDK5RAP2 expression during murine and human brain development correlates with cellular phenotype in MCPH3 patients, 10th Göttingen Meeting of the German Neuroscience Society, Göttingen, Germany, 13-16.3.2013 (Poster).

17   Kraemer N, **Issa L**, Neubert G, Seiler A, Ninnemann O, Kaindl AM. Effects of Cdk5rap2 Downregulation in Murine Embryonic Stem Cells correlate with cellular phenotype in MCPH3 patients, Jahrestagung der GNP, Innsbruck, Austria, 25.-28.04.2013 (Poster).

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