CEP41 is mutated in Joubert syndrome and is required for tubulin glutamylation at the cilium

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Tubulin glutamylation is a post-translational modification that occurs predominantly in the ciliary axoneme and has been suggested to be important for ciliary function[1,2]. However, its relationship to disorders of the primary cilium, termed ciliopathies, has not been explored. Here we mapped a new locus for Joubert syndrome (JBTS)[3], which we have designated as JBTS15, and identified causative mutations in *CEP41*, which encodes a 41-kDa centrosomal protein[4]. We show that CEP41 is localized to the basal body and primary cilia, and regulates ciliary entry of TTLL6, an evolutionarily conserved polyglutamylase enzyme[5]. Depletion of CEP41 causes ciliopathy-related phenotypes in zebrafish and mice and results in glutamylation defects in the ciliary axoneme. Our data identify *CEP41* mutations as a cause of JBTS and implicate tubulin post-translational modification in the pathogenesis of human ciliary dysfunction.

Joubert syndrome (JBTS MIM 213300) is characterized by cerebellar hypoplasia and neurological features, including ataxia, psychomotor delay and oculomotor apraxia with a pathognomonic 'molar tooth sign' on brain imaging. JBTS is frequently accompanied by various multi-organ signs and symptoms, including retinal dystrophy, nephronophthisis, liver fibrosis and polydactyly, conditions that are associated with disorders of the ciliopathy spectrum of diseases that include Meckel-Gruber Syndrome (MKS), Bardet-Biedl syndrome (BBS) and nephronophthisis (NPHP). Although several causative genes have been found for these disorders, they account for less than 50% of cases[6,7]. We recruited a consanguineous two-branch Egyptian family (MTI-429) with five affected members (**Fig. 1a,b** and **Table 1**). We excluded linkage to previously identified JBTS-associated loci using a panel of highly informative markers. Analysis of the family using the Illumina Linkage IVb SNP mapping panel identified a 5-Mb region of linkage at chromosome 7q31.33-32.3 with a peak multipoint logarithm of odds (LOD) score of 3.71, which we have defined as the JBTS15 locus. Haplotype analysis indicated that a candidate interval between the rs766240 and rs4728251 SNPs represented the peak of highest significance (**Supplementary Fig. 1a,b**).

To further narrow the interval, we reanalyzed MTI-429 samples with the denser Affymetrix 250K NspI SNP array by applying a linkage-free, identity-by-descent (IBD) model[8]. The combination of the two SNP linkage analyses identified a 2.8-Mb IBD interval between rs17165226 and rs2971773 that contains 26 genes (**Fig. 1c**). Direct sequence analyses of candidate genes within the interval led to the identification of a homozygous c.33+2T>G nucleotide change in *CEP41* (NM_018718), which was predicted to abolish the consensus splice-donor site from exon 1 of the *CEP41* gene (**Fig. 1d** and **Supplementary Fig. 2a**). To determine whether this mutation causes a splicing defect, we evaluated *CEP41* transcripts from primary fibroblasts that were isolated from

Figure 1 Identification of mutations in *CEP41* in affected persons linked to the JBTS15 locus. (**a**) The pedigree of MTI-429 shows two first cousin marriages with a total of five affected offspring (filled symbols). The symbol with a slash indicates an individual who is deceased. (**b**) Axial brain MRI images from individuals with *CEP41* mutations in the MTI-429, MTI-1491 (T1-weighted) and COR-98 (T2-weighted) families, showing the molar tooth sign (red arrows). (**c**) The JBTS15 locus spans 5.5 Mb at chromosome 7q31.33-32.3 (red box) defined by rs766240 and rs4728251. A denser SNP scan further narrowed the JBTS15 interval to a 2.8-Mb region encompassing rs17165226 and rs2971773 (black arrows). (**d**) *CEP41* genomic organization, depicting locations of identified base changes, including homozygous (red) splice-site mutations and heterozygous (blue) missense and nonsense mutations. Capital letters, exon sequences; small letters, intron sequences; asterisks, point mutations; underline, deletion. (**e**) RT-PCR confirmation of the splicing defect of *CEP41* in fibroblasts from MTI-429 subjects. Both *CEP41* mutant cell lines failed to produce *CEP41* mRNA compared with cells isolated from a control subject (WT, wild type). *GAPDH* was used as a control. Green arrows, positions of primers; asterisk, region of splice-site mutation in MTI-429.

MTI-429 family members (MTI-429-IV-1 and MTI-429-IV-6). The RT-PCR result showed an absence of mature *CEP41* mRNA products in cells from both individuals, which can probably be attributed to nonsense-mediated decay (**Fig. 1e**).

We next screened an additional 832 individuals with ciliopathy (720 with JBTS and 112 with MKS, many of whom were excluded for mutations in known ciliopathy genes) by directly sequencing *CEP41* and found two additional consanguineous families with homozygous mutations, including c.97+3_5delGAG in an Egyptian family with JBTS (MTI-1491) and c.423-2A>C in a Portuguese family with JBTS (COR-98) (**Fig. 1b–d** and **Supplementary Fig. 2a**). These mutations were predicted to abolish the consensus splice-donor site from exon 2 and the splice-acceptor site from exon 7, respectively. Moreover, we confirmed that the mutation in MTI-1491 led to skipping of exon 2, thereby generating a premature stop in exon 3 (**Supplementary Fig. 2b**). In addition to the individuals with JBTS, the MTI-1491 family included one family member with a phenotype consistent with BBS who lacked the pathognomonic molar tooth sign, and this individual was heterozygous for the c.97+3_5delGAG mutation (**Supplementary Fig. 2b–d**), suggesting that *CEP41* may modify other ciliopathy conditions. From our cohort screen of families affected with JBTS, we identified additional heterozygous *CEP41* mutations (c.83C>A, c.107T>C, c.265C>G, c.536G>A and c.1078C>T) that alter amino acid residues that are highly conserved among vertebrates or lead to a premature stop codon (**Fig. 1d**, **Supplementary Fig. 2e** and **Supplementary Table 1**). Each of the individuals with *CEP41* mutations was additionally sequenced at the known JBTS-associated genes, and four were found to harbor an additional heterozygous, potentially deleterious variant. It is notable that all homozygous mutations in *CEP41* were splice-site mutations and were identified only in subjects with JBTS, whereas heterozygous mutations were present in several ciliopathies, including BBS and MKS. Our findings suggest that constitutive disruptions of CEP41 result in JBTS, but CEP41 may also serve as a modifier in the broader class of ciliopathies.

The *CEP41* gene has been poorly characterized except for expression analysis in human organs, including brain, testis and kidney[9]. *CEP41* encodes a 41-kDa centrosomal protein that is predicted to contain two coiled-coil domains and a rhodanese-like domain (RHOD), which is structurally related to the catalytic subunit of the Cdc25 class of phosphatases[10]. However, we found that the CEP41 protein lacks phosphatase activity in an *in vitro* p-nitrophenylphosphate (pNPP) phosphatase assay (data not shown). The RHOD domain may therefore be an enzymatically inactive version similar to RHOD domains previously described in other proteins[10] that function in protein interactions.

We next examined *CEP41* gene expression at the mRNA level and CEP41 protein subcellular localization. In zebrafish, *cep41* was expressed in various ciliary organs, including Kupffer's vesicle, ear and heart, as well as brain and kidney, the regions that are predominantly affected in JBTS (**Fig. 2a** and **Supplementary Fig. 3**). In several ciliated cell lines, such as mouse inner medullary collecting duct cells (IMCD3) and human retinal pigment epithelial cells (hTERT-RPE1), endogenous CEP41 protein was predominantly noted at the centrioles and cilia (**Fig. 2b**). The cilia-associated expression and localization of CEP41 prompted us to assess a possible role for this protein in cilia-related function. Accordingly, we performed knockdown experiments using translation-blocking morpholino antisense oligonucleotides (MOs) in zebrafish. In embryos injected with *cep41* MO (morphants), we observed peripheral heart edema and tail defects along with ciliopathy-related phenotypes, including hydrocephalus, abnormal ear otolith formation and smaller eyes[11–14] (**Supplementary Fig. 4a,b**). We also found that injection of *cep41* MO induced a decrease in production of the protein, with dose-dependent phenotypic severity in the embryos (**Supplementary Fig. 4b,c**).

Cilia of Kupffer's vesicle, a structure that corresponds to the mammalian embryonic node, are essential to mediate lateral asymmetry[15]. Accordingly, defects in left-right asymmetry of the heart, a well-established ciliopathy phenotype in mammals, has been noted

in zebrafish[16–18]. To examine whether *cep41* depletion resulted in a heart laterality phenotype, we injected *cep41* MO into Tg(*myl7:egfp*) zebrafish, a myocardium-specific transgenic reporter line, and found defects such as inversion or failure to develop asymmetry of the ventricle and atrium (**Fig. 2c**). We also generated a *Cep41* knockout mouse line using a gene trap strategy (**Supplementary Fig. 5a–c**)

and characterized its phenotype at embryonic days 10–13 (E10-E13). The homozygous *Cep41*[Gt/Gt] embryos showed a range of phenotypes: malformed hindbrain, exencephaly, brain hemorrhage, dilated pericardial sac and lethality, as well as unexpected normal development in some homozygous mutants (**Fig. 2d**, **Supplementary Fig. 5d,e** and **Supplementary Table 2**). Although exencephaly, dilated heart

Table 1 Summary of clinical features of individuals with homozygous *CEP41* mutations

	MTI-429-IV-1	MTI-429-IV-2	MTI-429-IV-5	MTI-429-IV-6	MTI-429-IV-7	MTI-1491-V-2	MTI-1491-V-3	COR-98
Demographic information								
Family ID	MTI-429-IV-1	MTI-429-IV-2	MTI-429-IV-5	MTI-429-IV-6	MTI-429-IV-7	MTI-1491-V-2	MTI-1491-V-3	COR-98
Country of origin	Egypt	Egypt	Egypt	Egypt	Egypt	Egypt	Egypt	Portugal
Sex	M	M	F	M	M	F	F	M
Death	N	N	N	N	Died at 7 days	N	N	N
Documented consanguinity	Y	Y	Y	Y	Y	Y	Y	Y
Neurological signs								
Hypotonia/ataxia	Y	Y	Y	Y	Y	Y	Y	Y
Psychomotor delay	Y	Y	Y	Y	N/A	Y	Y	Y
Mental retardation	Mild	Borderline	Borderline	Borderline	N/A	Y	Y	Y
Oculomotor apraxia	Y	Y	N	N	N/A	Y	Y	N
Breathing abnormalities	N	N	N	N	Y	Y	Y	N
Head circumference	50th percentile	50th percentile	50th percentile	50th percentile	75th percentile	50th percentile	50th percentile	N/A
Ocular signs								
Retinopathy	N	N	N	N	U	N	N	Y
Other abnormalities	B ptosis	U ptosis and leukoma	U ptosis	N	N	U ptosis, squint, leukoma	B squint and leukoma	N
Coloboma	N	N	N	N	U	N	N	N
Renal signs								
NPHP/UCD	N	N	N	N	U	N	N	N
Kidney ultrasound	N	N	N	N	N	N	N	N
Other organs								
Liver abnormalities	N	N	N	N	M	N	N	N
Polydactyly	N	N	U postaxial	U postaxial	B postaxial	N	U postaxial	Y
Other abnormalities	GHD, MP	GHD, MP	N	MP	AG, MP, hypoplastic scrotum	N	N	N
MRI reading								
MTI	Y	Y	Y	Y	Y	Y	Y	Y
Other abnormalities	N	N	N	N	N	N	N	N
***CEP41* mutation analysis**								
Exon(s)	1					2		7
Hetero/Homozygous	Homozygous					Homozygous		Homozygous
Nucleotide change	c.33+2T>G					c.97+3_5delGAG		c.423-2A>C
Amino acid change	Splice site					Splice site		Splice site
Amino acid consequence	Premature stop					Premature stop		Predicted premature stop
Comprehensive JSRD genetic screening								
Linkage	Linkage to chromosome 7 (q31.33-q32.3)					Linkage negative to *AHI1*, *TMEM67* and *TMEM138-TMEM216*		Linkage negative to *AHI1*, *TMEM67* and *TMEM138-TMEM216*
Additional gene with PDSV	None					None		*CEP290*
Exon								31
Heterozygous/homozygous								Heterozygous
Nucleotide change								c.3626C>G
Amino acid change								p.Ser1209Cys
Amino acid consequence								Neutral to neutral
In NCBI SNP database								No

AG, ambiguous genitalia; B, bilateral; F, female; GHD, growth hormone deficiency; M, male; MP, micropenis; MTI, molar tooth on imaging; N/A, not available or not applicable; NPHP, nephronophthisis; PDSV, potentially deleterious sequence variant; U, unilateral; UCD, urinary concentrating defect.

Figure 2 *CEP41* is expressed in ciliated tissues and its loss recapitulates ciliopathy-related phenotypes in zebrafish and mouse. (**a**) Zebrafish *cep41* mRNA is expressed ubiquitously at gastrulation stages (6 h post-fertilization (h.p.f.)), but at later stages, it is expressed specifically in ciliated organs: Kupffer's vesicle (red box), inner ear (white boxes), brain (brackets), eyes (arrows), pronephric duct (black box) and heart (asterisks). A, anterior; D, dorsal; P, posterior; V, ventral. (**b**) CEP41 is predominantly localized to the basal body (arrowheads) and primary cilium (arrows) in ciliated IMCD3 and hTERT-RPE1 cells. Scale bars, 5 μm. Insets, co-localization of CEP41 with GT335, a marker of basal bodies and cilia. (**c**) Knockdown of *cep41* by injection with *cep41* MO causes heart laterality defects in Tg (*myl7*:*egfp*) zebrafish embryos. The *cep41* morphants show either loss of asymmetry (midline) or inversion of ventricle-atrium asymmetry (reversed) at 72 h.p.f. Error bars, s.e.m. *$P < 0.01$, **$P < 0.001$. A, atrium; V, ventricle. (**d**) *Cep41* gene-trap mice show altered embryonic morphogenesis. The range of phenotypes for *Cep41^{Gt/Gt}* embryos includes mild malformed hindbrain (arrowheads), exencephaly (brackets), hemorrhage in the head (asterisks), dilated pericardial sac (arrows), failure to rotate and lethality at E10–12. (**e**) Injection of human *CEP41* RNA into *cep41* morphants rescued ciliary phenotypes of pericardial edema (arrowhead), hydrocephalus (asterisk) and curved tail (arrows), completely or partially.

and embryonic lethality suggest possible ciliary roles of Cep41 in the mouse[16,19,20], the phenotypic variability, including the occurrence of normal development, suggests the presence of extragenic phenotypic modifiers. We attempted a genetic rescue using human *CEP41* in zebrafish *cep41* morphants and found partial rescue of the cilia-associated morphant phenotype (**Fig. 2e**). These data suggest that CEP41 potentially has an evolutionarily conserved role in ciliary function.

To explore the possible roles of CEP41, we examined primary cultured fibroblasts from human subjects (MTI-429-IV-1 and MTI-429-IV-6). We first tested whether *CEP41* mutant cells were devoid of CEP41 protein. Consistent with RT-PCR results (**Fig. 1e**), neither fibroblast line produced detectable CEP41, showing that these mutant cells are nearly null for CEP41 (**Fig. 3a,b**). To examine the effect of CEP41 loss on cilia assembly, we induced ciliogenesis using serum starvation–mediated cell cycle arrest in confluent cells and visualized cilia by co-immunostaining with antibodies to ARL13B (a cilia marker)[21] and GT335 (a centriole and cilia marker)[22]. In control fibroblasts with wild-type *CEP41*, cilia were evident in 70% of the total stained cells by 48 h and 72 h of serum starvation, and nearly all co-stained with both markers (**Fig. 3c** and **Supplementary Fig. 6**). However, in the *CEP41* mutant fibroblasts, cilia were stained positively with ARL13B but not with GT335 (**Fig. 3c**). We quantified this

effect and found that, whereas the percentage of ARL13B-positive ciliated cells in the mutant fibroblasts was approximately equal to that in control fibroblasts, the percentage of GT335-positive ciliated cells was substantially reduced in mutant fibroblasts (**Supplementary Fig. 6**). The antibody to GT335 was originally generated to recognize the glutamylated forms of tubulin (having both mono- and polyglutamylation)[23]. Therefore, the data suggest a potential role of CEP41 in regulating tubulin glutamylation.

Microtubules are the major structural scaffolds of the ciliary axoneme and undergo several post-translational modifications, including acetylation, detyrosination, glycylation and glutamylation (**Supplementary Fig. 7**). We therefore tested the effect of CEP41 deficiency on these tubulin post-translational modifications in human primary fibroblasts and found no defects other than in glutamylation in *CEP41* mutant cells (**Supplementary Fig. 8**). In addition, immunostaining using PolyE antibody (specific for polyglutamylated tubulin) in mutant fibroblasts indicated that CEP41 might regulate both tubulin mono- and polyglutamylation (**Supplementary Fig. 9**). Concordantly, we observed a defect in tubulin glutamylation as well as a mild reduction in glycylation of the olfactory placode cilia in zebrafish deficient in *cep41* relative to wild-type animals (**Fig. 3d** and **Supplementary Fig. 10**). In the human fibroblast lines, forced expression of exogenous CEP41 in mutant fibroblasts notably increased the

Figure 3 CEP41 is required for tubulin glutamylation at the ciliary axoneme. (**a**) Absence of CEP41 protein in *CEP41* mutant cells from human subjects. (**b**) Ciliary localization of endogenous CEP41 in human fibroblasts and loss of the protein in *CEP41* mutant cilia. Scale bar, 5 μm. (**c**) Cells with wild-type *CEP41* have both GT335-positive basal bodies (arrowheads) and cilia (arrows, marked by ARL13B), whereas cells with mutant *CEP41* show staining of GT335 only at the basal bodies. Scale bar, 5 μm. (**d**) Depletion of *cep41* causes glutamylation defects in zebrafish olfactory placode cilia (red arrows). Images of GT335-stained wild-type and *cep41* morphant embryos were taken in anterior view (head is up, tail is down), with quantification shown below. D, dorsal; V, ventral. (**e**) Exogenous CEP41 expression restores ciliary axoneme glutamylation in *CEP41* mutant cells. Arrows, primary cilia stained for CEP41 and GT335. Scale bar, 5 μm. Insets, merged images at higher magnification, with quantification shown below. Error bars, s.e.m. **P < 0.001. (**f**) Ultrastructural analysis of the pronephric ciliary axoneme at 72 h.p.f. in zebrafish embryos. Compared to wild-type embryos, *cep41* morphants have A-tubule–specific defects in the outer doublet microtubules. Arrows, A-tubules. Scale bars, 100 nm. One of nine outer doublet microtubules is magnified in the red box. The numbers of cilia, categorized as having normal and abnormal A-tubules according to the schematic were counted in both wild-type embryos and *cep41* morphants (n = 3 embryos each, >20 cilia per animal), with quantification shown below. Error bars, s.e.m. *P < 0.01.

percentage of cells with glutamylated cilia (**Fig. 3e**), suggesting that glutamylation of the cilium is dependent on the expression of CEP41. Furthermore, we found that cell lines from individuals with mutations in other JBTS-associated genes, including *TMEM216* and *INPP5E*, displayed no such glutamylation defects (data not shown), indicating that the tubulin glutamylation phenotype is not a nonspecific consequence of JBTS-causing mutations. Together, these data show that CEP41 is required for ciliary glutamylation but is not necessary for initial cilia assembly.

Recent studies have shown that defective tubulin post-translational modification is associated with altered ciliary axonemal structure[24–26]. Accordingly, we investigated whether the CEP41-dysfunctional cilia exhibiting glutamylation defects had an abnormal axonemal structure. Transmission electron microscopy showed that depletion of *cep41* resulted in obvious structural defects in zebrafish renal cilia. Specifically, we found that A-tubules of the outer doublet microtubules of the axoneme were collapsed and/or duplicated (**Fig. 3f** and **Supplementary Fig. 11**). Previous studies have suggested that ciliary structural disruption affects ciliary motility[27,28]; thus, we examined the cilia of Kupffer's vesicle and kidney in zebrafish *cep41* morphants

and found disabled motility of both types of cilia (**Supplementary Fig. 12** and **Supplementary Videos 1–6**). Our data suggest that CEP41 is involved in ciliary structural formation and motility by having an essential role in tubulin glutamylation at the cilium.

We next investigated how CEP41 modulates microtubule post-translational modifications at the ciliary axoneme. We noted that only microtubules of ciliary axonemes showed defective glutamylation, whereas those of centrioles were properly modified in the *CEP41* mutant human fibroblasts (**Fig. 3c**). Furthermore, the lack of a tubulin tyrosine ligase (TTL) domain in CEP41, which is required for enzymatic activity[5], implies that it is unlikely to serve as a glutamylase. The main enzymes mediating tubulin glutamylation are members of the conserved TTL-like (TTLL) family[5]. Among several identified TTLL factors, TTLL6 was found to show consistently strong localization to the basal body (the organizing structure at the base of cilium that is derived from a mother centriole) and the cilium (**Supplementary Fig. 13**). In addition, previous studies have suggested that TTLL6 may be involved in ciliary function in several organisms, including zebrafish[5,24,29]. We therefore examined the effects of *ttll6* deficiency using a *ttll6* translation-blocking MO[30] in zebrafish and observed

Figure 4 CEP41 interacts with TTLL6 and is required for TTLL6 localization to the cilium. (a) MO-mediated knockdown of zebrafish *ttll6* results in ciliary phenotypes, such as abnormal number and/or orientation of ear otolith (boxes), cystic kidney (arrowheads) and peripheral cardiac edema (asterisks) as well as curved tail (arrows) at the different dosages indicated and causes A-tubule–specific defects in the outer doublet microtubules. The numbers of defective cilia were counted in both wild-type embryos and *ttll6* morphants ($n = 3$ embryos each, >20 cilia per animal), with quantification shown below. (b) GFP-CEP41 and its associated proteins were immunoprecipitated with antibody to the Flag epitope recognizing Flag-tagged TTLL6 from the whole-cell extract (WCE) of cells transfected with this expression construct, with immunoprecipitation in WCE from cells transfected with empty GFP vector serving as a control. In the reciprocal co-immunoprecipitation experiment with antibody to GFP, the interaction between CEP41 and TTLL6 was confirmed. (c) Disturbed localization of TTLL6 to the cilium following the co-transfection of *Cep41* siRNA with GFP-TTLL6 into IMCD3 cells as determined by immunostaining with either GT335 or ARL13B antibody. Arrows, cilia; arrowheads, basal bodies. Scale bar, 5 μm. Cells expressing cilium-localized TTLL6 were counted only in siRNA-transfected cells and are quantified in the graph. Error bars, s.e.m. *$P < 0.01$, **$P < 0.001$.
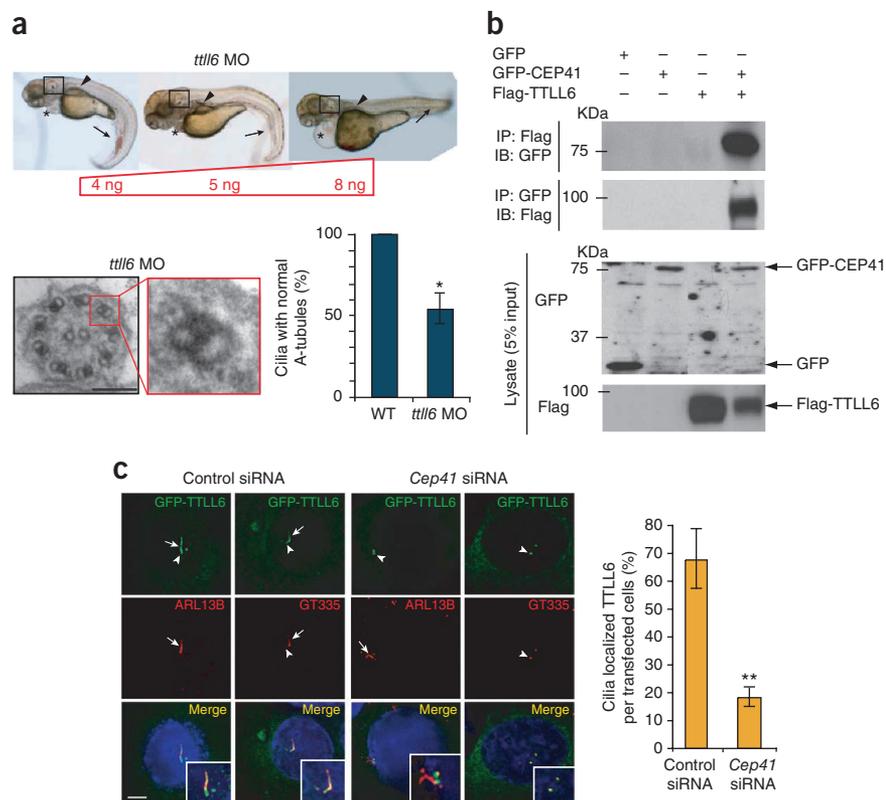
ciliopathy-related morphological phenotypes similar to, although less severe than, what we observed following *cep41* knockdown (**Fig. 4a**). Additionally, *ttll6* morphants showed A-tubule axonemal defects, similar to those of *cep41* morphants (**Fig. 4a**), along with diverse axonemal structural defects, which were consistent with a previous report[30] (data not shown). These data prompted us to test a possible functional relationship between CEP41 and TTLL6 by pairwise co-immunoprecipitation. We found that the two proteins were part of a complex (**Fig. 4b**). We next tested whether CEP41 might function to regulate the transport of TTLL6 between the basal body and the cilium as has been suggested for the *fleer* gene in zebrafish[29]. Following efficient knockdown of *Cep41* in mouse IMCD cells using siRNA (**Supplementary Fig. 14**), we found localization of TTLL6 restricted mainly to the basal bodies, indicating a block of TTLL6 entry into the cilium (**Fig. 4c**). These data suggest that CEP41 functions in tubulin glutamylation by mediating transport of TTLL6 between the basal body and cilium.

Our discovery of *CEP41* mutations in persons with JBTS provides the first evidence directly linking defective tubulin glutamylation at the cilium to a human ciliopathy. Consistent with previous studies implicating tubulin post-translational modifications in cilia function[23,24,31,32], our data suggest that CEP41-mediated ciliary glutamylation is essential for axonemal formation. Moreover, we found that CEP41 is required for the transport of TTLL6 to modulate tubulin glutamylation, although it remains to be determined how these molecules function together. Most likely, these proteins enter through the ciliary diffusion barrier at the transition zone[33,34] and are then transported along ciliary microtubules by intraflagellar transport motor proteins. Thus, examining whether CEP41 and TTLL6 form a complex with other factors at these locations will follow as a future study. Because tubulin glycylation is partially affected in *cep41* morphants and the phenotype of *cep41* morphants is more severe than

that of *ttll6* morphants, it is likely that CEP41 modulates other TTLL family members[5,30]. Evidence for this possibility has been described in recent studies that show the involvement of both tubulin glycylation and glutamylation, each regulated by different TTLL family members, in maintaining ciliary structure and motility[30,35–37].

URLs. USCS Human Genome Browser, http://www.genome.ucsc.edu/; ClustalW, http://www.ebi.ac.uk/Tools/msa/clustalw2/.

METHODS

Methods and any associated references are available in the online version of the paper at http://www.nature.com/naturegenetics/.

Note: Supplementary information is available on the Nature Genetics website.

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ONLINE METHODS

Research subjects. The MTI-429 family and additional families were recruited worldwide based upon the presence of at least one individual with a neuro-radiographically proven molar tooth sign associated with any JBTS or related disorder (JSRD) phenotype. Whenever possible, individuals underwent a full diagnostic protocol as previously reported[38], and a standardized clinical questionnaire was administered to assess the extent of multi-organ involvement. We used standard methods to isolate genomic DNA from peripheral blood of the affected and unaffected family members after obtaining informed consent from all participants. Human subject research was approved by the Ethics Boards of Leeds (East), CASA Sollievo della Sofferenza Hospital/CSS-Mendel Institute and Hôpital Necker–Enfants Malades, the Human Subjects Division at the University of Washington, the University of Michigan Institutional Review Board and the Human Research Protection Program at the University of California, San Diego.

Genome-wide screen and fine mapping. A 5K whole genome linkage SNP scan was performed with the MTI-429 family using the Illumina Linkage IVb mapping panel[39], and analyzed with easyLinkage-Plus software[40], which runs Allegro version 1.2c in a Windows interface to calculate multipoint LOD scores. Parameters were set to autosomal recessive with full penetrance and disease allele frequency of 0.001. Fine mapping on the pedigree was performed with the Affymetrix 250K NspI SNP array, and data were searched for common shared homozygous intervals from all affected family members using a custom script implemented in Mathematica (B.M., unpublished). This script identifies all homozygous intervals longer than 2 Mb for which there are no more than 1% heterozygous calls (to permit potential genotyping errors).

Mutation screening. Mutation screening for *CEP41* was performed by direct sequencing of the 11 coding exons and the adjacent intronic junctions in human subjects. The PCR products that were generated were treated with Exonuclease I (Fermentas) and shrimp alkaline phosphatase (SAP) (Promega), and both strands were sequenced using a BigDye Terminator v3.1 sequencing kit with an ABI3100 automated sequencer (Applied Biosystems). Information about the primers and the optimized PCR conditions used are provided in **Supplementary Table 3**. Segregation of the identified mutations was investigated in all available family members. None of the identified mutations in *CEP41* were found in 96 ancestrally matched controls (188 chromosomes) upon direct sequencing.

Bioinformatics. Genetic location is according to the March 2006 Human Genome Browser build hg18. The ciliary proteome was searched using web-based tools[41,42]. Protein sequence conservation was determined using ClustalW multiple amino acid sequence alignment (see URLs).

Cloning. Full-length human *CEP41* was cloned into the TOPO Zero Blunt vector (Invitrogen), and shuttled into EGFP- and HA- encoding vectors. Human and zebrafish CEP41 ORFs were amplified by RT-PCR and cloned into the pCS2+ vector in order to synthesize RNA for injection into zebrafish embryos. Mouse *Cep41* (a gift from A.T. Look) was amplified and cloned into the GST-, EGFP- and FLAG-encoding vectors for biochemical assays.

Generation of *Cep41* mutant mice. The Sanger Institute Gene Trap Resource (SIGTR) ES cell line (AW0157), derived from 129P2/OlaHsd mice carrying a gene-trap insertion[43] in intron 1 of *Cep41*, was obtained from the European Conditional Mouse Mutagenesis Program (EUCOMM) and injected into C57BL/6 recipient blastocysts at the Mouse Biology Program, University of California, Davis. High-percentage chimeras (≥70%) were bred to C57BL/6 mice for germline transmission. Gene-trapped mice were isolated by PCR for the β-galactosidase–neomycin fusion gene and genotyped by Southern blot analysis, with hybridization of ~10-kb (wild-type allele) or ~8-kb (gene-trapped allele) products (**Supplementary Fig. 5**).

Zebrafish experiments. AB wild-type zebrafish strains (ZFIN) were used for *in situ* hybridization carried out by standard protocols using digoxigenin (DIG)-labeled sense and antisense RNA probes for *cep41*. To knock down zebrafish *cep41*, we used a translation-blocking MO (Gene Tools), 5′-CATCTTCCAGC AGCAGAGCTTCGGC-3′, which was diluted to appropriate concentrations in deionized sterile water and injected into embryos at the one-cell or two-cell stage, which were obtained from natural spawning of zebrafish lines. To rescue the phenotypes in MO-injected embryos, we co-injected RNA transcribed *in vitro* with the SP6 mMessage mMachine kit (Ambion). For characterization of ciliary defects in zebrafish embryos, the morphological phenotype of either *cep41* or *ttll6* morphants were observed until 5 days post-fertilization (d.p.f.) and quantified under bright-field microscopy based on previously established criteria[44]. For protein blot analysis at 1–2 d.p.f., zebrafish control embryos and *cep41* morphants (~50 embryos with each genotype) were deyolked, and the embryos were lysed with RIPA buffer. For immunostaining, mouse antibody to acetylated-tubulin (Sigma), mouse antibody to GT335 (a gift from C. Janke) and rabbit PolyE antibody (a gift from M. Gorovsky) were used. Whole-mount zebrafish embryos, control embryos at 3 d.p.f. and *cep41* morphants were fixed in Dent's fixative (80:20 methanol:DMSO) at 4 °C overnight and incubated with primary antibody to GT335 (1:400), acetylated tubulin (1:400) or PolyG (1:300) and with anti-goat-mouse 594 (1:1,000, Life Technologies) secondary antibody in blocking solution diluted 1:2 (10% normal goat serum and 0.5% Tween-20 in PBS).

Cell culture and transfection. IMCD3, hTERT-RPE1, human fibroblasts and human embryonic kidney cells (HEK293) were grown in appropriate DMEM or MEM supplemented with 10–20% fetal bovine serum (FBS) at 37 °C in 5% CO_2. Control fibroblasts from healthy human females and males were obtained from the American Type Culture Collection (ATCC), and subject fibroblasts were isolated from skin biopsies and were propagated in culture (≤5 passages). Human fibroblasts were transfected using the Basic Nucleofector Kit for Primary Mammalian Fibroblasts (Lonza). Other cells were transfected at 60–80% confluency with plasmids or siRNA (see **Supplementary Table 4**) using Lipofectamine 2000 (Invitrogen). The transfected cells were incubated for 24–72 h with FBS or without FBS, dependent on the experimental purpose.

Fluorescence microscopy and transmission electron microscopy. Images of immunofluorescently stained cells were obtained on a Deltavision RT Deconvolution microscope (Olympus IX70) under the same parameters for each experiment. Acquired images were edited and analyzed using Adobe Photoshop CS. For electron microscopy, a standard protocol[45] was used except for the modification that tannic acid was included in the fixative to enhance the final contrast of the images. Formvar-coated slot grids (Electron Microscopy Sciences) were used for sections (60–70 nm) to maximize visibility of the tissue and cross-sectioning performed to observe cilia axonemal structure.

Live imaging of zebrafish embryos. Zebrafish embryos (12 h.p.f. for cilia in Kupffer's vesicle and 2.5 d.p.f. for the renal cilia) were transferred with their medium to glass-bottomed culture dishes (MatTek), and embryos at 2.5 d.p.f. were anesthetized in tricaine solution (~0.016 mg/ml). Images were acquired for 30s to 2 min intervals using a PerkinElmer UltraView Vox Spinning Disk Confocal microscope with EMCCD Hamamatsu 14 bit 1K × 1K camera and edited with Volocity imaging software (PerkinElmer).

Immunofluorescence and biochemical assays. For immunofluorescence, cells were fixed in 100% methanol at −20 °C for 10 min. Primary antibodies used for immunofluorescence were: rabbit antibody to CEP41, raised in rabbit to a purified bacterially expressed GST-fused CEP41 protein (PRF&L), mouse antibody to acetylated-tubulin, mouse antibody to GT335, rabbit PolyE antibody and rabbit antibody to ARL13B (a gift from T. Caspary). We used Alexa 488– or Alexa 594–conjugated secondary antibodies (Molecular Probes) and Hoechst 33342 nuclear dye. All antibodies were diluted in 4% normal donkey serum in PBS. Primary antibodies were incubated with samples overnight at 4 °C and secondary antibodies were applied for 1 h at room temperature. For immunoblotting, cells were lysed with RIPA buffer 3 d after transfection and boiled with SDS sample buffer. Equal amounts of lysate were loaded for paired experiments. Primary antibodies used for protein blotting were: rabbit antibody to GFP (Roche), mouse antibody to Flag (Sigma) and mouse antibody to α-tubulin (Sigma). Bound antibodies were detected using horseradish peroxidase–conjugated secondary antibodies (Pierce). For co-immunoprecipitation, WCEs were prepared from confluent HEK293 cells transiently transfected with 14 μg of plasmid DNA in 10-cm² tissue culture dishes. WCE supernatants

were processed for immunoprecipitation experiments by using 2 µg of primary antibody and protein A/G mixed agarose beads (Pierce).

Statistical analysis. The χ^2 statistic was computed manually with the P value assigned for 1 degree of freedom in the characterization of the mouse embryonic phenotype (**Supplementary Table 2**). For other studies, Student's two-tailed non-paired t tests were carried out to determine the statistical significance of differences between samples. $P < 0.05$ was considered statistically significant for all tests.

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