CDK5RAP2 interaction with components of the Hippo signaling pathway may play a role in primary microcephaly

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Received: 5 July 2016 / Accepted: 28 November 2016 / Published online: 21 December 2016 © The Author(s) 2016. This article is published with open access at Springerlink.com

Abstract Autosomal recessive primary microcephaly (MCPH) is characterized by a substantial reduction in brain size but with normal architecture. It is often linked to mutations in genes coding for centrosomal proteins; however, their role in brain size regulation is not completely understood. By combining homozygosity mapping and whole-exome sequencing in an MCPH family from Pakistan, we identified a novel mutation (XM_011518861.1; c.4114C > T) in CDK5RAP2, the gene associated with primary microcephaly-3 (MCPH3), leading to a premature stop codon (p.Arg1372*). CDK5RAP2 is a component of the pericentriolar material important for the microtubule-organizing function of the centrosome. Patient-derived primary fibroblasts had strongly decreased CDK5RAP2 amounts, showed centrosomal and nuclear abnormalities and exhibited changes in cell size and migration. We further identified an interaction of CDK5RAP2 with the Hippo pathway components MST1 kinase and the transcriptional regulator TAZ. This finding potentially provides a mechanism through which the Hippo pathway with its roles in the regulation of centrosome number is linked to the centrosome. In the patient fibroblasts, we observed higher levels of TAZ and YAP. However, common target genes of the Hippo pathway were downregulated as compared to the control with the exception of BIRC5 (Survivin), which was significantly upregulated. We propose that the centrosomal deficiencies and the altered cellular properties in the patient fibroblasts can also result from the observed changes in the Hippo pathway components which could thus be relevant for MCPH and play a role in brain size regulation and development.

Keywords Centrosome · MCPH · Hippo pathway · YAP/TAZ · MST1

Introduction

Autosomal recessive primary microcephaly (MCPH; MIM 251200) is a rare heterogeneous developmental congenital brain disorder characterized by a reduced occipitofrontal circumference of the head. Mutations in MCPH genes reduce the population of neurons in each of the six layers of the cerebral cortex during development leading to reduced thickness of the cerebral cortex. Patients with mutations at MCPH loci show moderate to severe microcephaly. Some
of the notable phenotypes of microcephaly patients are a decrease in head size and brain volume, seizures and mental retardation, but no motor deficit (Kaindl et al. 2010). According to the OMIM data base, 16 genes have been identified as cause of MCPH. Several of the MCPH-associated proteins localize to centrosomes; however, there is still little information available relating to centrosomal mutations and brain size regulation. The centrosome is the main microtubule-organizing center in the cell and is involved in many different cellular processes, particularly during cell division, cell migration and differentiation (Bornens 2012; Conduit et al. 2015).

Cyclin-dependent kinase 5 regulatory subunit 2 (CDK5RAP2) is a pericentriolar structural component functioning in γ-tubulin ring complex (γTuRC) attachment and in the microtubule-organizing role of the centrosome (Kraemer et al. 2011; Fong et al. 2008, 2009). Mutations of CDK5RAP2 are the cause of both primary microcephaly and Seckel syndrome (Bond et al. 2005; Yigit et al. 2015). CDK5RAP2 is a 215-kDa protein originally identified as CDK5 regulatory kinase 1 (CDK5R1)-binding protein. Near the N terminus it contains the γTuRC-binding site followed by an EB1-binding domain, the CDK5R1-interaction domain, a domain responsible for pericentrin binding and for Golgi complex association and several SMC domains along the molecule. It has many roles, among others it regulates mitotic spindle positioning, asymmetric centrosome inheritance, centriole replication, DNA damage signaling, and also has a spindle checkpoint function (Barr et al. 2010; Zhang et al. 2009; Barrera et al. 2010; Lizarraga et al. 2010).

A mouse mutant, Hertwig’s anemia (an) mouse, provided the first mechanistic link between the centrosome function of CDK5RAP2 and primary microcephaly. In this mouse, which suffers from severe hypoproliferative anemia and leukopenia and shows a high level of spontaneous aneuploidy in primary cultures of fetal cells, the underlying mutation was identified in Cdk5rap2. Similar to human the mice exhibited microcephaly with neurogenic defects including proliferative and survival defects in neuronal progenitors (Lizarraga et al. 2010). Phenotypes of the patients that result from CDK5RAP2 mutations are sensorineural hearing loss, intellectual disability and a reduced occipital frontal head circumference (Pagnamenta et al. 2012; Issa et al. 2013). Therefore, from the human phenotype and the mouse mutant, an influence of CDK5RAP2 on the regulation of brain size during fetal development is apparent. Whether mutations in CDK5RAP2 and the decrease in neuronal cell density are associated with altered signal transduction pathways is not really known although phosphorylation by LRRK1, a kinase that regulates the orientation of mitotic spindles, has been reported. This phosphorylation may affect the formation of the CDK5RAP2–γTuRC complex (Hanafusa et al. 2015). We recently reported that CEP161, the CDK5RAP2 ortholog of Dictyostelium discoideum, binds to hippo-related kinase Hrk-svk and inhibits its kinase activity thereby presumably leading to inactivation of the pathway. We further found that it colocalizes with MST1 at the centrosome (Sukumaran et al. 2015).

The Hippo signaling pathway functions to restrict growth in adult tissues and modulates cell proliferation, differentiation, and migration in developing organs. Thus, it is a tumor-suppressive pathway. It primarily affects the number of cells produced whereas it has only minor effects on tissue patterning (Halder and Johnson 2011; Yu et al. 2015). The center of the Hippo pathway is formed by a kinase cassette consisting of two related serine/threonine kinases, mammalian STE20-like protein kinase 1 (MST1 and MST2), that are homologous to D. melanogaster Hippo (HPO), and large tumor suppressor 1 (LAT51) and LATS2 together with the adaptor proteins Salvador homologue 1 (SAV1) and MOB kinase activator 1A (MOB1A) and MOB1B (Udan et al. 2003; Harvey et al. 2003). They limit tissue growth by facilitating LAT51- and LATS2-dependent phosphorylation of the transcriptional activators Yes-associated protein (YAP) and transcriptional co-activator with PDZ-binding motif (TAZ) which promotes 14-3-3 binding resulting in their retention in the cytosol (Kanai et al. 2000).

YAP and TAZ function through regulation of the activity of several families of transcription factors such as Transcriptional Enhancer Factor Domain (TEAD) and Similar to Mothers Against Decapentaplegic (SMAD) family members. TEADs are key mediators of growth and presumably are responsible for the tumorigenic potential of YAP and TAZ. The genetic program that is regulated by these factors and promotes tissue growth is not well defined (Hong et al. 2005). Hippo signaling is also crucial for regulating the size of the mammalian liver; however, it does not appear to regulate the size or growth of other mammalian tissues to the same degree (Dong et al. 2007; Song et al. 2010).

In this study, we report the identification of a novel mutation in CDK5RAP2 in an MCPH family. We further investigate a potential interaction of CDK5RAP2 with the Hippo signaling pathway and use patient-derived fibroblasts to study whether Hippo signaling is affected by the mutation. We identified MST1 as a CDK5RAP2 interaction partner and found that CDK5RAP2 has an impact on components of the Hippo signaling pathway such as YAP and TAZ. YAP/TAZ plays important roles in development in general and also in brain development as demonstrated in vertebrates (Piccolo et al. 2014). It was also shown that when YAP/TAZ is inhibited the expansion of neural progenitor cells is limited (Lavado et al. 2013). A role for MST1 at the centrosome and particularly in centriole formation has been shown previously (Hergovich et al. 2009).
We find that MST1 knockdown has effects on centrosome nucleus distance, whereas the association of CDK5RAP2 with the centrosome appeared unperturbed. Based on our findings, we propose that aside from its role as a centrosomal component, CDK5RAP2 might have an additional role in the regulation of the brain size through its interaction with MST1 which impacts on the activity of the Hippo signaling pathway.

Materials and methods

Subjects

Approval of this study was obtained from the ethics review board of the Medical Faculty, University of Cologne and the National Institute for Biotechnology and Genetic Engineering in Faisalabad, Pakistan, according to the Declaration of Helsinki protocols. After getting consent from the parents, venous blood was obtained from both affected persons, parents and one from a normal individual of the MCP105 family.

Linkage analysis

DNA was extracted from peripheral blood samples using standard methods. All the available individuals from the family were genotyped using highly polymorphic microsatellite markers spanning the regions of seven known MCPH loci. Later on, the identified homozygosity at the MCPH3 locus was corroborated by the genotyping of two affected individuals with the Axiom® Genome-wide CEU Array according to the manufacturer’s protocols (Affymetrix, Santa Clara, CA). Assuming autosomal recessive mode of inheritance, full penetrance, consanguinity and a disease allele frequency of 0.0001, we performed linkage analysis as described previously (Hussain et al. 2013).

Genomic DNA sequencing

One microgram DNA of affected individual VI-2 was used for whole-exome sequencing (WES). SeqCap EZ Human Exome Library v2.0 kit from NimbleGen (Roche NimbleGen Inc., Madison, WI 53719, USA) was used which needs a DNA probe for sequencing and the sample was run on an Illumina HiSeq™ 2000 sequencing instrument. The detailed procedure for WES carried out here was also described previously (Hussain et al. 2013). VARBANK database (https://varbank.ccg.uni-koeln.de) designed at the Cologne Center for Genomics (CCG) was used to filter data and to prioritize variants. Sanger sequencing was employed to sequence the targeted region of exon 27 of CDK5RAP2 harboring the mutation. Primer sequences to amplify exon 27 are enlisted in Supplementary Table 1.

Cell culture and transfection experiments

HeLa and HEK293T cells were grown in Dulbecco’s modified Eagle’s medium (DMEM) supplemented with 10% fetal bovine serum, penicillin and streptomycin (100 μg/ml), L-glutamine and non-essential amino acids at 37 °C and 5% CO₂. Transfection was done using Lipofectamine 2000 (Invitrogen) according to the manufacturer’s instructions (HeLa) or Polyethyleneimine (PEI, Polysciences, cat No 23966-2) for HEK293T cells. HeLa cells were used for immunofluorescence analysis, HEK293T cells for protein analysis. Transfected cells were incubated for 24–48 h post-transfection. A CDK5RAP2 plasmid was obtained from addgene (pRCMV Cep215 (Nigg CW493), 40). It was used for generation of truncated versions and point mutations which were introduced by site-directed mutagenesis. The following mutant versions encoding shortened polypeptides were generated: CDK5RAP2-C corresponding to nonsense mutation c.246T > A, p.Y82* (residues 1–82) (Park et al. 2011), CDK5RAP2-C1 (residues 1–580) encompasses the γTuRC and SMC domains, CDK5RAP2-C2 (residues 1–1271) and CDK5RAP2-C3 (residues 1–1372) encompass the γTuRC, SMC and EB1 domain. GFP-MST1 and Flag-TAZ are described in Habbig et al. (2011); Flag-hnRNPF was obtained from Dr. Ping Li. MST1 knockdown was achieved in HeLa cells by siRNA using MST1 siRNA (Set I S25-911-05, SignalChem, Richmond, BC, Canada). RNAi transfection was performed using Interferin (Polyplus) as transfection reagent and siRNAs at a concentration of 1 to 10 nM. The protocol provided by the manufacturer was followed. 72 h after transfection cells were fixed or harvested. The efficiency of the knockdown was assessed by western blot analysis. For control, cells were treated with siRNA control oligos (Li and Noegel 2015).

Patient cell culture and analysis

Primary fibroblasts were established from a biopsy of patient VI-2. The detailed procedure has been previously described (Hussain et al. 2013). The fibroblasts from patient (passages 3 and 4) and respective controls (passages 4 and 6) were cultured in Dulbecco’s Modified Eagle’s Medium (DMEM, PAA supplemented with 10% fetal bovine serum (FBS, Biochrom), L-Glutamine (PAN Biotech) and antibiotics (Penicillin/Streptomycin, PAN Biotech). Care was taken to carry out all experiments with cells at similar densities and passage number.

Cell cycle and growth analysis Cells were trypsinized and 5 × 10⁵ cells dissolved in DMEM were stored on ice.
After incubation with Nuclear-ID™ Red DNA stain (Enzo Life Sciences), diluted 1:250, for 5 min at 37 °C, the cells were sorted immediately using the BD FACSAria III cell sorter (BD Bioscience USA). Cell sorting was carried out at the CMMC facilities. For analysis of growth, cells were trypsinized and counted.

**Cell size determination** For cell size determination, the cells were trypsinized and photos immediately taken with a microscope using a 10× objective. Diskus software was used for size measurements.

**Cell migration assay** For cell migration experiments, cells were seeded at equal densities in Ibidi culture inserts placed in eight-well Ibidi μ-slides. Next day, the inserts were removed and cells were allowed to migrate into the 500-μm gap generated by the insert and images were captured at 15-min intervals for 24 h with a Leica microscope (Leica DMi6000 B, LAS AF software version 2.0.2 build 2038) equipped with a camera (Hamamatsu) and using a 10×/0.25 NA dry objective and magnification of 1.6×. The slides with fibroblasts were placed in a humidified 5% CO2 atmosphere. For 37 °C warm air incubation the microscope and objectives were encased (heater and ventilation ON).

Later, processing and analysis was performed using Image J ‘Manual Tracking’ and ‘Chemotaxis tool’. To study polarization, cells were fixed at the indicated time points and stained for Golgi and centrosome with 58 K Golgi protein (Abcam) and pericentrin-specific antibodies (Abcam), respectively. Golgi and centrosome polarity was determined for the first row of cells by drawing a wound-facing 120° sector on the cells. In general, the experiments were carried out at least three times or as indicated.

**RNA isolation and cDNA generation for quantitative RT-PCR analysis**

Total RNA was extracted from cells grown in a monolayer in cell culture dishes with a kit following the instructions of the supplier (Promega, Heidelberg, Germany). First-strand cDNA synthesis was performed using M-MLV reverse transcriptase RNase H Minus-kit from Promega. Each sample for real-time RT-PCR analysis contained 200 ng of cDNA, SYBR Green Master Mix and 0.4 μM of each primer. The PCR amplification and real-time fluorescence detection were performed with the Opticon III instrument (MJ Research) using the QuantitectTM SYBR1 green PCR kit (Qiagen, Hilden, Germany). As quantification standard defined concentrations of annexinA7 cDNA were used for amplification. PCR amplification was carried out according to the manufacturer’s instruction and all PCR products were amplified in a linear cycle. GAPDH mRNA was employed as an internal standard, and expression of each gene was determined by RT-PCR and normalized against WT GAPDH mRNA levels. Data are the mean ± SD from three samples per group of four independent experiments. All primers are listed in Supplementary Table S1.

**Immunofluorescence analysis**

For immunofluorescence, cells were grown on 12-mm coverslips and fixed with 3% paraformaldehyde (5 min, RT), followed by permeabilization with 0.5% Triton X-100 for 3 min (RT). Further steps were done as described (Husain et al. 2013). Imaging was done by confocal laser scanning microscopy (Leica TCS-SP5). Images were processed using TCS-SP5 software. Antibodies against the following proteins were used: CDK5RAP2 (pAb, 06-1398, Millipore), anti-Myc (mAb 9E10), β-actin (mAb A2228, Sigma), α-tubulin (mAb YL1/2), γ-tubulin (mAb, T6557, Sigma), YAP/TAZ (mAb, 8418, Cell Signaling), MST1 (pAb, 3682, Cell Signaling), phospho-MST1/MST2 (pAb, 3681, Cell Signaling), GM130 (mAb, 618822, abcam), 58 K (mAb, ab27043, Abcam), pericentrin (pAb, ab4444, Abcam), γ-tubulin (mAb GTU-88, Sigma), Nesnpr-1 (SpecII; (Taranum et al. 2012), BIRC5 (mAb, ab76424, Abcam), Ki67 as cell proliferation marker (mAb, M 7249, DAKO), anti-PH3 rabbit polyclonal IgG to stain mitotic cells (06-570, Upstate Biotechnology) and Cleaved Caspase-3 (ASP175)-specific polyclonal antibodies as apoptosis marker (No 9661, Cell signaling).

**Immunoprecipitation**

For immunoprecipitation experiments, HEK293T cells were lysed in 10 mM Tris/HCl, pH 7.4, 50 mM NaCl, 0.5% NP40, protease inhibitor cocktail, 0.5 mM PMSF, 0.5 mM EDTA, 1 mM Benzamidine and incubated for 30 min at 4 °C (to ensure complete cell lysis) followed by a centrifugation step at 16,000 rpm for 10 min at 4 °C. The supernatants were either incubated with GFP-trap beads (ChromoTek, Martinsried, Germany) or with Flag-trap beads (Sigma, St. Louis, USA, Catalog Number A2220) for 2 h at 4 °C. GFP-trap beads were washed with a different wash buffer (10 mM Tris/HCl, pH 7.4, 50 mM NaCl, protease inhibitor cocktail (Roche), 0.5 mM PMSF, 0.5 mM EDTA, 1 mM Benzamidine). The beads were resuspended in SDS sample buffer, incubated at 95 °C for 5 min and the proteins were separated by SDS-PAGE and analyzed by western blotting using anti-GFP mAb K3-184-2 (Noegel et al. 2004) and Flag-tag specific rabbit polyclonal Ab (Sigma).

**Protein extraction from mammalian cells**

Mammalian cells were trypsinized and washed with ice-cold 1x PBS plus protease inhibitor (DTT, Benzmidine and PMSF at 1 mM each). After centrifugation at 15,000 rpm at 4 °C the pellet was resuspended in lysis
buffer (50 mM Tris/HCl, pH 7.5, 50 mM NaCl, 1% Noni-
det P-40, protease inhibitor cocktail (PIC; Sigma) and fur-
ther protease inhibitors DTT, Benzamidine and PMSF. The
sample was denatured in SDS sample buffer at 95 °C for
5 min. The samples were used for SDS-PAGE and western
blot analyses.

**Western blotting**

For immunoblotting, equal amounts of total cell protein
were separated by SDS-PAGE (12% and 3–12% acrylamide
gradient gels) and subsequently transferred to nitrocellu-
lose membranes. For protein transfer, semi-dry (45 min, 12
Volt) or wet blotting (overnight, 15 Volt) transfer was used.
Subsequently, the membrane was blocked with 12.5 ml 1%
blocking solution [1% milk powder in NCP buffer (NCP
contains per liter, 10×: 12.1 g Tris, 87.0 g NaCl, 5.0 ml
Tween 20, pH 8.0)] under constant shaking for 1 h. After
blocking, the membrane was incubated with primary anti-
body solution for either overnight (+4 °C) or 1 h (RT). The
membrane was washed three times with NCP for 15 min
and incubated with the corresponding appropriate horserad-
ish peroxidase-coupled secondary antibodies (1:10,000) for
1 h, and the membrane was washed three times with NCP.
Antigen–antibody complexes were detected using the ECL
western blotting detection solution. The protein bands were
visualized using X-ray films. After imaging, the membrane
was stripped with 0.2 M NaOH for 15 min. The stripped
membrane was washed twice with NCP for 15 min. After
washing the membrane, the membrane was blocked with
blocking solution for 1 h at room temperature and used for
antibody incubation.

**Data analysis and statistical evaluation**

Unless otherwise stated, images were processed using
ImageJ 1.47d (NIH), Adobe Photoshop CS version 8.0,
and figures assembled using CorelDraw Graphics Suite
X4. Data analyses and statistical evaluations were carried
out using Microsoft Excel or GraphPad Prism; the number
of independent experiments, mean values, standard errors,
and p values (Student’s t test) are indicated in the figure
legends.

**Results**

**Clinical data**

We ascertained a six-generation consanguineous Pakistani
family with two affected individuals born to healthy con-
sanguineous parents (Fig. 1a, b). At the time of clinical
investigation, the affected female (VI-1) was 16 years old
and had a head circumference of -5 SD compared to the
average population of respective gender and age whereas
her brother (VI-2) showed -9 SD at the age of 12 years.
The patients had mild intellectual disability but had no
other neurological symptoms or skeletal anomalies. Typical
signs of Seckel syndrome like beaked nose and proportion-
ote short stature were not seen in our patients (Yigit et al.
2015).

**Linkage analysis**

All the available individuals from the family were geno-
typed using microsatellite markers flanking seven of
the known MCPH loci. Homozygous alleles around the
MCPH3 locus suggested linkage to this genomic region
data not shown). To verify these data, both affected
members were genotyped using the Axiom® CEU SNP
Array (Affymetrix). Analysis of the genotyping data con-
ﬁrmed linkage on chromosome 9 (LOD score 2.4). Hap-
lotype analysis revealed that a homozygous region on
chromosome 9 spanning a 1-cM interval limited by the
markers AX-11201712 and AX-11374355 (122,801,186-
124,152,939; hg19) includes the MCPH3 locus (Fig. 1c,
CDK5RAP2 position is boxed).

**Identification of the disease-causing mutation
in CDK5RAP2**

To find the causal variant and exclude additional vari-
ants in other known MCPH-associated genes, the DNA
sample of one affected individual (VI-2) was subjected to
whole-exome sequencing (WES), which revealed a novel
homozygous nonsense mutation in exon 27 of CDK5RAP2
(c.4114C > T; p.Arg1372*; NM_018249.5). The muta-
tion results in a truncated protein lacking the C-terminal
CDK5R1 domain and the region responsible for pericen-
trin binding and Golgi association. The DNA variant was
validated by Sanger sequencing. As expected, both patients
were homozygous and the parents were heterozygous for
the mutation (Fig. 1d). WES did not reveal homozygous or
compound heterozygous variants in any one of the other
known MCPH-associated genes.

**Characterization of CDK5RAP2 patient fibroblast cells**

Immunofluorescence analysis with polyclonal antibodies
speciﬁc for CDK5RAP2 showed in control fibroblasts a
puncta staining in the vicinity of the nucleus which colo-
ralized with γ-tubulin indicating its presence at the centro-
some. Furthermore, CDK5RAP2 staining was also seen
around the centrosome on tubule-like structures, which
have previously been identiﬁed as Golgi membranes
(Wang et al. 2010). In patient fibroblasts, we detected
among the affected individuals. The homozygous region is sur-
ing the typical signs of primary microcephaly. c Constructed haplo-
types to depict the homozygous segment on chromosome 9 shared
among the affected individuals. The homozygous region is sur-
rrounded by SNP markers AX-11001712 and AX-11374355 situated
at the upstream and downstream regions of CDK5RAP2, respectively.

The haplotype corresponding to each SNP marker is shown alongside
the chromosomal region and the haplotype within the boxed region
demonstrates the genomic region of CDK5RAP2. d Sanger traces
of the relevant region of CDK5RAP2 obtained with DNA samples
of individual VI-1 and its heterozygous parent IV-1. The mutation
c.4114C > T is absent from the wild-type trace

in some but not all cells a faint signal for CDK5RAP2 which colocalized with γ-tubulin. Furthermore, in cells exhibiting CDK5RAP2 staining, tubule-like structures were not observed (Fig. 2a). In western blot analysis, the CDK5RAP2-specific polyclonal antibodies detected in lysates from control fibroblasts a protein at ~250 kDa. The signal was rather faint and in the patient lysates the CDK5RAP2 amounts were below detection level empha-
sizing a reduction or absence of the protein. We also did not detect a truncated protein (expected at ~155 kDa) in the patient’s lysate (Fig. 2b). GAPDH was the loading control. In quantitative real-time PCR experiments (qRT-PCR) with primers derived from the 5′ and 3′ ends of the transcript, we found a significant reduction in the CDK5RAP2 mRNA levels in the patient sample (5′ primer, −60% of control; 3′ primer, −42% of control) (Fig. 2c).

In further characterization of the patient-derived fibro-
blasts, we focused on nuclear and centrosomal aspects to
understand the role of CDK5RAP2 in cell division and cell
cycle. Control fibroblasts were mostly mononucle-
ated, with their centrosome located close to the nucleus in
a distance of <10 µm. By contrast, ~8% of the patient cells
had multiple nuclei, which might be the result of a cyto-
kinesis defect, and the centrosome nucleus distance was
increased to >10 µm in 20% of the cells as compared to
4% in the control and >15 µm in 9% of patient cells (1%
for control cells) (Fig. 3a, b). The centrosome–nucleus
ratio was altered in 11% of the patient cells and 3% of the cells
showed more than 2 centrosomes per nucleus (Fig. 3c). We
also observed that the nuclei in ~7% of the patient cells had
abnormal shapes. The abnormalities were classified as lob-
ulated (2.3%), misshapen (1.8%), micronuclei (2.5%) and
distorted (0.5%) (Fig. 3d). Nearly no such abnormalities
were noted in control fibroblasts.

Analysis of the mitotic stages was performed in control
and patient fibroblasts that were immunolabeled with mAb
YL1/2 for staining of the tubulin network. The centrosome
was detected with γ-tubulin-specific antibodies. Mutant
and control fibroblasts progressed through mitosis in a
similar fashion (Fig. 4a, b). The spindle fibers were less
prominent in the patient cells and astral microtubules were
not always visible (Fig. 4b, arrow, metaphase). However,
this could be due to sample preparation as variable stain-
ing was also seen in control cells (see metaphase cell). Loss
of astral microtubules had been observed upon knockdown
of CDK5RAP2 but there are also conflicting results (Lucas
and Raff 2007; Fong et al. 2008). By contrast, in interphase
cells the tubulin staining appeared enhanced. Western blot
analysis confirmed increased levels for γ-tubulin (relative
intensities normalized to GAPDH levels: control, 0.546;
patient, 1.173; P, 0.035; three independent experiments).
At the transcript level, we also observed increased amounts
in the patient (see below, Fig. 7e). We further performed
FACS analysis to study cell cycle progression. Albeit we
noted a tendency towards reduced length of the S and M
phases in the mutant, the differences were not statistically
significant (Table 1). Growth behavior was very similar and
growth curves paralleled each other. Furthermore, in immu-
nofluorescence analysis using markers for cell prolifera-
tion and apoptosis we did not observe differences (data not
shown).

Since the centrosome regulates cell motility and cell
polarity, we performed migration assays. The patient cells
migrated with an average speed of 0.8 µm/min whereas
the control cells traveled at an average speed of 0.35 µm/min
(Fig. 5a). We also assayed the ability of the fibroblasts to
reorient and migrate into a wound. In cell scratch assays,
we found that the mutant cells closed the gap faster than
the control which is presumably due to their increased
migration speed. The gap closure also indicated that the
cells were able to polarize and reorient. This was further
tested by submitting the cells to immunofluorescence anal-
ysis seven hours after introduction of the scratch and
staining for the centrosome and the Golgi apparatus which are
both structures that reorient when cells polarize. We found
that in the majority of the cells from control and patient the
Golgi apparatus and the centrosome were located in front
of the nucleus facing the gap. A comparable number of
cells had their Golgi and centrosome in the back (Fig. 5b),
and in some cells the centrosome and the Golgi were not
colocalizing (12% in control, 29% in patient cells; Fig. 5b,
arrow in patient sample).

A visual inspection of the patient fibroblasts had already
indicated a smaller cell size. In further analyses, we con-
firmed this notion and found that patient cells were signifi-
cantly smaller. Approximately, 50% of the cells had a diam-
eter below 20 µm whereas in case of the control fibroblasts
~50% had a diameter between 20 and 29 µm (Fig. 5c). For
these experiments, cells were trypsinized to obtain rounded
cells as described in the Materials and Methods section.
The smaller cell size was confirmed by determining the
area where we measured for the majority of wild-type cells
(42%) an area between 400 and 600 µm, whereas for the
In earlier work on CEP161, the \textit{D. discoideum} ortholog of CDK5RAP2, we identified a link to Hippo signaling. CEP161 bound to the Hippo kinase Hrk-svk, and the N-domain of CEP161 inhibited the kinase activity. Overexpression of CEP161 resulted in reduced growth, defects in development and further deficiencies (Sukumaran et al. 2015). To study whether human CDK5RAP2 has a similar activity, we first investigated whether there exists a similar interaction in the human system and then analyzed the Hippo pathway in WT and patient fibroblasts.

To probe an interaction between CDK5RAP2 and Hippo pathway components, we expressed GFP-tagged MST1 in HeLa cells and used CDK5RAP2-specific antibodies for immunoprecipitation. GFP-MST1 was found in the immunoprecipitate. GST-antibodies which were used as centrosomal marker and the nuclei were stained with DAPI. Scale bar 10 µm.

**Fig. 2** CDK5RAP2 is not detected in patient fibroblasts. **a** Localization of CDK5RAP2. CDK5RAP2 was detected with polyclonal antibodies, γ-tubulin detected with monoclonal antibodies was used as centrosomal marker and the nuclei were stained with DAPI. Scale bar 10 µm. **b** Detection of CDK5RAP2 in whole cell lysates. Whole cell lysates from control and patient fibroblasts were probed with polyclonal anti-CDK5RAP2 antibodies. The panel below represents the corresponding GAPDH levels. The signal at ~100 kDa is non-specific. **c** CDK5RAP2 transcript abundance in control and patient cells as analyzed by quantitative RT-PCR. Primers were derived from 5′ and 3′ regions of the cDNA sequence. The differences were significant (\(P < 0.001\)).
control did not precipitate the protein (Fig. 6a). Detection of endogenous MST1 in the immunoprecipitate was prevented due to co-migration of MST1 (~55 kDa) with the IgG heavy chain of the antibodies used in the immunoprecipitation. To identify the interacting domain within CDK5RAP2, Myc-tagged full-length CDK5RAP2 and C-terminally truncated CDK5RAP2-C (residues 1–82), –C1 (residues 1–580), –C2 (residues 1–1271) and –C3 (residues 1–1372) were coexpressed with GFP-MST1 in HEK293T cells (Fig. 6b). Myc-CDK5RAP2-C2 harboring the γTuRC domain, SMC and EB1-binding domain was the shortest protein that could precipitate GFP-MST1. GFP alone did not coprecipitate with the CDK5RAP2 proteins (Fig. 6c). The localization of the Myc-tagged truncated proteins was revealed by immunofluorescence studies carried out in HeLa cells. Full-length CDK5RAP2 as well as C1 and C2 were present as a dot near the nucleus colocalizing with pericentrin (Fig. 6d). This is in agreement with previous work by Barrera et al. (2010) who reported that the first 435 amino acids were sufficient for targeting the centrosome. In the C1- and C2-expressing cells, we noted an increased centrosome number which could be due to the overexpression of the protein.

We expanded our studies on the MST1–CDK5RAP2 interaction and carried out an immunofluorescence analysis where we observed that GFP-MST1 was present in the cytosol and also showed an enhanced staining near the nucleus where it colocalized with Myc-CDK5RAP2-C2 detected with mAb 9E10 and pericentrin as centrosomal marker (Fig. 6e). MST1 localization at the centrosome has also been shown in previous work (Hergovich et al. 2009). We then depleted MST1 from HeLa cells using siRNA and studied whether this has an effect on centrosome position, centrosome number and recruitment of CDK5RAP2 to the centrosome. A significant reduction of MST1 levels was observed by western blot analysis (reduction to 83.7% of normal levels with 1 nM siRNA; reduction to 54.6% with 10 nM siRNA; β-actin levels served as control) confirming the knockdown of MST1 (Fig. 6f). The centrosome nucleus distance was enhanced and 36.5% of the cells had the centrosome more than 6 μm away from the nucleus as compared to 14.5% for control cells (Fig. 6g, h). CDK5RAP2 stayed associated with the γ-tubulin-positive centrosome and the typical “Golgi-like” staining was observed (Fig. 6h). The centrosome number was not significantly affected.

Based on these data, we turned to control and patient fibroblasts to explore the Hippo pathway and studied the transcript levels of the core Hippo signaling components TAZ, YAP and MST1. The qRT-PCR studies showed significantly higher transcript levels of TAZ and YAP in the patient fibroblast cells, those of MST1 were not significantly altered (Fig. 7a). The qRT-PCR data were further supported by analyzing the protein levels using pAb YAP/TAZ, which showed concordant increase in the expression levels of the ~70 kDa YAP (twofold, mean of three experiments) and ~50 kDa TAZ (threelfold, mean of four experiments) as compared to the control (a representative experiment is shown in Fig. 7b). In support of these findings, overexpression of CDK5RAP2 in HEK293T cells led to reduced amounts of coexpressed Flag-tagged TAZ protein (see below, Fig. 7g). There were no significantly changed MST1 and phosphorylated MST1 protein levels in the patient cells as compared to the control (Fig. 7c and data not shown). In immunofluorescence studies, we observed in control cells a weak staining for YAP/TAZ in the cytoplasm and in the nucleus, patient cells exhibited a slightly enhanced staining in both compartments (Fig. 7d).

**Impact of the Hippo pathway at the transcriptional level**

The Hippo pathway acts primarily in an inhibitory fashion on the transcription of genes involved in cell proliferation through inactivating YAP/TAZ. We, therefore, determined the levels of commonly used YAP/TAZ target genes, namely CTGF, BIRC5 (Survivin), CYR61 and AMOTL2, by qRT-PCR and found that in the patient fibroblasts the transcript amounts of all genes were significantly lower as compared to the wild type. The levels of CTGF were most affected and reduced to about half of wild-type levels. The increased levels of YAP and TAZ apparently are not leading to increased levels of their downstream targets (see also discussion). An exception was BIRC5 for which we found a strong increase (~fivefold) (Fig. 7e). The protein levels of BIRC5 were also significantly increased. In western blots, we easily detected the protein in patient cell lysates whereas in the control nearly no signal was seen (Fig. 7e, right panel). The data suggest that in fibroblasts the transcriptional regulation by the Hippo pathway functions in the absence of CDK5RAP2.

**CDK5RAP2 interacts with TAZ**

We also tested whether CDK5RAP2 can interact with TAZ and co-transfected HEK293T cells with Myc-CDK5RAP2, Myc-CDK5RAP2-C1 and -C2 and FLAG-TAZ and precipitated FLAG-TAZ using FLAG-trap beads. In the precipitate, we detected Myc-CDK5RAP2, CDK5RAP2-C1 and -C2 indicating that the binding site is located between residues 1 and 580 which encompass the γTuRC domain (Fig. 7f). It thus is different from the binding site of MST1 which is precipitated by C2 as the shortest protein. qRT-PCR analysis using RNA obtained from HEK293T cells overexpressing Myc-CDK5RAP2 showed a highly significant decrease in the transcript levels of TAZ in relation to
A  

DAP/Neprin-1

B  

Altered centrosome position

DAP/Pericentrin

45.5 μm

C  

Multiple centrosome

DAP/Pericentrin

D  

Lobulated nucleus  Misshapen/Micronuclei  Distorted nucleus

DAP/Neprin-1

Number of nucleates/cell in %

Ratio of nucleus:cell

<1 μm  1-10 μm  10-15 μm  >15 μm

Number of cells in %

Nucleus-centrosome distance

<1:1  1:1  2:1  >2:1

Ratio of centrosome:nucleus per cell

Number of centrosome/nucleus in %

Lobulated  Misshapen  Micronuclei  Distorted

Nuclear abnormalities

Number of cells in %
The microtubule network in mitotic cells appeared more delicate and astral microtubules were less pronounced or absent. Studies by Fong et al. (2008) had shown that CDK5RAP2 loss inhibited centrosomal microtubule nucleation and led to the formation of anastral mitotic spindles although there are also conflicting reports (Lucas and Raff 2007). This activity of CDK5RAP2 is based on the presence of the γTURC domain which mediates γTURC attachment and nucleation of microtubules. Reduced levels of CDK5RAP2 or its absence might, therefore, be responsible for our observations. At the protein level, we observed a significant increase in tubulin in interphase which was paralleled by increased transcript levels. In summary, the analysis of the patient cells yielded data that seem to agree with those obtained from the analysis of other MCPH cells with respect to centrosomal and nuclear abnormalities.

We report further a connection of CDK5RAP2 to the Hippo pathway by showing that the protein can interact with two components of the pathway, the kinase MST1 and the transcriptional regulator TAZ. In the mutant fibroblasts we found increased YAP/TAZ levels. This did, however, not result in increased transcription of YAP/TAZ target genes (see also below). In fact their transcript levels were significantly reduced. For the patient situation this might be important for cell proliferation. An exception was the BIRC5 gene for which we found strongly increased transcript levels accompanied by increased protein levels. BIRC5/Survivin might account for some properties of the patient fibroblasts. BIRC5 has several roles. It promotes cell proliferation and prevents apoptosis. It was also identified as component of a chromosome passenger protein complex (CPC), a complex which is essential for chromosome alignment and segregation during mitosis. CPC has different locations. It is present at the centromere during prometaphase and at the midbody during cytokinesis and is involved in the organization of the spindle by associating with polymerized microtubules. In addition to the Hippo pathway, BIRC5 is regulated by other developmental signaling pathways such as the Wnt/β-catenin, the Hedgehog and the Notch pathway (Altieri 2015). In the murine embryonic brain, conditional deletion of survivin leads to apoptosis of neuronal precursor cells in the CNS and in newborn mutants a marked reduction in the size of the brain was observed (Jiang et al. 2005).

A link between an MCPH protein and the Hippo pathway is very appealing. During the last couple of years it has become clear that Hippo signaling regulates stem cell and progenitor pools in mammals. This has mainly
Fig. 4 Mitotic stages in control (a) and patient cells (b). Confocal images of control and patient cells of the indicated mitotic stages are shown. Nuclei were stained with DAPI, centrosomes were detected with mouse mAb against γ-tubulin and microtubules were stained with rat mAb YL1/2. The arrow in B, metaphase, points to a spindle pole without prominent astral microtubules. Scale bar 10 µm.
been studied for the intestine but it appears to be relevant for other tissues as well (Camargo et al. 2007; Cao et al. 2008). For mammalian brain development, an involvement of the Hippo pathway has been described and in particular

Table 1 Cell cycle progression of control and patient fibroblasts as studied by FACS analysis

|                | Control     | Patient     | P values |
|----------------|-------------|-------------|----------|
| G0/G1 phase    | 78.16 ± 4.95| 84.8 ± 3.59 | 0.3185   |
| S phase        | 11.85 ± 3.01| 8.80 ± 2.24 | 0.4473   |
| M phase        | 6.98 ± 1.57 | 3.75 ± 0.34 | 0.0958   |

The data are mean values in percent derived from four independent experiments. The differences were not statistically significant (two-tailed P values)

On the other hand, MST1 has also a centrosomal role as it was recently reported that MST1 signaling controls a role for YAP/TAZ in the regulation of self-renewal and expansion of tissue progenitor cells has been proposed. An important component in this event is the tumor suppressor neurofibromatosis 2 (NF2, merlin) which limits the expansion of neural progenitor cells (NPCs) in the mammalian dorsal telencephalon through suppression of YAP activity as reported for mouse (Lavado et al. 2013, 2014). It was also shown that knockdown of LATS1/2 or expression of dominant-negative MST2 caused neuroepithelial proliferation. The authors, therefore, concluded that in vertebrates MST1/2 and LATS1/2 regulate neural progenitor proliferation and survival through inhibiting the activity of YAP (Cao et al. 2008).

Fig. 5 Cell migration, cell polarity and cell size analysis. a Analysis of speed during migration in μm/min (***P < 0.001). 32 cells each were analyzed. b Cell polarity analysis. Cells migrating into a scratch wound were fixed after 7 h of migration and stained for the Golgi (anti 58 K, mAb), centrosome (anti-pericentrin, pAb) and the nuclei (DAPI). The cells were migrating towards the lower right (location of the wound). The arrow points to a centrosome which is not colocalizing with the Golgi apparatus. Scale bar 10 μm. c Cell size of control and patient cells in micrometers. ~220 and 390 cells were analyzed, respectively
CDK5RAP2 and the link to Hippo pathway components. a Co-immunoprecipitation of CDK5RAP2 and GFP-MST1. CDK5RAP2 was immunoprecipitated from HeLa cell lysates expressing GFP-MST1. GFP-MST1 and CDK5RAP2 were detected with GFP-specific mAb K3-184-2 and polyclonal CDK5RAP2 antibodies, respectively. GST-specific polyclonal antibodies were used for control. b Schematic of Myc-tagged CDK5RAP2 proteins. The amino acid residues and the domains are indicated. c CDK5RAP2 interaction with MST1. Myc-tagged CDK5RAP2 proteins were coexpressed in HEK293T cells with GFP-tagged MST1 and Myc-tagged polypeptides precipitated with Myc antibodies. GFP-MST1 was detected in the precipitates with mAb K3-184-2. GFP was used for control. d Immunofluorescence analysis of HeLa cells expressing Myc-tagged CDK5RAP2 proteins. Myc was recognized by mAb 9E10, pericentrin was detected with polyclonal antibodies, nuclei were stained with DAPI. The boxed area is enlarged at the right. Scale bar 10 µm. e Immunofluorescence analysis of HeLa cells expressing Myc-tagged CDK5RAP2 and GFP-MST1. Myc antibodies mAb 9E10 detected Myc-CDK5RAP2, GFP-specific mAb K3-184-2 detected GFP-MST1, the centrosome was detected with pericentrin-specific antibodies, DNA was stained with DAPI. Scale bar 10 µm. f Knockdown of MST1 with siRNA in HeLa cells leads to reduced MST1 protein amounts and an increased centrosome nucleus distance. Detection of the centrosome was with γ-tubulin-specific antibodies. G Bar graph analysis of the centrosome nucleus distance. The distance from the nucleus was determined using the Leica LAS AF lite program. The number of cells in percent is given. For control 58 cells and for the knock down 66 cells were evaluated. h Immunofluorescence analysis of control and MST1 knockdown cells staining for γ-tubulin and CDK5RAP2. Cells treated with 1 nM siRNA are shown. Scale bar 10 µm

Although we have found increased YAP/TAZ levels, we did not observe enhanced target gene transcription. Increased nuclear YAP is not necessarily a sign for increased YAP activity. This was concluded from experiments in which a Yap (S112A) knock-in mutation in the endogenous Yap locus of mice was generated which is an activating mutation and which resulted in normal mice (Chen et al. 2015). S112 phosphorylation is required for cytoplasmic translocation and binding to 14-3-3. We also found an interaction of CDK5RAP2 with TAZ. The binding site appears to be different from the one of MST1 and the implications of this interaction are not clear at present. How the enhanced YAP/TAZ transcript levels are achieved in the patient cells is unclear. There exist only few studies on the regulation of YAP gene expression. So far β-Catenin/TCF4, miRNAs, ETS transcription factors and cJUN have been shown to be involved (Liu et al. 2010; Konsavage et al. 2012).

It was reported that loss of Mst1/2 or Lats1/2, or activation of YAP-TEAD in neural progenitor cells leads to a marked expansion of neural progenitors, partially due to upregulation of cell cycle re-entry and stemness genes, and a concomitant block to differentiation by suppressing key genes. Conversely, YAP/TAZ loss of function results in increased cell death and precocious neural differentiation (Cao et al. 2008). Furthermore, bone marrow-derived mesenchymal stem cells depleted of TAZ show decreased osteogenesis (Hong et al. 2005). In addition, as mentioned previously, YAP and TAZ proteins are important in brain development (Lavado et al. 2013). In neuronal cells, loss of CDK5RAP2 might lead to insufficient TAZ-dependent proliferative signaling under certain conditions leading to a reduced size of the brain.

Our analysis was carried out in primary fibroblasts and it is not clear whether the status of Hippo signaling resembles the one in the embryonic brain. In addition, it has been revealed that cell polarity, cell adhesion, cell contacts and mechanical cues, soluble factors and also the metabolic status of the cells are impacting on the Hippo pathway and they all can vary in different situations (Yu et al. 2015). Combining all findings, we propose a dual role for CDK5RAP2 and Hippo pathway components. In neuronal cells, loss of CDK5RAP2 leads to insufficient YAP/TAZ-dependent proliferative signaling under certain conditions resulting in reduced size of the brain which is in agreement with our findings of reduced expression of YAP/TAZ target genes in patient cells (Fig. 8). This idea is also supported by the observation that there is a significant reduction in brain size in MCPH patients with a mutation in CDK5RAP2 leading to a non-functional protein (Kraemer et al. 2011). We presume...
that there is a requirement for perfectly balanced amounts of YAP/TAZ in neural stem and progenitor cells which are involved in controlling correct expansion of the progenitor pool and timely differentiation and that this might be ensured by a crosstalk between CDK5RAP2 and the Hippo pathway. Second, CDK5RAP2 provides a physical binding site for MST1 (and also TAZ) (Fig. 8). This interaction is important for the integrity of the centrosome with its important roles in neurogenesis (Chavali et al. 2014).

Acknowledgements We thank Berthold Gaßen for providing antibodies, Sebastian Klein for help with experiments and Ramesh Rijal for help with RT-PCR. SK was a member of the CECAD Graduate School. Part of this work was carried out for the PhD thesis of SK. FACS cell sorting was carried out by Dr. G. Rappl of the CMMC facilities.

Compliance with Ethical Standards

Funding This study was funded by grant C6 from the CMMC (Center for Molecular Medicine Cologne) to PN and AAN and grant C05 from CECAD (Cologne Excellence Cluster on Cellular Stress Responses in Aging-Associated Diseases).

Conflict of interest PN declares that he has a conflict of interest. He is a founder, CEO, and shareholder of ATLAS Biolabs GmbH. ATLAS Biolabs GmbH is a service provider for genomic analyses.

Ethical approval All procedures performed in studies involving human participants were in accordance with the ethical standards of the institutional and/or national research committee and with the 1964 Helsinki declaration and its later amendments or comparable ethical standards. Informed consent was obtained from all individual participants included in the study. This article does not contain any studies with animals performed by any of the authors.
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