CHAPTER 6

Deficiency of the centrosomal protein CEP170 is associated with microcephaly

Anneke Haitjema¹, Jesper A. Balk¹, Eileen G. Daniels¹, Davy A.P. Rockx¹, Anneke B. Oostra¹, Sander R. Piersma², Gea Beunders³⁴, Marian M. Weiss⁴, Sarina G. Kant⁵, Egbert Bakker⁵, Connie R. Jimenez², Hans Joenje¹, Astrid M. van der Sar⁶, Josephine C. Dorsman¹

¹Department of Clinical Genetics, section Oncogenetics, VU University Medical Center, van der Boechorststraat 7, 1081 BT Amsterdam, The Netherlands.
²OncoProteomics Laboratory, CCA 1-60, Department of Medical Oncology, VUmc-Cancer Center Amsterdam, VU University Medical Center, De Boelelaan 1117, 1081 HV Amsterdam, The Netherlands.
³Department of Complex Trait Genetics, CNCR, VU University, de Boelelaan 1085, 1081 HV Amsterdam, The Netherlands.
⁴Department of Clinical Genetics, VU University Medical Center, van der Boechorststraat 7, 1081 BT Amsterdam, The Netherlands.
⁵Department of Clinical Genetics, Leiden University Medical Center, Albinusdreef 2, 2333 ZA Leiden, The Netherlands
⁶Department of Medical Microbiology and Infection Control, VU University Medical Center, van der Boechorststraat 7, 1081 BT Amsterdam, The Netherlands.

Submitted for publication.
Abstract

Primary autosomal recessive microcephaly (MCPH) and Seckel syndrome (SCKS) are associated with microcephaly without malformations in other organ systems. The various MCPH-SCKS-related proteins play roles in a variety of molecular processes including those that control spindle orientation, microtubule dynamics and DNA damage response. However, how these processes interconnect is unclear. The recent discovery of WD repeat-containing protein 62 (WDR62), also known as MCPH2, has broadened the molecular basis underlying the clinical phenotype of MCPH-SCKS spectrum disorders. Molecular network characterization of WDR62 is largely lacking. Here we report an endogenous interaction between WDR62 and CEP170, a centrosomal protein linked to DNA damage response pathways. In line with this, subcellular fractionation studies have indicated that both WDR62 or CEP170 localize primarily in the cytoplasm and membranes, while depletion experiments for WDR62 and CEP170 indicate that their mutual interactions are not required for their subcellular localization and/or stabilization. The chromosomal breakage assay did not provide evidence for a direct role of neither WDR62 nor CEP170 in DNA repair. The human chromosomal region, 1q43-1q44, in which CEP170 is located, has been associated with microcephaly. We show that knockdown of cep170 in a zebrafish model caused an MCPH-like phenotype associated with increased apoptosis at the early embryo stage. Furthermore the data suggest a possible role for cep170 in vasculature development. We conclude that CEP170 can contribute to microcephaly in humans and warrants further studies on the possible role of CEP170 in vasculature development which might affect microcephaly patients who carry chromosomal aberrations affecting CEP170.

Introduction

Microcephaly is characterized by reduced head circumference of at least 2 standard deviations (SD) below ethnically, age- and sex-related mean. A reduced head circumference at birth is thought to be caused by the reduced number of generated cerebral cortical neurons during embryonic neurogenesis. Microcephaly is the main characteristic in primary autosomal recessive microcephaly (MCPH) and Seckel syndrome (SCKS). The MCPH-SCKS spectrum disorders do not have malformations in any other organ systems (Verloes et al. 1993). In addition to the small head, abnormal brain structure, craniosynostosis, cognitive impairment, and short stature are some of the variable characteristics seen in the MCPH-SCKS spectrum disorders.

Currently, molecular genetics has revealed 12 MCPH loci (MCPH1-12 [MIM 251200, 604317, 604804, 604321, 608716, 608393, 612703, 614673, 614852, 615095, 615414, and 616080]) and 8 SCKS loci (SCKL1-8 [MIM 210600, 606744, 608664, 613676, 616080]).
Deficiency of the centrosomal protein CEP170 is associated with microcephaly

613823, 614728, 614851, and 615807]), corresponding to 18 genes, 1 gene remains to be identified and 17 genes have been described of which 2 genes are causative for MCHP and SCKL. The MCHP and SCKL genes are highly expressed in the proliferating neuroepithelium of the developing brain. The corresponding MCHP-SCKS proteins have roles in diverse molecular processes, including spindle orientation, microtubule dynamics, cell cycle regulation, and DNA damage response (Mahmood et al. 2011); however it is unclear if there is a common process that underlies the cellular mechanisms resulting in microcephaly (Novorol et al. 2013).

The relation of MCHP-SCKS proteins with DNA damage response is of special interest since microcephaly is also a common feature in syndromes related to DNA repair such as Xeroderma pigmentosum (XP [MIM 278700, 610651, 278720, 278730, 278740, 278760, and 278780]) and Fanconi anemia (FA [MIM 227650, 300514, 227645, 605724, 227646, 600901, 603467, 614082, 609053, 609054, 614083, 614087, 610832, 613390, 613951, and 615272]). This and the role of MCHP-SCKS proteins in DNA damage response suggests a possible network between MCHP-SCKS and DNA repair proteins.

The discovery of WD repeat-containing protein 62 (WDR62), also known as MCPH2, one of the frequently affected genes in MCHP, which localization is indicative of a role in the centrosome and proliferative cell divisions, broadens the clinical phenotype with the occurrence of lissencephaly (smooth brain), schizencephaly (split brain), and polymicrogyria (many small gyria) (Bilgüvar et al. 2010; Nicholas et al. 2010; Yu et al. 2010; Kousar et al. 2011; Murdock et al. 2011; Bhat et al. 2011; Bacino et al. 2012; Sajid Hussain et al. 2012). Molecular characterization of WDR62 has focused mainly on cellular localization (Bilgüvar et al. 2010; Nicholas et al. 2010; Yu et al. 2010; Bhat et al. 2011); information on the functional network in which WDR62 is operating is largely lacking. Further molecular and functional characterization of WDR62 would provide insight into its role in the molecular pathway defects connected with microcephaly syndromes.

To obtain additional functional molecular information for WDR62, we used a proteomic approach as starting point. We found an endogenous interaction between WDR62 and CEP170. Subsequently, we studied the phenotypes that could be connected to disruption of CEP170 in either humans or zebrafish.

Material and Methods
Cell culture. HeLa S3 cells (a human epithelial carcinoma cell line) and LN9SV cells (a human SV40 immortalized fibroblast cell line; kindly provided by Prof. Martin. J. Digweed; Institute of Human Genetics, Charité Berlin, Germany), were cultured in Dulbecco's modified Eagle medium (DMEM) containing 4.5 g/L D-Glucose supplemented with 10% fetal bovine serum (FBS). Cells were regularly tested for mycoplasma.
**Cell lysis.** Whole cell extracts were prepared in lysis buffer (50 mM Tris-HCL, pH 7.5, 250 mM NaCl, 5 mM EDTA, 0.1% Triton X-100, supplemented with protease inhibitors). Protein concentration was determined with a protein assay (Protein Standard II and BioRad Protein Assay Dye Reagent Concentrate; BioRad) based on the Bradford method (Bradford 1976).

**Immunoprecipitation.** For immunoprecipitation HeLa S3 extracts (2500 µg of protein per isolation) were incubated 3 h at 4°C with the following primary rabbit polyclonal antibodies: anti-WDR62, mapping to a region between residue 750 and 800 (GeneID 284403; ProteinID NP775907.4; A301-559A; Bethyl), anti-WDR62, mapping to a region between residue 900 and 950 (GeneID 284403; ProteinID NP775907.4; A301-560A; Bethyl), anti-FAK (c-903; SantaCruz), and anti-GFP (sc-8334; SantaCruz). Pull-downs were performed with protein A Sepharose CL-4B (50% slurry; GE Healthcare) for 1 h at 4°C.

**Electrophoresis and Coomassie Blue staining.** Immunoprecipitation extracts were loaded on a pre-cast NuPAGE 4-12% w/v Bis-Tris 1.5-mm minigel (Invitrogen). Electrophoresis was carried out at 150 V in NuPAGE MES SDS running buffer (Invitrogen). Following electrophoresis, the gel was fixed (50% ethanol and 3% phosphoric acid), stained (34% methanol, 3% phosphoric acid, 15% ammonium sulphate, and 0.1% Coomassie Blue G-250 (BioRad)), and subsequently destained in Milli-Q water.

**In-gel digestion.** The whole gel was processed for in-gel digestion (Shevchenko et al. 1996). Briefly, the whole gel was washed and dehydrated twice in 50 mM ammonium bicarbonate (ABC), pH 7.9, and ABC containing 50% acetonitrile (ABC/ACN). Subsequently, cysteine bonds were reduced with 10 mM DTT for 1 h at 56°C and alkylated with 50 mM iodoacacetamide in the dark for 45 min. at room temperature. After two consecutive wash/dehydration cycles, the gel was sliced and each slice was diced into cubes. Gel pieces were washed once with ABC/ABN and dried for 10 min. in a vacuum centrifuge. The gel pieces were incubated overnight with Trypsin Gold (mass spectrometry grade; Promega) at 25°C. The peptides were extracted once in 1% formic acid followed by two washes in 50% ACN in 5% formic acid. The volume was reduced to 50 µl in a vacuum centrifuge at 50°C prior to LC-MS/MS analysis as described (Warmoes et al. 2012).

**LC-MS/MS.** Peptides were separated by an Ultimate 3000 nanoLC-MS/MS system (Dionex LC-Packings, Amsterdam, The Netherlands) equipped with a 20 cm × 75 µm ID fused silica column custom packed with 3 µm 120 Å ReproSil Pur C18 aqua (Dr Maisch GMBH, Ammerbuch-Entringen, Germany). After injection, peptides were trapped at 6 µl/min on a 10 mm × 100 µm ID trap column packed with 5 µm 120 Å ReproSil Pur C18 aqua at 2% buffer B (buffer A: 0.5% acetic acid in MQ; buffer B: 80% ACN + 0.5% acetic acid in MQ) and separated at 300 nl/min in a 10–40% buffer B gradient in 60 min (90 min
Deficiency of the centrosomal protein CEP170 is associated with microcephaly inject-to-inject). Eluting peptides were ionized at a potential of +2 kV into a Q Exactive mass spectrometer (Thermo Fisher, Bremen, Germany). Intact masses were measured at resolution 70,000 (at m/z 200) in the orbitrap using an AGC target value of 3 × 106 charges. The top 10 peptide signals (charge-states 2+ and higher) were submitted to MS/MS in the HCD (higher-energy collision) cell (4 amu isolation width, 25% normalized collision energy). MS/MS spectra were acquired at resolution 17,500 (at m/z 200) in the orbitrap using an AGC target value of 2 × 105 charges and an underfill ratio of 0.1%. Dynamic exclusion was applied with a repeat count of 1 and an exclusion time of 30 s.

**Protein identification.** MS/MS spectra were searched against the Uniprot human reference proteome FASTA file, release January 2014, no fragments; 42104 entries using MaxQuant 1.4.1.2. (Cox and Mann 2008). Enzyme specificity was set to trypsin and up to two missed cleavages were allowed. Cysteine carboxamidomethylation (Cys, +57.021464 Da) was treated as fixed modification and methionine oxidation (Met, +15.994915 Da) and N-terminal acetylation (N-terminal, +42.010565 Da) as variable modifications. Peptide precursor ions were searched with a maximum mass deviation of 4.5 ppm and fragment ions with a maximum mass deviation of 20 ppm (default MaxQuant settings). Peptide and protein identifications were filtered at an FDR of 1% using the decoy database strategy. Proteins that could not be differentiated based on MS/MS spectra alone were grouped to protein groups (default MaxQuant settings).

**Protein quantitation.** Each sample was separated in 5 fractions that were subjected to nano-LC-MS/MS. Proteins were (label-free) quantified by spectral counting i.e. the sum of all MS/MS spectra for each identified protein (Liu et al. 2004). For quantitative analysis across samples, spectral counts for identified proteins in a sample were summed across all 5 fractions and were normalized to the sum of spectral counts for that sample. This gives the relative spectral count contribution of a protein to all spectral counts in the sample. When comparing different biological samples, these normalized spectral counts were used to calculate ratios. In this way, we were able to correct for loading differences between samples. Differential analysis of samples was performed using the beta-binominal test (Pham et al. 2010), which takes into account within- and between-sample variations, giving fold-change values and associated p-values for all identified proteins. Protein cluster analysis of the differentially expressed proteins was performed using hierarchical clustering in R. The protein abundances were normalized to zero mean and unit variance for each individual protein. Subsequently, the Euclidean distance measure was used for protein clustering.

**siRNA experiments.** Knockdown experiments were performed using siGENOME SMARTpool siRNAs (Dharmacon; Thermo Scientific) against WDR62 (M-031771-01), CEP170 (M-021258-02), FANCD2 (M-016376-02), and Non-Targeting #2 (D-001206-
Knockdown experiments were performed with Lipofectamine® 2000 transfection reagent (Invitrogen) according to the manufacturer's protocol.

**Subcellular protein fractionation.** Cell fractionation was performed using a Subcellular Protein Fractionation Kit (Thermo Scientific) according to the manufacturer's instructions.

**Protein analysis.** Proteins were separated on 3-8% Tris-Acetate NuPAGE gradient gels (Invitrogen) and proteins were separated at 150 V. Proteins were transferred to Immobilon-P Transfer membranes (Millipore) and the membranes were blocked with 5% dry milk in TBST (10mM Tris HCl pH8.0, 150 mM NaCl, 0.05% Tween-20). After blotting the membranes were incubated with the following primary antibodies: anti-p300 (sc-585; Santa Cruz), anti-Tubulin (B-5-1-2; sc-23948; Santa Cruz), anti-HAGH (glyoxalase II; C-17; sc-51091; Santa Cruz), anti-Histone H3 (#9715; Cell Signaling), anti-CEP170 (A301-023A and A301-024A; Bethyl), anti-WDR62 (A301-559A and A301-560A; Bethyl). After washing with TBST, the membranes were incubated with peroxidase-conjugated goat anti-rabbit, peroxidase-conjugated goat anti-mouse, or peroxidase rabbit anti-goat depending on the primary antibody (DakoCytomation). Proteins were visualized with ECL Western blotting detection reagents (Amersham Biosciences). Blots were visualized and analyzed using Image Lab 3.0 software (Biorad).

**Chromosomal breakage test.** Metaphases of siRNA-transfected HeLa cells cultured in the absence or presence of MMC (120 nM for 24 hrs) were evaluated for chromatid-type aberrations as described previously (Oostra et al. 2012). Simultaneously, cells were collected for Western blot analysis to confirm knockdown. A 2-sample Chi-square test was used to determine the differences between the treated and untreated cultures by using the percentages of aberrant metaphases.

**Patients, SNP arrays, array CGH, FISH, and MLPA analysis.** Genomic DNA of patients 1 and 2 (Leiden University Medical Center) was extracted from whole blood by using a Gentra Puregene DNA Purification Kit system (Gentra Systems, Minneapolis, USA), following the manufacturer's protocol. Genomic DNA of patient 3 (VU University Medical Center) was extracted from whole blood by an Autogen Automated Nucleic Acid Extraction robot, following the manufacturer’s protocol (Autogen Inc. Holliston, MA, USA). Genomic DNA of patients 1 and 2 was analyzed using the Affymetrix GeneChip Human Mapping 262 K NspI, 238 K StyI arrays (Affymetrix, Santa Clara, USA) according to the manufacturer’s protocol and data were analyzed as described previously (Gijsbers et al. 2009). Genomic DNA of patient 3 was analyzed by array CGH (Agilent 180K), FISH (probe for the subtelomere for the long arm of chromosome 1 [D1S3738, Telvysion 1q, Vysis] and a BAC probe RP11-551G24 for chromosome band 1q44 [BlueGnome]), and Multiplex Ligation-dependent Probe Amplification (MLPA, MRC Holland, kits P036B and P070) were performed following the manufacturer’s instructions.
Deficiency of the centrosomal protein CEP170 is associated with microcephaly

**Patient Ethics statement.** The study was approved by the Leiden University Medical Center Clinical Research Ethics Board and the Institutional Review Board of the VU University Medical Center adhering to Dutch law and the World Medical Association Declaration of Helsinki.

**Zebrafish husbandry.** Zebrafish embryos were collected from a laboratory breeding colony maintained and bred at 26°C according to standard protocols (zfin.org). Embryos were routinely raised at 28°C and staged based on hours post fertilization (hpf) (Kimmel et al. 1995). Lines used in this study included Casper zebrafish (White et al. 2008) and Tg(Fli1:GFP)y1 Casper zebrafish (Lawson and Weinstein 2002a; Lawson and Weinstein 2002b). All procedures involving zebrafish embryos were performed in compliance with local animal welfare laws.

**Ethics statement zebrafish.** Zebrafish lines were handled in compliance with the local animal welfare regulations. The breeding of adult fish was approved by the local animal welfare committee (DEC) of the VU University Medical Center and adhered to in accordance with the international guidelines specified by the EU Animal Protection Directive 2010/63/EU. All experiments in this study were performed on embryos/larvae before the free-feeding stage and therefore outside the scope of the experimentation law according to the EU Animal Protection Directive 2010/63/EU.

**Morpholino injections.** Splice-blocking (SB) and transcriptional initiation blocking (TB) morpholinos against Cep170 (Reference sequence XM_680542.5) were designed and obtained via Gene Tools, LCC (Golzio et al. 2012) (SB Cep170 5'-GTCATCCTGATGGACAGAGAAATAA-3'; and TB Cep170 5'-GAAACCAGGACGTGAGGCTCATCTC-3'). Morpholinos were reconstituted as 1 mM stock solutions in water and 1 nl of diluted morpholino (5 or 10 ng) was injected into the yolk of 1-to 2-cell stage embryos essentially as described previously (Benard et al. 2012).

**CEP170 pSC2+ constructs.** Two splice variants of human CEP170 mRNA construct were generated. The alpha-CEP170 (NM_014812.2) pSC2+ construct was established by amplification of cDNA derived from LN9SV (wildtype). The gamma-CEP170 (NM_001042405.1) pSC2+ construct was established using the pEGFP CEP170 construct as template (Guarguaglini et al. 2005) [Addgene Plasmid #41150]). For both PCR reactions a forward oligo gccaccATGAGCTTAACATCCTGGTTTTTGGT, was combined with a reverse oligo containing a Xho1 site GGATctcgagTCATTCTTGTACTGTAACATCTTCCTCTTC. Gel electrophoresis analyses on 1% agarose gels were performed and PCR products were separated at 100 V. Gel fragments were purified using a gel purification kit (Qiagen) according to the manufacturer's instructions. Subsequently, the DNA sequences of the purified PCR products from the gel were checked via Sanger sequencing. Finally, the PCR products were cloned into the pCS2+ vector and the final construct was checked again via Sanger sequencing.
RNA rescue and overexpression. For RNA rescue and overexpression experiments, the pSC2+ constructs containing human wild-type alpha-CEP170 and gamma-CEP170 mRNAs were transcribed in vitro according to the manufacturer’s protocols using the Ambion SP6 Message Machine Kit (Ambion). One nl of H2O-diluted capped mRNA (50 or 100 pg) was injected in the 2 cell stage morphant embryos. Injected embryos were kept overnight at 28°C in E3 medium containing 1.67% E3 medium stock (5 mM NaCl, 0.17 mM KCl, 0.33 mM CaCl2.H2O, and 0.33 mM MgSO4.7H2O) and 0.003% methylene blue in milliQ to allow further development. Eggs were transferred after 24 hours to egg-water (60 µg/ml Instant Ocean® Sea Salt). For the duration of imaging, embryos were kept under anesthesia in egg-water containing 0.02% Tricaine (MS-222; Sigma-Aldrich). Embryos were scored at 28 hpf, or 4 days post fertilization (dpf) and classified on the basis of relative eye size, anterior-posterior body axis length and curvature compared with age-matched controls of the same embryo clutch, into four groups: normal, small-eyed, severe mutant, and dead. All experiments were repeated three times.

Detection of apoptotic cell death. Apoptotic cell death was determined via Acridine Orange (acridinium chloride hemi-[zinc chloride], Sigma-Aldrich; AO) staining. Embryos at 28 hpf were dechorionated and placed in 5 µg/ml of AO in egg-water (60 µg/ml Instant Ocean® Sea Salt). The embryos were stained for 10 minutes and then washed with egg water for 3 times. Before examination, the embryos were anesthetized with 0.02% Tricaine (MS-222; Sigma-Aldrich) and embedded in 1% low-melting point agarose (Boehringer Mannheim, 12841221-01) dissolved in egg-water. All embryos were examined with a dissecting Leica MZ16FA fluorescence stereomicroscope. Brightfield and fluorescence images were generated with a Leica DC500 (DFC420C) camera.

Results

WDR62 interacting proteins
To identify human proteins interacting with WDR62, co-immunoprecipitation followed by mass spectrometry was performed in HeLa cells using human WDR62 as bait using two commercial rabbit polyclonal antibodies against human WDR62 (GeneID 28440). One antibody recognized a region between residue 750 and 800, and the second antibody a region between residue 900 and 950 (Figure 1A). In order to rule out nonspecific protein interactions, two non-related antibodies raised in rabbit were used as controls. As candidates proteins that were present in both WDR62 pull-downs and absent in both control pull-downs were selected. Three proteins fitted the criteria (Table 1) of which, as expected, the highest number of unique peptides belonged to the bait WDR62. The two other proteins, present in both WDR62 pull-downs and absent in
Deficiency of the centrosomal protein CEP170 is associated with microcephaly

the control pull-downs, were CEP170 (20 unique peptides for the WDR62 pull-down antibody 750-800 and 12 unique peptides for the WDR62 pull-down antibody 900-950) and TUFM (2 unique peptides for the WDR62 pull-down antibody 750-800 and 2 unique peptides for the WDR62 pull-down antibody 900-950). TUFM is a mitochondrial Tu translation elongation factor of 50 kDa (452 aa; GenID 7284) and is functioning in mitochondrial protein translation. CEP170 is a centrosomal protein of 170 kDa (1584 aa; GenID 9859), a component of the centrosome, and plays a role in microtubule organization. Based on cellular localization and molecular function CEP170 is similar to WDR62 and therefore we focused on investigating CEP170.

We validated the endogenous interaction of WDR62 with CEP170, identified via mass spectrometry, with reciprocal co-immunoprecipitation experiments combined with Western analysis. These experiments were performed, using the two different WDR62 antibodies (raised against different parts of the WDR62 protein; Figure 1A) and two distinct CEP170 antibodies (raised against different parts of the CEP170 protein; Figure 1B), followed by Western blot analysis. Both WDR62 antibodies co-immunoprecipitated CEP170 (Figure 1C). Vice versa, the two CEP170 antibodies co-immunoprecipitated WDR62. These studies thus confirm an endogenous interaction between WDR62 and CEP170.

Figure 1. WDR62 protein-protein interaction.
(A) Schematic representation of WDR62 protein (1523 aa; NCBI ID: NP_001077430) indicating the position of two independent antibodies (recognizing aa 750-800 and aa 900-950) used for the co-immunoprecipitation experiments. Green blocks indicate WD40 repeats. (B) Schematic representation of CEP170 protein (1584 aa; isoform alpha; NCBI ID: NP_055627.2) indicating the position of two independent antibodies (recognizing aa 900-950 and aa 1534-C-terminus). The red block indicates the forkhead-associated domain (FHA). (C) Endogenous reciprocal co-immunoprecipitation between WDR62 and CEP170 in HeLa S3 cells. Input represents 10% of the total cell extract uses for each immunoprecipitation. FAK was used as control antibody.
WDR62 and CEP170 are primarily localized in the cytoplasm and membranes

The subcellular localization of WDR62 and CEP170 in HeLa cells and whether their localization and/or stability were interdependent was subsequently studied. To this end subcellular fractionation studies in combination with depletion via siRNA were performed. Both WDR62 and CEP170 localized primarily in the cytoplasmic compartment; a significant fraction of both proteins was also found in the membrane fraction. After CEP170 depletion the stabilization and/or localization of WDR62 were not changed (Figure 2A). Vice versa, we neither detected changes after depletion of WDR62 on stabilization and/or cellular localization of CEP170. Thus, our data suggest that the interaction between WDR62 and CEP170 is not related to their stability and/or localization. However, our subcellular fractionation studies indicate that WDR62 and CEP170 both localize to cytoplasmic and membrane fractions, suggesting possible similar molecular roles in both of these cellular components.

WDR62- and CEP170-depleted cells do not harbour chromosomal aberrations

In addition to its role in microtubule organization, CEP170 is phosphorylated upon DNA damage, via ATR or ATM, (Matsuoka et al. 2007) and interacts with PLK1 (Guarguaglini et al. 2005). CEP170 may thus have a role in the DNA damage response. Moreover, WDR62 share several features with proteins of the Fanconi anemia (FA)/BRCA pathway of genome maintenance (Haitjema et al. 2013). We therefore hypothesized that CEP170 and WDR62 may play a role in DNA repair.

To test this, we used a chromosomal breakage assay routinely used to test for defects in the FA proteins. In this assay the number of microscopic visible chromosomal breaks is determined in the presence or absence of a DNA crosslinking agent, such as mitomycin C (MMC). HeLa cells were depleted for WDR62, CEP170, or FANCD2 (FA protein; positive control) via siRNAs specific for WDR62 and CEP170 respectively, in the absence or presence of MMC (Figure 2B – left panel). Alongside, their protein levels in the protein extracts via Western blotting was analyzed (Figure 2B – right panel). The results indicate that the protein levels of the depleted samples (WDR62, CEP170, and FANCD2) were reduced when compared to samples treated with the control non-

---

**Table 1. WDR62 protein-protein interactions.**

<table>
<thead>
<tr>
<th>Protein</th>
<th>Accession Number</th>
<th>MW (kDa)</th>
<th>WDR62 (750-800)</th>
<th>WDR62 (900-950)</th>
<th>FAK (control)</th>
<th>GFP (control)</th>
</tr>
</thead>
<tbody>
<tr>
<td>WDR62</td>
<td>IPI00470483</td>
<td>167</td>
<td>54</td>
<td>45</td>
<td>0</td>
<td>0</td>
</tr>
<tr>
<td>CEP170</td>
<td>IPI00647185</td>
<td>180</td>
<td>20</td>
<td>12</td>
<td>0</td>
<td>0</td>
</tr>
<tr>
<td>TUFM</td>
<td>IPI00027107</td>
<td>50</td>
<td>2</td>
<td>2</td>
<td>0</td>
<td>0</td>
</tr>
</tbody>
</table>
Deficiency of the centrosomal protein CEP170 is associated with microcephaly. Cells with knockdown of FANCD2 displayed a significant increase of the number of breaks in the presence of MMC when compared to the negative control (p-value 0.01). However, both CEP170 (p-value 0.21) and WDR62 (p-value 0.66) depleted cells were not significantly sensitive to MMC. In addition, we analyzed the metaphases for premature chromosome condensation (PCC), which is a hallmark for MCPH1 depleted cells (Trimborn et al. 2004). However, no evidence for PCC was found after siRNA depletion of either WDR62 or CEP170. Thus, no direct role of WDR62 and/or CEP170 in genome maintenance could be detected.

Figure 2. Excluded molecular mechanism WDR62-CEP170 interaction. (A) Subcellular localization of WDR62 and CEP170 combined with depletion via siRNA in HeLa cells. In wild-type cells WDR62 and CEP170 both localize primarily to the cytoplasmic compartment (CE). In addition a significant fraction of WDR62 and CEP170 was found in the membrane fraction (ME). Depletion of WDR62 did not change the stabilization and/or localization of CEP170. Vice versa, after depletion of CEP170 the stabilization and/or localization of WDR62 were not changed. CE: cytoplasmic extract; ME: membrane extract; NE: nuclear extract; CB: chromatin-bound extract; PE: pellet extract; Tubulin: control cytoplasmic fraction; Hexokinase: control membrane fraction; p300: control nuclear fraction. (B) Chromosomal breakage assay. Left panel: HeLa cells were depleted for WDR62, CEP170, FANCD2 (positive control), and control oligo (negative control) via siRNAs in the absence (white bars) or presence (black bars) of MMC. Right panel: Corresponding protein levels in the protein extracts were analyzed via Western blotting. Cells with knockdown of FANCD2 showed a significant increase of the number of breaks in the presence of MMC when compared to the control oligo (p-value 0.01). However, both CEP170 (p-value 0.21) and WDR62 (p-value 0.66) were not significantly sensitive to MMC.
**Micro- and macrocephaly patients with deletions or amplification in CEP170 region**

We subsequently determined whether CEP170 could be linked to human microcephaly. CEP170 resides in the chromosomal region 1q44. The region has been linked to microcephaly before (see Figure 3) but CEP170 has not yet been identified as a causal gene in the interrogated patient populations. We therefore investigated two Dutch cohorts, from the Leiden University Medical Center and the VU University Medical Center. Within these cohorts of unclassified patients 3 patients were identified with deletions and/or duplications within the genomic region of CEP170, 1q43-44 (Table 2 and Figure 3). Patient 1 presented with microcephaly (MIC) and was found to have a deletion of 1.8 Mb in the 1q43-44 region. Patient 2 also presented with microcephaly and was found to have a duplication of 368 kb which disrupted the CEP170 gene only. However, patient 2 has additional chromosomal abnormalities at 6p12.2, and 9p24.1, of which the status in the parents is unknown. Therefore, the microcephaly phenotype could be connected to the CEP170 disruption, but could also be caused by other mutations. Interestingly,

---

**Figure 3. Schematic representation of chromosomal region 1q43-q44 harbouring CEP170.**

Upper panel depicts the ideogram of chromosome 1 indicating chromosomal region 1q43-q44 in a red box. Middle panel depicts CEP170 and neighboring genes located at 1q43-q44. A dark grey vertical bar indicates the CEP170 region in our cohort of patients (1-3). Patients 1 and 2 both present with microcephaly (MIC) and patient 3 presents with a macrocephaly phenotype (MAC). In the patients 1 and 2 the CEP170 gene is disrupted, in patient 3 CEP170 is entirely duplicated. Patient 1 has a de novo deletion. Patient 2 has in addition to the indicated duplication affecting CEP170 additional chromosomal abnormalities at 1q43-44 (241,569,929-241,740,770)x3 (not shown), 6p12.2 (52,611,314-52,701,561)x1, 9p24.1 (6,689,708-6,998,161)x0, the status of these aberration(s) in the parents is unknown. Patient 3 has in addition to indicated 5 Mb duplication an additional 1q44 microdeletion (not shown) the status of the chromosomal aberration(s) in the parents is also unknown. The yellow bar indicates a region of deletion and blue bars indicate regions of duplication. Light grey vertical bars indicate proposed regions for microcephaly (MIC), abnormalities of the corpus callosum (ACC), and seizures (SZR) accounting for ~90% of cases described in literature (Ballif et al. 2012).
Deficiency of the centrosomal protein CEP170 is associated with microcephaly

Patient 3 with a duplicated region of 5 Mb in the 1q43-44 region presented with macrocephaly. Although the 3 patients presented with either micro- or macrocephaly, common additional features were (mild) intellectual disability and upslanted palpebral fissures of the eye and/or hypertelorism with small eyes. The human genetic findings thus suggest that CEP170 could be a candidate gene involved in head (and eye) development.

Knockdown of zebrafish cep170 causes an MCHP-like phenotype

The reciprocal endogenous interaction between WDR62 and CEP170, the centrosomal role of CEP170, the comparable cellular localization of WDR62 and CEP170, and the chromosomal localization, underscores the hypothesis that CEP170 could be involved in microcephaly. In order to study the possible role of CEP170 in microcephaly we used the zebrafish model, which is an established model (Golzio et al. 2012; Novorol et al. 2013).

To investigate the role of CEP170 in a zebrafish model we first identified the zebrafish orthologue. There have been three splice variants of human CEP170 described (Guarguaglini et al. 2005). The longest CEP170 transcript (alpha) encodes a protein of 1584 aa (NCBI ID: NP_055627.2) and the shortest (gamma) encodes a protein of 1460 aa (NCBI ID: NP_001035864). The CEP170 protein contains an FHA domain near the N-terminus. Biochemical studies on γCEP170 have shown that the C-terminus is important for centrosome and microtubule localization (Guarguaglini et al. 2005). The zebrafish orthologue of human αCEP170 protein was identified (NCBI ID: XP_685634.5) along with its corresponding 4516 bp cep170 gene (NCBI ID: XM_680542.5). The zebrafish cep170 protein sequence (1481 aa) shares between 50-59% aa identity with human αCEP170 protein. Both the forkhead associated (FHA) domain and C-terminus are conserved between human and zebrafish (Figure 4A).

We designed a translation-blocking (TB) and a splice-blocking morpholino (MO) directed against the intron1-exon2 splice site of the cep170 zebrafish orthologue. Injection of 5 ng of cep170 SB MO resulted in a marked reduction of cep170 mRNA between ~80% at 1 dpf until ~40% at 6 dpf (Figure S1). We therefore chose to screen the phenotype at approximately 4 dpf similar to other microcephaly phenotype studies in zebrafish (Golzio et al. 2012; Novorol et al. 2013). The reduction of cep170, via either SB or TB MO, resulted in a consistent phenotype with a marked reduction in head and eye size (Figure 4B – top three pictures), which was first noted at 28 hpf and clearly visible at 4 dpf. The consistent microcephaly phenotype induced by either cep170 SB or TB MO ruled out an off-target effect of the MOs. An increase in the amount of SB or TB MO (10 ng) resulted in a shift towards more severe phenotypes (Figure 4C and 4D). Overall the phenotypes of the cep170 morphants closely resemble those of wdr62 morphants and are consistent with a MCPH-like phenotype (Novorol et al. 2013).
<table>
<thead>
<tr>
<th>Patient</th>
<th>1</th>
<th>2</th>
<th>3</th>
</tr>
</thead>
<tbody>
<tr>
<td>Age (year)</td>
<td>47</td>
<td>43</td>
<td>13</td>
</tr>
<tr>
<td>Sex</td>
<td>M</td>
<td>M</td>
<td>F</td>
</tr>
<tr>
<td>Age at evaluation</td>
<td>45</td>
<td>41</td>
<td>9</td>
</tr>
<tr>
<td>Reason for diagnosis</td>
<td>Microcephaly, intellectual disability</td>
<td>Microcephaly, intellectual disability</td>
<td>Mild developmental delay, recurrent urinary tract infections, torticollis, and mild dysmorphic features</td>
</tr>
<tr>
<td>Frontal occipital circumference</td>
<td>-2.5 SDS</td>
<td>-2.6 SDS</td>
<td>+2 SDS</td>
</tr>
<tr>
<td>Weight</td>
<td>BMI 17.2</td>
<td>BMI 21.9</td>
<td>+1.5 SDS</td>
</tr>
<tr>
<td>Height</td>
<td>+1.6 SDS</td>
<td>-2.8 SDS</td>
<td>0 SDS</td>
</tr>
</tbody>
</table>

**Cranio-facial dysmorphism**

| Head | Microcephaly, plagiocephaly with asymmetry of the face | Microcephaly | Mild prominent forehead, high hairline |
| Facial dysmorphic features | Upslanted palpebral fissures, thin nasal bridge with broad nasal base, large mouth with thin upper lip | Small eyes with mild hypertelorism, upslanted palpebral fissures | Hypertelorism with narrow eye palpebral fissures, heterochromia iridium, mild pointed chin, high placed eyebrows |

**Nervous system**

| Developmental | Intellectual disability | Intellectual disability | Intellectual disability (mild) |
| Seizures | - | Suffered from seizures for which medication was given, after age 25 medication was stopped and seizures did not recur. | Dizziness |
| Behavioral issues | Minor (mood changes, extremely anxious of fireworks) | Autism spectrum disorder | Poor concentration |
| Structural brain abnormalities | No brain imaging available | No brain imaging available | No brain imaging available |

**Other**

| Heart | Murmur |
| Orthopedics | Long, narrow feet | Asymmetry of the body due to hemiparesis (unknown cause) | Hip dysplasia |
Deficiency of the centrosomal protein CEP170 is associated with microcephaly

Zebrafish MCPH-like phenotype caused by increase in apoptosis at an early embryonic stage

Examination of 28 hpf embryos revealed the presence of many round cells resembling dying cells in the morphant embryos compared to untreated age matched embryos (Figure 5). To determine if these rounded cells are undergoing apoptosis we performed an Acridine Orange staining. A clear increase in the number of apoptotic cells was visible throughout the CNS and spinal cord of cep170 morphants at 28 hpf (Figure 5) which might explain the reduction in head and eye size.
Additional morphological abnormalities were observed in some but not all *cep170* morphant embryos including cardiac oedema, curvature of the tail, reduction in Anterior-Posterior axis or completely shortened and curved embryos. Interestingly, at 48 hpf morphant embryos displayed an inflated hindbrain ventricle which together with the cardiac oedema suggested problems with the development of vasculature (Figure 6). Therefore, we decided to use the *Tg(Fli1:GFP)y1* Casper zebrafish line to examine vasculature development after *cep170* knockdown. The *Tg(Fli1:GFP)y1* Casper zebrafish line has been specially established to study vascular development in zebrafish embryos (*Fli1* is an endothelial cell marker expressed during vascular development in zebrafish embryos) (Lawson and Weinstein 2002a; Lawson and Weinstein 2002b). In zebrafish injected with *cep170* MO the vasculature development was clearly delayed at 28 hpf when compared to control zebrafish (Figure 6). At 4 dpf the final segmental artery of the *cep170* morphants were less dense and structured than the final segmental artery of the controls at 4 dpf. The results suggest a possible role for *cep170* in vasculature development. In total, the data suggest that the reduction in *cep170* expression gives rise to a phenotype associated with altered vasculature development and microcephaly.

**Discussion and conclusion**

WDR62 was considered as a suitable bait to expand the protein network important for microcephaly. In this study we uncovered CEP170 as a binding partner of WDR62. CEP170 constitutes a centrosomal protein linked to DNA damage response pathways (Guarguaglini et al. 2005). In line with the observed interaction between WDR62 and CEP170, the proteins could be found in the same cellular compartments, while their localization and stability was not interdependent. The gene location of CEP170 at 1q43-q44 supported a possible role in microcephaly. An unequivocal link between
Deficiency of the centrosomal protein CEP170 is associated with microcephaly

CEP170 gene disruption could, however, not be established. To test a possible connection, a zebrafish model was established which clearly showed that loss of CEP170 could interfere with head, and also eye size.

WDR62 has been unambiguously linked to microcephaly in humans (Bilgüvar et al. 2010; Nicholas et al. 2010; Yu et al. 2010; Kousar et al. 2011; Murdock et al. 2011; Bhat et al. 2011; Bacino et al. 2012; Sajid Hussain et al. 2012). Conversely CEP170 is present in a chromosomal region linked to microcephaly (MIC), agenesis of the corpus callosum (ACC [MIM 217990]), and seizures (SZR; Figure 6). Attempts have been made to connect these phenotypes to specific genes. Reported cases could be linked to the genes AKT3 (MIC [MIM 611223]), ZNF238 (ACC [MIM 608433]), FAM36A (SZR [614698]), C10ORF199 (SZR [MIM 612337]), and HNRNPU (SZR [MIM 602869]) (Ballif et al. 2012). CEP170 in combination with ZNF238 has been implicated as the possible causative gene for ACC (Nagamani et al. 2012). However, a recently described patient with ACC had a deletion including ZNF238 and not CEP170 (Perlman et al. 2013). Therefore, at least ACC could be caused by ZNF238 mutation. However, based on these data CEP170 could still be a candidate gene contributing to head size in humans.

Therefore we investigated cohorts from 2 Dutch clinical genetics centers for co-occurrence of microcephaly and aberrations in the CEP170 chromosomal region. Two patients with microcephaly were identified (Table 2 and Figure 3). Patient 1 carried a deletion in the 1q43-q44 region and exhibited similar characteristics as described previously (Ballif et al. 2012). Patient 2 had instead of a deletion, a duplication which disrupted the CEP170 gene only. This supported a possible role for CEP170 in human
microcephaly. Nevertheless, patient 2 had additional chromosomal abnormalities at 6p12.2, and 9p24.1, of which the status in the parents is unknown. Therefore, also in these cohorts, CEP170 could not be unequivocally linked to head size.

It has been recently reported that KCTD13, depending on copy number variants (CNVs), results in either micro- or macrocephaly – a mirrored phenotype (Golzio et al. 2012). We therefore investigated the same two independent Dutch cohorts for macrocephaly combined with DNA gains in the CEP170 region. Notably, one patient associated with a 5 MB duplication within the 1q43-q44 region, presented with macrocephaly. Therefore, the gene(s) in the region 1q43-q44 that cause(s) microcephaly could hypothetically also cause macrocephaly depending on CNVs.

The protein-protein interaction data combined with the human genetics data support the hypothesis that CEP170 disruption could be important for head size. We therefore tested this in zebrafish. The generated cep170 morphant zebrafish were indeed associated with the microcephaly phenotype, and have in addition small eyes. This phenotype was remarkably similar to the previously-described zebrafish wdr62 morphant (Novorol et al. 2013). Together this reinforces the proposition that CEP170 together with WDR62 could contribute to head size. RNA rescue and overexpression experiments so far with 2 cDNAs from CEP170, type alpha-CEP170 and gamma-CEP170, in zebrafish did, nevertheless, not result in a reproducible and robust phenotype. It is thus unclear whether CEP170 (or WDR62) expression levels can result in mirrored phenotypes dependent on either up- or down-regulation. Of note, small eyes were also a characteristics observed in our patient cohort, although it is uncertain if the eyes are small or the eyes appear small due to narrow palpebral fissures. Further investigation of the patients is necessary in order to link the phenotype observed in the zebrafish morphant with the human phenotype.

Importantly, our zebrafish studies indicated that the phenotype associated with cep170, besides microcephaly, was also characterized by delayed and structurally impaired vasculature development. This phenotype has not been described for the zebrafish wdr62 morphant, (Novorol et al. 2013). Interestingly, patients with typical vascular disorders such as Sturge-Weber syndrome (SWS [MIM 185300]) and the associated Klippel-Trenaunay-Weber syndrome (KTW [MIM 149000]) were described with, among other characteristics, mental retardation, polymicrogyria, seizures, and micro- or macrocephaly (Hall et al. 2007). However, vascular malformations have so far not been reported in WDR62 patients (Memon et al. 2012). Nonetheless, this suggests that cellular processes causing micro- and/or macrocephaly are closely related, and/or overlap, with the vasculature development process.
Deficiency of the centrosomal protein CEP170 is associated with microcephaly

Since WDR62 and CEP170 can bind, and both have been linked to microcephaly and eye size, these likely contribute together to a cellular process especially critical in cells from the neuronal lineage. In non-neuronal HeLa cells, both proteins were primarily found in the cytoplasm and in the membrane fraction, while their location or stability was not interdependent. The observed cytoplasmic location is in line with the proposed centrosomal role of both CEP170 and WDR62 (Guarguaglini et al. 2005; Bhat et al. 2011). Moreover, reduction of the levels of CEP170 and WDR62 was not associated with chromosomal breaks; thus no direct or indirect connection with DNA repair was observed. The zebrafish wdr62 morphant showed increased levels of mitotic and apoptotic cells, while the apoptosis seemed to be secondary to the mitotic defects (Novorol et al. 2013). Also the zebrafish cep170 morphant in this study showed these increased levels of apoptotic cells. Overall, the findings support a model in which perturbation of a basic process during mitosis contributes to microcephaly.

Our study suggests a molecular connection between WDR62 and CEP170, thereby expanding the molecular network involved in microcephaly. In addition, our data emphasize that genes associated with microcephaly could possibly also be important for vasculature development.

Supplemental Data
Supplemental Data includes Material and Methods and one figure.

Acknowledgements
We thank the patients and their parents for their participation in the study. We would like to thank Marjo den Broeder for supplying us the pCS2+ vector. We also thank the Laboratory of Diagnostics Genome Analysis, Leiden University Medical Center and the Genome diagnostic laboratory of the VU University Medical Center for conventional karyotyping and FISH experiments.

Web Resources
The URLs for data presented herein are as follows:
Online Mendelian Inheritance in Man (OMIM), http://www.omim.org/
References


Material and Methods S1

RNA extraction, cDNA synthesis and qRT-PCR analyses. Total RNA was extracted according to the manufacturer's protocol (High pure Isolation Kit, Roche) from 5-10 zebrafishes (1 and 6 dpf) and cDNA was prepared (iScript cDNA synthesis kit, Biorad). The mRNA levels were quantified by real-time quantitative polymerase chain reactions (SYBR Green reaction kit, Lightcycler 480, Roche). The forward primer against cep170 is located in exon 1 (5’- GGGATAAAACTGACAGCGAAA-3’) and the reverse primer in exon 2 (5’-CCCACAAAGATCATCTCACG-3’), the target of the SB MO. Relative gene expression was calculated via the $2^{-\Delta\Delta C_T}$ method, normalized against the zebrafish housekeeping gene beta-actin (5’-CGAGCAGGAGATGGGAACC-3’ and 5’-CAACGGAAACGCTCATTGC-3’). Knockdown efficiency was calculated comparing the zebrafish injected with cep170 SB MO to the control zebrafish.

Supplemental Figure 1. Knockdown efficiency of cep170 SB MO injected zebrafish.
Deficiency of the centrosomal protein CEP170 is associated with microcephaly