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Citation for published version:
Stephen, LA, Tawamie, H, Davis, GM, Tebbe, L, Nürnberg, P, Nürnberg, G, Thiele, H, Thoennes, M, Boltschäuser, E, Uebe, S, Rompel, O, Reis, A, Ekici, AB, McTeir, L, Fraser, AM, Hall, E, Mill, P, Daudet, N, Cross, C, Wolfrum, U, Jamra, RA, Davey, MG & Bolz, HJ 2015, 'TALPID3 controls centrosome and cell polarity and the human ortholog KIAA0586 is mutated in Joubert syndrome (JBTS23)' eLIFE, vol 4, no. September. DOI: 10.7554/eLife.08077

Digital Object Identifier (DOI):
10.7554/eLife.08077

Link:
Link to publication record in Edinburgh Research Explorer

Document Version:
Peer reviewed version

Published In:
eLIFE

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DOI: http://dx.doi.org/10.7554/eLife.08077
Cite as: eLife 2015;10.7554/eLife.08077

Received: 13 April 2015
Accepted: 19 September 2015
Published: 19 September 2015

This PDF is the version of the article that was accepted for publication after peer review. Fully formatted HTML, PDF, and XML versions will be made available after technical processing, editing, and proofing.

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TALPID3 controls centrosome and cell polarity and the human ortholog \textit{KIAA0586} is mutated in Joubert syndrome (\textit{JBTS23})

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\textbf{Short title:} \textit{KIAA0586 (TALPID3)} is mutated in Joubert syndrome
Key words: Joubert syndrome, intellectual disability, TALPID3, KIAA0586, cilia, ciliopathy, centrosome, cell polarity, centriolar satellites
Joubert syndrome (JBTS) is a severe recessive neurodevelopmental ciliopathy which can affect several organ systems. Mutations in known JBTS genes account for approximately half of the cases. By homozygosity mapping and whole-exome sequencing, we identified a novel locus, JBTS23, with a homozygous splice site mutation in KIAA0586 (alias TALPID3), a known lethal ciliopathy locus in model organisms. Truncating KIAA0586 mutations were identified in two additional JBTS patients. One mutation, c.428delG (p.Arg143Lysfs*4), is unexpectedly common in the general population, and may be a major contributor to JBTS. We demonstrate KIAA0586 protein localization at the basal body in human and mouse photoreceptors, as is common for JBTS proteins, and also in pericentriolar locations. We show that loss of TALPID3 (KIAA0586) function in animal models causes abnormal tissue polarity, centrosome length and orientation, and centriolar satellites. We propose that JBTS and other ciliopathies may in part result from cell polarity defects.
INTRODUCTION

Joubert syndrome (JBTS) is a rare ciliopathy characterized by a specific midhindbrain malformation presenting as ‘molar tooth sign’ on axial MRI. Patients typically have a perturbed respiratory pattern in the neonatal period and pronounced psychomotor delay. Depending on the genetic subtype, there may be additional retinal degeneration, nephronophthisis, liver fibrosis and skeletal abnormalities (such as polydactyly). JBTS is genetically heterogeneous, with recessive mutations reported in more than 20 genes encoding proteins related to the function of cilia and associated structures (1, 2).

Cilia are axoneme-based organelles which protrude into the extracellular milieu, anchored to the cell by a modified centriole (basal body). They are present in virtually every cell type (3). Non-motile ‘primary’ cilia play essential roles in mechanotransduction, chemosensation and intracellular signal transduction, including Hedgehog (Hh), PDGFα and WNT pathways, in embryonic development and adult tissue homeostasis (4). In addition, highly modified and specialized cilia constitute the light-sensitive outer segments of retinal photoreceptor cells. Dysfunction of cilia, centrioles of basal bodies and centrosomes can lead to a spectrum of developmental single- or multi-organ disorders termed "ciliopathies" (5).

*KIAA0586 (TALPID3; MIM #610178, MIM #000979-9031)* is essential for vertebrate development and ciliogenesis. The KIAA0586 (TALPID3) protein is localized at the centrosome in human, chicken, mouse and zebrafish cells (6-8), and in particular at the distal end of the mother centriole – the basal body of cilia (9). In model organisms, *KIAA0586* null mutations cause failure of basal body docking and loss of cilia, leading to early embryonic lethal phenotypes (6, 10-12). KIAA0586 (TALPID3) binding partners include PCM1, Cep120 and CP110, which interact with a known JBTS protein, CEP290 (13).

Here, we report three JBTS families with loss-of-function mutations in *KIAA0586*. Using animal models, we demonstrate that *TALPID3 (KIAA0586)* is not only essential for
transduction of Hedgehog signaling but plays an important role in centrosomal localization, orientation and length. Finally, and beyond its established requirement for ciliogenesis, TALPID3 (KIAA0586) plays a key role in cell and tissue polarity.
METHODS

Patients
Blood samples for DNA extraction were obtained with written informed consent. All investigations were conducted according to the Declaration of Helsinki, and the study was approved by the institutional review board of the Ethics Committees of the University of Erlangen-Nürnberg, the University of Bonn, and the University Hospital of Cologne.

Genetic analysis of human JBTS families
In accordance with the Human Gene Nomenclature Committee (HGNC), we have used KIAA0586/KIAA0586 for designation of the human gene and protein, respectively. In accordance with the Chicken Gene Nomenclature Committee (CGNC), we use TALPID3/TALPID3 for designation of the chicken gene and protein, respectively. Although the current gene symbol for the mouse gene is 2700049A03Rik (protein: 2700049A03RIK), we use Talpid3/Talpid3 as the gene and protein names, respectively. Where we refer to a generic conclusion on the function of the orthologs of KIAA0586, we use KIAA0586. As in previous publications, the chicken model is referred to as talpid3, and the mouse model is referred to as Talpid3−/−. The nomenclature of human KIAA0586 mutations refers to reference sequence NM_001244189.1 (corresponding protein: NP_001231118.1). The Exome Aggregation Consortium (ExAC) database (Cambridge, MA, USA; http://exac.broadinstitute.org), which aggregates numerous databases including the current versions of the Exome sequencing project (ESP, (14)) and the Thousand Genomes Project (TGP, (15)) was last accessed on July 11, 2015 for presence and frequency of identified variants in healthy individuals.
Family 1: Genotyping and homozygosity mapping were performed in Family 1 (MR026) as previously reported (16). DNA from patient MR026-01 underwent exome capture and whole-exome sequencing (WES) using the SureSelect Human All Exon 50 Mb Kit (Agilent technologies, Santa Clara, USA) and a SOLiD4 instrument (Life Technologies, Carlsbad, USA) as described previously (17). Of the targeted regions, 73.2% were covered at least 20x, and 83.4% at least 5x. To validate the results, we also conducted WES in the likewise affected sibling, MR026-04, analogous to previously described disease gene identification approaches (18, 19). 96% of the target sequence were covered at least 20x.

Family 2: Samples from the index patient, MD1, and her parents underwent WES at GeneDX (Gaithersburg, MD).

Family 3: WES and mapping of reads for the index patient (G2) and both parents were carried out as previously described (20, 21). In brief, filtering and variant prioritization was performed using the varbank database and analysis tool (https://varbank.ccg.uni-koeln.de) of the Cologne Center for Genomics. In particular, we filtered for high-quality (coverage >15-fold; phred-scaled quality >25), rare (MAF (minor allele frequency) ≤0.001) variants (dbSNP build 135, the 1000 Genomes database build 20110521, and the public Exome Variant Server, NHLBI Exome Sequencing Project, Seattle, build ESP6500). To exclude pipeline-related artifacts (MAF≤0.01), we filtered against variants from in-house WES datasets from 511 epilepsy patients. The Affymetrix genome-wide Human SNP Array 6.0 utilizing more than 906,600 SNPs and more than 946,000 copy number probes was used for genome-wide detection of copy number variations in patient G2. Quantitative data analyses were performed with GTC 3.0.1 (Affymetrix Genotyping Console) using HapMap270 (Affymetrix) as reference file. In the index patient (G2), all coding KIAA0586 and KIF7 exons were Sanger-sequenced in search of a second mutation. In addition, we amplified and sequenced all KIAA0586 exons from cDNA (derived from whole blood mRNA, PAXgene Blood RNA
Tube, PreAnalytiX, Hombrechtikon, Switzerland) in search of potential hints of aberrant splicing due to extra-exonic variants. Continuous PCR-amplification of KIF7 exons from whole-blood mRNA was not successful. The sample of patient G2 was analyzed by genome-wide CGH (Affymetrix 6.0 SNP array) to exclude structural alterations adjacent to or within KIAA0586, KIF7, CEP41, KIF14 or WDPCP. Confirmation of the identified mutations and segregation analyses were carried out by Sanger sequencing.

**RT-PCR**

In Family 1, we isolated mRNA using the RNeasy kit (QIAGEN, Hilden, Germany) from lymphoblastoid cell lines that have been established based on standard protocols from patients MR026-01 and MR026-04. We transcribed mRNA to cDNA using SuperScriptII reverse transcriptase and random primers (Invitrogen). To test if the KIAA0586 mutation c.2414-1G>C impairs splicing, we used two pairs of primers (KIAA0586_exprF1, 5´-TCCATCTCCTAAGTCCAGACCAC-3´ and KIAA0586_expR1, 5´-TCCAGTGGTCACGGAGG-3´, located in exons 16 and 19, and KIAA0586_exprF2, 5´-TCAGGTACATTGGAAGGTCATC-3´ and KIAA0586_expR2, 5´-AACTGGCAGGATGGGTCATC-3´, located in exons 17 and 21; NM_001244189.1), and standard PCR methods. Electrophoresis on standard agarose gel followed by cutting out the DNA bands, purifying the DNA using QIAquick gel extraction kit (QIAGEN), and Sanger sequencing were performed.

**Animal models**

Eggs were obtained from talpid3 flock (MG Davey; talpid3 chicken lines are maintained at the Roslin Institute under UK Home Office license 60/4506 [Dr. Paul Hocking], after ethical review). Mice were maintained at the Human Genetics Unit, Western General, Edinburgh,
under UK Home Office license PPL 60/4424 [Ian Jackson]. The Talpid3+/Kif7+/- line was produced by crossing of the previously described Talpid3+/ knockout mouse line (10) and the reported Kif7+/- mouse line (22). Animal experiments carried out at the JGU Mainz corresponded to the statement of the Association for Research in Vision and Ophthalmology (ARVO) as to care and use of animals in research. Adult mice were maintained under a 12 hour light–dark cycle, with food and water ad libitum.

**Incubation and dissection of animal models**

Chicken eggs from talpid3 flock were incubated at 38 °C until 12 days at the latest, staged as per Hamburger and Hamilton 1951 (23), dissected into cold PBS and fixed in 4% PFA/PBS. Mouse timed matings were established between Talpid3+/ mice (10) and Kif7+/- mice (22) and confirmed by vaginal plug. Pregnant females were sacrificed at day 10 of pregnancy, embryos dissected and used to make mouse embryonic fibroblasts or between day 12-16 of pregnancy and embryos were dissected in cold PBS, decapitated and fixed immediately in 4% PFA, or pups were sacrificed between 7-21 days after birth by lethal injection. Brains were dissected into 4% PFA/PBS.

**Chicken and mouse genotyping**

Embryos used in comparisons were dissected as family groups and genotyped after analysis. Tissues were collected on dissection, lysed in 10 mM Tris (pH8), 10 mM EDTA (pH 8), 1% SDS, 100 mM NaCl and 20 mg/ml proteinase K at 55 °C overnight before DNA extraction using Manual Phase Lock Gel Tubes (5 Prime) for phenol/chloroform extraction. For chicken TALPID3, sequencing primers used were 5’-TCATCCATAGCTCTGCCG-3’ (forward) and 5’-CCATCAAACCAACAGCTCAG-3’ (reverse). For mouse Talpid3, PCR primers were 5’-TGCCATGCAGGGATCATAGC (forward), 5’-GAGCACACTGGAGGAAAGC-3’
(reverse) and 5’-GAGACTCTGGCTACTCATCC-3’, 5’-
CCTTCAGCAAGAGCTGGGGAC-3’, respectively. For mouse Kif7, PCR primers were- 5’-
CACCACCATGCCTGATAAAAC-3’ (P1 forward), 5’-
CTATCCCCAATTCAAAGTAGAC-3’ (P1 reverse), 5’-
CCAAATGTGTCAGTTTCATAGC-3’ (P2 forward), 5’-TTCTCACCCAAGCTCTTTATCC-
3’ (P2 reverse).

Histology

Fixed samples from mouse brain and chicken legs were embedded in paraffin, sectioned and
stained in haematoxylin and eosin as described previously (24).

Wholemount RNA in situ hybridisation

Mouse and chicken embryos were rehydrated through a methanol gradient and in situ
hybridization carried out for chicken β-catenin (codons 1–127) as previously described (25).

In ovo knockdown of Kif7 in chicken

The following Kif7 sequences were targeted for knockdown: Target 1:
TTATCGACGAGAAGCCTCAAt, Target 2: cATCCAGAACAAAGCCTGGTG, Target
3: gTCCTCTAAACACTAAAGACCTT, Target 4: gACAGATGACATAGTCCGTGTG to
which 22mer sequences were designed in Genscript and cloned into pRFPRNAiC (26)
(Dundee Cell Products, UK). Embryos were electroporated at stage 12HH (as described (7)),
observed for RFP expression at stage 24HH, fixed and prepared for sectioning and
immunohistochemistry at stage 22HH as below. Tissue from embryos was collected and
genotyped.
Cell culture and immunocytochemistry

Mouse embryonic fibroblasts (MEFs) were prepared from E10.5 eviscerated and decapitated embryos. Cells were dissociated in trypsin/versin and maintained to passage 2 as per (27) and serum removed from media for 48 hours to induce ciliogenesis. RPE1 cells (ATCC) were grown in DMEM-F12, 10% FCS Gold, 50 μl hygromycin, 5 ml L-glut. IMCD3 (mouse inner medullary collecting duct cells) cells were grown in DMEM-F12 10% FCS. To induce ciliogenesis, RPE1 and IMCD3 cells were starved in DMEM:F12 or Opti-MEM I (Life Technologies) for 72 h. Cells were fixed with methanol at -20°C for 2-5 minutes. After washing in PBS, cells were immunolabeled with polyclonal antibodies against acetylated tubulin (Sigma-Aldrich T7451), pericentrin-2 (Santa Cruz sc-28145) and KIAA0586 (Atlas HPA000846) before incubation with appropriate secondary antibodies conjugated to Alexa 488 (Molecular Probes A21206), CF 568 (Biotrend 20106-1), and CF 640 (Biotrend 20177) and with DAPI (Roth 6335.1).

Immunohistochemistry

Eyes from a healthy human donor (#199-10; 56 years of age, dissection 29 hours post mortem) were obtained from the Department of Ophthalmology, University Hospital of Mainz, Germany, according to the guidelines of the declaration of Helsinki. After sacrifice, eyeballs from adult C57BL/6J mice were dissected, cryofixed in melting isopentane, cryosectioned and immunostained as previously described (28). Cryosections were incubated with monoclonal antibodies to centrin-3 as a molecular marker for the ciliary apparatus of photoreceptor cells as previously characterized (29), and polyclonal antibodies against KIAA0586 (Atlas HPA000846). Washed cryosections were incubated with appropriate antibodies conjugated to Alexa 488 (Molecular Probes A21206) and Alexa 568 (Molecular Probes A11031)) in PBS with DAPI (Roth 6335.1) to stain the nuclear DNA and mounted in
Mowiol 4.88 (Hoechst, Germany). Specimens were analyzed in a Leica DM6000B deconvolution microscope (Leica, Germany). Image contrast was adjusted with Adobe Photoshop CS using different tools including color correction. For section immunocytochemistry on chicken tissue, chicken embryos were dissected into PBS, fixed, sectioned and stained as described (11), except for CEP164, in which an antigen retrieval step was undertaken (incubation in 0.1% BME/PBS for 5 min., incubation in 55°C PBS for 4 hours). For bone sections, legs were dissected at E12. For immunocytochemistry, cells were fixed as above. Antibodies were used against: acetylated α-tubulin (Sigma-Aldrich T7451), γ-tubulin (Sigma-Aldrich T5192; T5326), TGN46 (Abcam ab16059), PCM1 (Abcam ab72443), AZI1 (kind gift of Jeremy Reiter, UCSF), centrin-3 (29), KIAA0586 (Atlas HPA000846, ProteinTech 24421-1-AP), CEP164 (ProteinTech 22227-1) RFP (Life Technologies R10367), GFP (Life Technologies A-21311), Pax7 (Developmental Studies Hybridoma Bank (DSHB)), ISLET1 (DSHB), NKX2.2 (DSHB), Phalloidin (Life Technologies A12379), Anti-mouse (Life Technologies A11017), anti-rabbit (Life Technologies A21207). Imagining was undertaken on a Zeiss LSM 710 or a Nikon Air confocal microscope or Leica DMLB.

Conventional transmission electron microscopy (TEM)

Chicken embryos were dissected into PBS at 8 days of incubation, avoiding contamination with yolk, heads were removed and placed into 4% PFA, 2.5% glutaldehyde in 0.1 M cacodylate buffer. The choroid plexus was immediately removed and placed into fresh fixative (as previous) for 24 hours. Tissue was prepared and visualized for TEM as described previously (30), and axoneme/basal body structure was compared to what was observed and reported previously (31).

Immunoelectron microscopy analysis
Anti-KIAA0586 antibody (Atlas HPA000846) was used for pre-embedding labeling in mouse retinas as previously described (32, 33). Ultrathin sections were cut on a Leica Ultracut S microtome and analyzed with a Tecnai 12 BioTwin transmission electron microscope (FEI, The Netherlands). Images were obtained with a charge-coupled device SIS Megaview3 SCCD camera (Surface Imaging Systems, Herzogenrath, Germany) and processed with Adobe Photoshop CS.

**Cell polarization and cilia length measurements**

Angles of proliferation, migration, orientation and localization were calculated using Axiovision Angle3 software, and cilium length was measured using Zen software (Zeiss, Oberkochen, Germany). Scratch assays were carried out in wildtype and Talpid3−/− MEFs grown to confluence and serum starved (DMEM + 0.5% FCS) for 48 hours with a p10 pipette tip. Medium was then renewed and MEFs incubated for four hours before fixation in ice-cold methanol prior to immunofluorescence. Angles of orientation were then taken as a measurement of the angle from the centre of the nucleus, through the centre of the leading edge (towards the wound, identified by phalloidin staining for F-actin) and again through the centre of the Golgi apparatus (identified by TGN46 antibody staining). Tiled Z stacks of the scratch/wound were analyzed for greater accuracy (Figure 3O).

The expected orientation of the stereocilia of the basilar papilla hair cells were taken as being at 90° to the abneural edge of the basilar papilla. The angle of orientation was taken by drawing a line through the cell perpendicular to the abneural edge and a second from the centre of the cell, intersecting with both the perpendicular line and centre of the actin bundle. The internal angle was taken to be the angle by which cell orientation deviated from the expected. Cilium length was measured using Zen software (Zeiss, Oberkochen, Germany).
RESULTS

Clinical description of patients with *KIAA0586*-associated JBTS

The diagnosis of JBTS was based on the presence of a molar tooth sign in all three families.

Family 1 (Figure 1A) is a consanguineous Kurdish family from northeast Syria. The two affected siblings were examined at the age of 6 years and 10 months (MR026-01) and 2 years and 2 months (MR026-04), respectively. Pregnancy, delivery, and birth parameters of both children were unremarkable. In the neonatal period, both were hypotonic and weepy. Motor and speech development in MR026-01 were delayed, and his IQ was estimated to be between 50 and 70. Further symptoms were severe myopia, scoliosis, brachydactyly, distinct facial characteristics, and recurrent febrile seizures. Height was reduced (108 cm, -2.6 SD), weight was normal (22 kg, -0.27 SD), and head circumference was increased (57 cm, +2.3 SD).

MR026-04 had not reached any milestones, and at the age of 7 years, she was wheelchair-bound. Cognitive abilities were weaker than in her brother, with an IQ estimated to be below 35. MR026-04 had similar physical characteristics as her brother, severe muscular hypotonia, prolonged and therapy-resistant seizures since the age of 14 months, and hypothyroidism. At the time of examination, her height was 91 cm (1 SD), weight was 11.5 kg (-0.7 SD), and there was macrocephaly (head circumference of 59 cm, +8 SD).

Family 2 (Figure 1B) is of North American origin. Patient MD1 was born at 34 3/7 weeks gestation following preterm premature rupture of membranes at 26 weeks. At birth, patient MD1 was found to have cardiac defects including a patent ductus arteriosus (PDA), patent foramen ovale (PFO) and a 3/6 ventricular septal defect (VSD) causing persistent pulmonary hypertension 24 hours after birth. The PDA and PFO resolved, and VSD was at 2/6 within 22 days. At 7 months, MD1 was found to have a superior vena cava duplication. At 2 years of age, MD1 has hypotonia which inhibits motor actions, although she can crawl proficiently,
uses sign language and single words, and can self-feed by hand and with utensils. In addition, she had type I bilateral Duane syndrome with no abduction in either eye, narrowing of the palpebral fissure of the inturned eye, was farsighted, had thin tooth enamel, held her jaw sideways in a cross-bite pattern, and had long fingers with a slight clinodactyly of the 5th finger. She had a broad forehead, arched eyebrows, ptosis of the right eye, and a triangle shaped mouth. Her receptive language was good. There was intermittent hyperpnea/apnea during awake periods. Patient MD1 had no liver, kidney, or eye abnormalities at 2 years of age.

Family 3 (Figure 1C) is of German origin: Patient G2 displayed a relatively mild JBTS phenotype with developmental delay and behavioral abnormalities, but no dysmorphic signs of renal, retinal, skeletal or liver systems. His symptoms were described previously (Figure 1C, (34)).

Mutations of KIAA0586 cause JBTS

We have identified KIAA0586 mutations in three JBTS families (Figure 1A-D). Genome-wide SNP genotyping in Family 1 identified eight homozygous chromosomal candidate regions with a total range of 67.1 Mb. By WES, the homozygous mutation c.2414-1G>C in intron 17, affecting the invariant consensus of the exon 18 acceptor splice site, was found in the index patient, MR026-01, and his affected sister, MR026-04. Segregation analysis in the family was compatible with causality (Figure 1A). The mutation was absent from 372 healthy Syrian controls, including 92 of Kurdish origin, and not listed in the ExAC database.

In patient MD1 from Family 2, WES identified compound heterozygosity for the KIAA0586 mutations c.428delG (p.Arg143Lysfs*4; rs534542684; MAF of 0.39% in ExAC db) and c.2512C>T (p.Arg838*), each inherited from a healthy parent (Figure 1B), and both resulting in premature stop codons. Because the coiled coil domain, which is essential for KIAA0586
function in mouse, chicken and zebrafish (residues 531-571 and residues 497-530 in human and chicken KIAA0586, respectively; Figure 1E), would be lost in a truncated protein derived from the c.428delG mutation, we consider it a loss-of-function mutation (as is the case for the talpid3 chicken mutation which introduces a frameshift 3′ to c.428 in the chicken ortholog, Figure 1E). Like the Talpid3/TALPID3 null mutations in mouse and chicken, c.428delG is clearly recessive because the father of the patient is a healthy carrier. The c.2512C>T (p.Arg838*) mutation is predicted to lead to nonsense-mediated decay (NMD) or a truncated protein, but with preservation of the essential coiled coil domain.

The simplex patient of Family 3, G2, was a known carrier of a heterozygous N-terminal frameshift mutation in exon 3 of the JBTS12 gene KIF7, c.811delG (p.Glu271Argfs*51) (34). WES of the family trio (patient G2 and his parents) additionally identified the c.428delG (p.Arg143Lysfs*4) mutation in KIAA0586, in the patient (Figure 1C). We hypothesized that disease in patient G2 could be due to biallelic mutations either in KIF7 (JBTS12) or in KIAA0586 (JBTS23), assuming that the "missing mutation" has escaped detection by sequencing due to an extra-exonic localization. Genome-wide CGH (Affymetrix 6.0 SNP array) did not reveal structural alterations adjacent to or within KIF7 (34) or KIAA0586, thereby largely excluding a large deletion or duplication. PCR amplification and subsequent sequencing of KIAA0586 exons from cDNA did not reveal aberrant splicing as a potential hint for a deep intronic splice site mutation. Because KIF7 and KIAA0586 both encode modulators of GLI processing and c.428delG KIAA0586 and c.811delG KIF7 likely represent recessive loss-of-function mutations, we investigated the possibility of a potential epistatic effect predisposing to JBTS. No such interactions were identified in mouse and chicken experiments (details are fully described in Figure 1- figure supplement 1). Therefore, unidentified mutations are likely to be involved, either mutations in KIF7, KIAA0586 (e.g. deep intronic mutations or alterations in non-coding regulatory regions which would both not
be covered by WES), or biallelic mutations in another (yet unknown) JBTS gene. WES revealed further heterozygous missense variants in three known recessive ciliopathy genes in patient G2 (Figure 1C), all affecting evolutionarily conserved residues of the respective proteins: 1. c.536G>A (p.Arg179His, rs140259402; MAF of 0.001647% in ExAC db) in CEP41, the gene associated with JBTS15 (35). 2. c.3181A>G (p.Ile1061Val; MAF of 0.01155% in ExAC db) in KIF14, a gene associated with a lethal fetal ciliopathy phenotype (36). 3. c.1333G>C (p.Ala445Pro, rs61734466; MAF of 0.6609% in ExAC db) in WDPCP, the gene associated with Bardet-Biedl syndrome type 15 (BBS15), and a putative contributor to Meckel Gruber syndrome (37). All variants were of paternal origin and rare in the general population except the WDPCP allele, which had been maternally inherited and which has been annotated homozygously in five healthy individuals (ExAC db), indicating that this is a benign variant. Genome-wide CGH (Affymetrix 6.0 SNP array) did not show structural alterations adjacent to or within CEP41, KIF14 or WDPCP. In addition, we searched the WES data of patient G2 for heterozygous putative loss-of-function (that is, truncating) variants in genes with documented ciliary function. This revealed a paternally inherited frameshift variant, c.206_207insA (p.Ser70Valfs*3), in PLA2G3, the gene encoding phospholipase A2. In a functional genomic screen, PLA2G3 was found to be a negative regulator of ciliogenesis and ciliary membrane protein targeting (38). The p.Ser70Valfs*3PLA2G3 variant is relatively common, but has not been documented in homozygous state in healthy individuals (MAF of 0.4060 in ExAC db).

We also filtered for known JBTS genes carrying at least two rare variants in patient G2, but we did not identify such a constellation. When applying this to all genes captured in the WES approach, there was also no potentially causative double heterozygosity in a gene of known or probable ciliary function. Filtering for homozygous rare and likely pathogenic variants was negative, compatible with lack of consanguinity in the parents of patient G2.
Consequences of the *KIAA0586* mutation c.2414-1G>C on mRNA level

The c.2414-1G>C mutation affects the invariant consensus of the acceptor splice site of exon 18. RT-PCR and Sanger sequencing of the fragments amplified from cDNA revealed three aberrant splicing products due to usage of alternative exonic acceptor splice sites at AG motifs within exon 18 and due to skipping of exon 18 (Figure 1D,E): a 13-bp deletion that results in a premature termination codon (alternative acceptor splice site at c.2425/2426AG), a 108-bp in-frame deletion (alternative acceptor splice site at c.2520/2521AG), and a 188-bp deletion due to skipping of exon 18 that results in a premature stop codon. These aberrant transcripts were present in the cDNA from both patients, but not in the cDNA of a healthy control individual (Figure 1D). The mutant mRNA molecules are likely to be degraded by NMD. If the mutant transcripts were stable, the essential coiled coil domain (Figure 1E), which mediates centrosomal localization and function of KIAA0586 protein (7, 8), would be preserved.

KIAA0586 localizes to the basal body of cultured cells and photoreceptor cells of human and mouse retina

KIAA0586 is a centrosomal protein and localizes to the basal body and the adjacent centriole of primary cilia in human RPE1, IMCD3 cells (Figure 2A) and other cell types (8, 9). Immunofluorescence analysis of the retina of *wildtype* C57BL/6 mice allowed us to allocate KIAA0586 expression to different retinal layers, namely the photoreceptor layer, the outer and inner plexiform layer, and the ganglion cell layer (Figure 2B). Co-staining with the ciliary marker centrin-3 (29) demonstrated KIAA0586 localization in the ciliary region at the joint between the inner and outer segment of photoreceptor cells in cryosections through the mouse retina and the retina of a human donor eye (Figure 2B,C,E). Higher magnification
revealed that KIAA0586 specifically localized at the basal body (mother centriole) and the adjacent centriole as well as between the two centrioles, but not in the connecting cilium of mouse and human photoreceptor cells (Figure 2D,F). These findings were confirmed by immunoelectron microscopy of KIAA0586 labeling on sections through mouse photoreceptor cilia (Figure 2G,H). Immunostaining was found at centrioles and in the pericentriolar region in the apical inner segment of photoreceptor cells. The spatial distribution of KIAA0586 labeling at the ciliary base of photoreceptor cells is summarized in the scheme of Figure 2J.

**Loss of TALPID3 (KIAA0586) causes abnormal tissue and cell polarity**

The talpid3/Talpid3-/- phenotype in model animals has thus far been attributed to the role of TALPID3 in ciliogenesis and the subsequent loss of Hh-dependent patterning. However, the patients in this study did not display any overt defects typical for impaired Hh signalling such as polydactyly or hypotelorism, which have been described in other JBTS patients (2).

Talpid3 chicken embryos also have polycystic kidneys (7), a phenotype that is frequently ascribed to a loss of oriented cell division (39, 40), as well as cell migration defects (10), which may also occur due to loss of cell polarity (39, 40). To investigate if tissue and cell polarity is impaired by a loss of TALPID3, we first examined the patterning of the skin and the inner ear, two highly polarized tissues independent of Hh signaling. At E10, embryonic chicken feather buds express $\beta$-catenin in an oriented manner, with a larger domain in the anterior part of the bud (Figure 3A,C). While 88% of wildtype feather buds at E10 are oriented in this manner (n=117/133), only 21% of stage-matched talpid3 feather buds were (n=38/179), whilst 22% of talpid3 buds were oriented in the wrong direction (n=39/179) and 57% had failed to show any orientation of $\beta$-catenin expression (n=102/179; Figure 3C’). Talpid3 feather buds also frequently merged (29% of buds; asterisk, Figure 3B’). Thus, the skin of talpid3 embryos did not show the characteristic rostral-caudal polarization of wildtype
skin. The hair cells (HCs) of the inner ear (known as the basilar papilla (BP) in chicken), have a highly polarized structure determined by the non-canonical Wnt-PCP signaling pathway. In the wildtype chicken, as in mouse, individual HCs exhibit an orientated actin-based stereocilia bundle, the apex of which lies at the abneural side of the cell, where within an actin-free ‘bare zone’, a single kinocilium (a microtubule-based true cilium) forms (arrow, Figure 3D). HCs are frequently used to assess how cell polarity and ciliogenesis are perturbed in mouse mutants (4). The HCs of talpid3 embryos formed actin filament bundles (curved line, Figure 3E), but no kinocilium, demonstrating that, as with other tissues studied, loss of TALPID3 impairs ciliogenesis. Furthermore, although stereocilia were present in talpid3 HCs, stereocilia bundles frequently lacked polarity compared to wildtype HCs as indicated by either centrally located stereocilia bundles in SEM or actin filaments throughout the cell (talpid3 n=1086/1195; wt n= 258/502; Figure 3D,E,F,G,L). Orientation of the polarized stereocilia bundles that did form in talpid3 HCs, was also abnormal (Figure 3E,G,N). The orientation of stereocilia was determined in relation to their position to the abneural side of the BP (Figure 3F,G,M). 73% of stereocilia of wildtype cells (n=244) were oriented within 40° of the expected angle (90°, compared to 38% of talpid3 cells (n=237; Figure 3 M'). Thus, talpid3 HCs showed disrupted polarity.

Loss of TALPID3 causes abnormal intracellular organization

Loss of TALPID3 prevents basal body docking (7), which we have previously suggested to be due to failure of centrosome migration (12). The migration and subsequent localization and docking of the centriole is crucial to establish polarity and placement of the actin bundle in the HC (41), and we therefore hypothesized that disturbed cell polarity may result from defective centrosome migration in talpid3 HCs. Using antibodies against γ tubulin to determine the localization of the centriole within the actin-negative abneural bare zone in the
HCs, 95% of wildtype HCs exhibited a basal body (centrosome) within the abneural bare zone (n=632 from 7 samples, Figure 3H,J,N). In contrast, only 49% of talpid\(^3\) cells exhibited a centriole within the bare zone (either abneural or abnormally polarized; n=219 from 6 samples. Figure 3I,K,N), thus demonstrating that the intracellular organization of talpid\(^3\) cells was frequently abnormal. Furthermore, and in agreement with the failure of correct polarization of the stereocilia, centrioles were frequently observed on the neural side of talpid\(^3\) HCs (Figure 3Kii). We conclude that failure of centriolar migration in talpid\(^3\) cells results in abnormal cell polarization and stereocilia formation in HCs. Because 49% of talpid\(^3\) cells did exhibit a centriole correctly localized yet ciliogenesis was completely disrupted, the failure of ciliogenesis may not only be due to impaired centriolar migration.

Directional cell migration is also intimately linked to the localization of the centrosome between the leading edge of the migrating cell and the Golgi apparatus. Talpid3\(^{-/-}\) MEFs show abnormal cell migration (10), and we therefore examined if the orientation of the Golgi apparatus to the leading edge of migrating cells was also disrupted by a loss of Talpid3 in mouse, in an in vitro scratch assay (Figure 3O,P). The angle between the leading edge and Golgi was taken as the angle of orientation, with an angle of 0\(^\circ\) suggesting perfect alignment of the Golgi to the leading edge of the migrating cell (Figure 3Q). The angle of orientation was within 40\(^\circ\) in 69% of wildtype cells and 55% of talpid3\(^{-/-}\) cells, whilst 20% of talpid3\(^{-/-}\) cells exhibited orientation angles greater than 60\(^\circ\) compared to 11% of wildtype cells (Figure 3Q'; wildtype cells=132, talpid3\(^{-/-}\) cells=117 from two experiments; Figure 3O-Q), suggesting a reduction in intracellular polarization of the Golgi apparatus to the leading edge in the Talpid3\(^{-/-}\) MEFs (Figure 3O,P). Thus, KIAA0586 (TALPID3) plays an essential role in the internal organization and polarization of cells, likely through its action on the centrosome.
Abnormalities of intracellular organization, centriole maturation and centriolar satellite dispersal in the neuroepithelium

JBTS primarily affects the brain of the patients. The choroid plexus is a highly polarized multiciliated neuroepithelium in which we have previously shown, as now in HCs, a failure of centrosome migration in \textit{talpid}^3\_mutant chickens (12). To determine if \textit{talpid}^3\_mutant neuroepithelia exhibits cell polarity defects, we examined the intracellular organization of choroid plexus cells in E8 \textit{talpid}^3\_mutant chickens. \textit{Wildtype} choroid plexus cells exhibited a distinctive polarization with an apical, centriolar zone (CZ, Figure 4A) above a separate zone of mitochondria (MZ, Figure 4A); the most apical mitochondria were found an average of 7 \(\mu\)m from the apical surface (Figure 4C). In contrast, the mitochondria in \textit{talpid}^3 choroid plexus are found in the most apical zone, an average of 3 \(\mu\)m from the apical surface (m, Figure 4B), and centrioles are present throughout the cell (asterisk in Figure 4B). We conclude that the neuroepithelium has an abnormal intracellular organization of centrosomes and mitochondria and therefore, like the HCs and migratory fibroblasts, is not correctly polarized. Although we have previously suggested that a failure of centrosome migration to the apical surface is the primary reason that cilia fail to form (12) our analysis of the HCs suggest an additional requirement for TALPID3 during ciliogenesis, independent of the centriole migration. We therefore investigated the maturation of the mother centriole, crucial for the basal body to dock to the membrane and initiate ciliogenesis. Subdistal appendages were identified in approximately 40\% of \textit{wildtype} and \textit{talpid}^3 centrioles (\textit{wt} \(n=35\), \textit{talpid}^3 \(n=48\), Figure 4D,E,G), whereas distal appendages were noted in 28\% of \textit{wildtype} centrioles and only 6\% of \textit{talpid}^3 centrioles (Figure 4D-G). To determine if there was a loss of distal appendages, we examined localization of CEP164, a protein known to localize to the distal appendages of the mature mother centriole, the basal body. CEP164 localized correctly at the mother centriole and not at the sister centriole, in both \textit{wildtype} and \textit{talpid}^3 cells of the
neuroepithelium and fibroblasts (Figure 4H-M). However, CEP164 puncta were smaller, disorganized and frequently orientated away from the apical cell surface in *talpid*<sup>3</sup> cells (Figure 4K,L). This confirmed our previous EM analysis (7) and data in this study, which demonstrated that centrioles frequently failed to migrate or orientate correctly in *talpid*<sup>3</sup> cells. Smaller sized CEP164 puncta also suggested that distal appendages were not formed normally in *talpid*<sup>3</sup> cells. As abnormal or absent distal appendages can result in elongation of the centriole due to improper capping, centriolar length was studied in *wildtype* and *talpid*<sup>3</sup> choroid plexus cells (Figure 4N-Q). Centrioles in *wildtype* tissue were on average 0.7 µm in length compared to 0.9 µm in the *talpid*<sup>3</sup> chicken, suggesting that *talpid*<sup>3</sup> centrioles may indeed fail to undergo complete maturation and are subsequently elongated (Figure 4R).

In human cells, KIAA0586 is also required for centriolar satellite dispersal (9). Compatible with this, we observed electron-dense condensations around the centrioles in the neuroepithelium of *talpid*<sup>3</sup> chicken, which were absent from *wildtype* centrioles (basal body; 80% of wildtype cell exhibited electron-dense clear area around the centriole, whereas only 21% of *talpid*<sup>3</sup> cells did; *wt* n=35, *talpid*<sup>3</sup> n=48; Figure 5A,D,G). To determine if these were centriolar satellites, we examined the localization of PCM1, a marker for centriolar satellites. Compared to *wildtype* centrioles (Figure 5B,C), PCM1 puncta were larger around *talpid*<sup>3</sup> centrioles (Figure 5E,F), possibly reflecting an increase in centriolar satellites. Because we observed KIAA0586 immunostaining around the pericentriolar region (Figure 2G,H), we used the centriolar satellite marker AZI1 in human RPE1 cells to determine if KIAA0586 localized to centriolar satellites (Figure 5H-J), but found that KIAA0586 and AZI1 did not colocalize. Thus, as observed in human cell lines, TALPID3 is essential for centriolar satellite dispersal. As TALPID3 protein does not localize to the centriolar satellites, we assume that this is an indirect consequence of *TALPID3* deficiency.
We conclude that KIAA0586 (TALPID3) is essential for several distinct roles in centriole function, including centriole migration and orientation which can subsequently affect cell and tissue polarity and ciliogenesis, centriole maturation which affects docking of the basal body and ciliogenesis and through an indirect mechanism, centriolar satellite dispersal, which may also affect ciliogenesis.
DISCUSSION

JBTS is a genetically heterogeneous condition, caused by mutations in several genes related to the structure and function of cilia (1). Through homozygosity mapping and WES, we identified a novel disease locus (JBTS23), defined by mutations in the KIAA0586 gene, encoding a centrosomal protein (42) (Figure 1), which is supported by simultaneous concurrent studies (43, 44). We used Talpid3/TALPID3−/− mouse and chicken models to understand the corresponding pathomechanisms causing the phenotypes of these patients, and discovered centrosome abnormalities and loss of cell polarity.

We confirm localization of KIAA0586 at centrosomal structures at the basal bodies and the adjacent daughter centrioles of primary cilia of mouse and human photoreceptor cells as well as in pericentriolar regions (Figure 2). KIAA0586 has previously been associated with recessive ciliopathy phenotypes in mouse (10, 24), chicken (11, 30) and zebrafish (6). These animal models have either naturally occurring or induced 5' mutations which disrupt an essential coiled coil domain, resulting in loss of protein function, consecutive loss of Hh signaling and early embryonic lethality. The talpid3 chicken is a thoroughly examined animal model with polydactyly, holoprosencephaly, abnormal neural tube patterning, polycystic kidneys, liver fibrosis, short ribs and endochondral bones with defective ossification (11, 24, 45, 46).

The c.428delG (p.Arg143Lysfs*4) mutation was identified in heterozygous state in patient MD1, in trans to a nonsense mutation (Figure 1B), and in a patient G2 who is also heterozygous for a KIF7 (JBTS12) frameshift mutation and variants in three other known ciliopathy genes (Figure 1C) (34). Our experiments did not indicate epistatic interaction between KIAA0586 and KIF7, and a secondary occult mutation in either gene cannot be excluded. The c.428delG mutation results in a premature termination codon in five human KIAA0586 isoforms, causing either a major protein truncation 5’ to the essential coiled coil
domain, or NMD. It is comparable to the talpid\(^3\) chicken loss-of-function mutation which introduces a frameshift in the orthologous region (Figure 1E). The c.428delG mutation is annotated in dbSNP (rs534542684), and its MAF in the general population is surprisingly high (0.39%, 378 out of 96534 alleles in the ExAC db), reminiscent of the most common deafness (c.35delG in \(GJB2\); 0.60% in the ExAC database) or cystic fibrosis (p.Phe508del in \(CFTR\); 0.67% in the ExAC database) mutation. In two concurrent studies reporting \(KIAA0586\) mutations in JBTS patients, c.428delG represented the most prevalent mutation (43, 44). While c.428delG was clearly enriched in patients with biallelic \(KIAA0586\) mutations in both studies (present in 20 of 24 such patients), only two were homozygous. Despite its commonness, c.428delG was neither observed in homozygous state in healthy individuals in the TGP, ESP, or ExAC databases. Such rarity of homozygosity could indicate that it causes embryonic lethality, early death or severe illness leading to underrepresentation of the respective samples. Embryonic lethality in talpid\(^3\) chicken and \(\text{Talpid3}^{-/-}\) knockout mice would support such an interpretation. On the other hand, c.428delG was not found in a simultaneous study that reports biallelic \(KIAA0586\) mutations in early lethal ciliopathies (47). Of note, a very recent study on rare human knockouts identified in the genomes of 2,636 healthy Icelanders lists one individual of 57 years with homozygosity for c.428delG (48). This could either be due to protective modifiers or a low mutational load in the ciliome of the respective person. Assuming the latter, c.428delG\(_{KIAA0586}\) could represent a hypomorphic allele that increases susceptibility to develop JBTS, with more severe mutations required either \textit{in trans} (in heterozygous carriers, as in most patients reported by Roosing et al. and Bachmann-Gagescu et al. (43, 44)), or in other genes (in homozygous carriers) for disease manifestation. The presence of a heterozygous potentially deleterious \(C5orf42\) (\(JBTS17\)) variant in the only c.428delG\(_{KIAA0586}\)-homozygous patient reported by Bachmann-Gagescu et al. (44), and the co-occurrence of such variants in four ciliopathy genes in patient G2
(including a truncation in the JBTS gene KIF7) support the categorization of c.428delG_KIAA0586 as a hypomorphic mutation of incomplete penetrance. Of note, no secondary KIAA0586 mutation was identified in c.428delG-heterozygous JBTS patients in the two other studies (43, 44), which could be due to the contribution of other genes. The homozygous mutations c.2414-1G>C (Family 1) and c.2512C>T (p.Arg838*, Family 2), would not disrupt the 5’ functionally essential coiled coil domain in the consecutive KIAA0586 protein, and partial function may be maintained (possibly due to preserved, albeit truncated, KIAA0586 protein). We have shown that KIAA0586 has several functions in the centriole, and this may be mediated by different protein residues. The occurrence of retinal degeneration in JBTS depends on the genetic subtype, but is variable even within a family. The localization of KIAA0586 at the ciliary base of retinal photoreceptor cells corresponds to other JBTS proteins. Proteins of the periciliary compartment at the base of the photoreceptor cilium are thought to be critical for the handover of cargo from the dynein-mediated transport through the inner segment to the kinesin-powered anterograde intraflagellar transport in the ciliary compartment (33, 49). KIAA0586 may be part of the protein networks implicated in these processes. The lack of retinal disease in the patients described herein may be due to the less strongly developed structure of the distal appendages and/or the possible functional redundancy in the cilia of retinal photoreceptor cells. Nevertheless, patients with KIAA0586-related JBTS should be investigated for signs of retinal degeneration, and given that mutations in the JBTS gene CEP290 may cause non-syndromic Leber congenital amaurosis (50), KIAA0586 represents a candidate gene for isolated retinopathies.

Loss of KIAA0586 (TALPID3) function in animal models results in a failure to produce both primary and motile cilia. Previously it has been suggested that this is due to a failure of the centrosome to migrate apically or dock at the plasma membrane (7, 12). The subsequent
failure of cilia formation results in abnormal Hh signaling and disrupted GLI processing (11). Most patients with KIAA0586-related JBTS exhibit few classical Hh phenotypes such as polydactyly (this study, (43, 44)), unlike the corresponding mouse, chicken and zebrafish models (6, 10, 11). We show that, independent of Hh signaling, cell and tissue polarity are disrupted upon loss of TALPID3. JBTS is characterized by cerebellar hypoplasia and loss of decussation of neuronal projections from the cerebellum (1). While Hh signalling is required for controlling the growth of the embryonic cerebellar primordia (51), the failure of decussation has been proposed to result from defective axonal guidance (1), a process depending on centrosome-guided cell polarity (52). Furthermore, we have shown that in inner ear HCs, cell polarity and ciliogenesis, albeit closely linked, are differentially affected in talpid3 cells. Thus, loss of decussation may reflect loss of polarity.

We propose that KIAA0586 exerts a role in intracellular trafficking and cell polarity distinct from its role in docking of the centriole. Talpid3 cells have abnormal microtubule dynamics (7). Microtubules are required for the recruitment of satellites and proteins in the distal centriole (53, 54), a process known to be impaired by loss of KIAA0586. Abnormal cell polarity in talpid3 cells may be due to the effect of TALPID3 on microtubule dynamics and a direct role in centrosome organization: Microtubules are essential for intracellular trafficking, cellular structure and polarity. We have shown that localization of the centrosome, mitochondria and Golgi is disrupted in talpid3 cells. Moreover, Rab8, a GTPase which binds to the Golgi and is required for vesicular trafficking and ciliogenesis (55-57), is mislocalized in KIAA0586-depleted cells (9). In JBTS patients with mutations in AHII (JBTS3), encoding an interactor of RAB8 (58), non-ciliary trafficking from the Golgi and ciliogenesis are impaired. Of note, Golgi mislocalization in the talpid3 choroid plexus is similar to what has been observed in Ahi1−/− mice (58). This suggests a similar pathogenesis of JBTS23 and JBTS3, with defective cell polarity, intracellular trafficking, and Hh signaling.
KIAA0586 interacts with CP110 and Cep120 (9), distal centriolar proteins implicated in centriole duplication and maturation, and ciliogenesis. The predominant expression of Cep120 on the daughter centriole throughout most of the cell cycle depends on Kiaa0586, as indicated by high expression of Cep120 on both centrioles and absence of CP110 from the mother centriole prior to ciliogenesis in Talpid3/− cells (59). Although equal expression of KIAA0586 on the mother and daughter centrioles has been reported (this study, (9)) there is evidence that KIAA0586 predominantly localizes at the mother centriole (8). In addition, loss of chicken KIAA0586 (TALPID3) causes centriole elongation whereas overexpression of Cep120 causes elongation of the mother centriole, suggesting that KIAA0586 (TALPID3) may control centriole length through depletion or suppression of Cep120 on the mother centriole. Similarly, depletion of CP110 also increases centriole length (53), suggesting that KIAA0586 regulates centriolar length through controlling CP110 localization and centriolar capping of the distal mother centriole. Loss of other centriolar proteins, such as OFD1, likewise results in elongated centrioles and loss of distal appendages (60). Based on the colocalization of KIAA0586, CP110 and Cep164, it has been proposed that KIAA0586 regulates ciliary vesicle docking adjacent to Cep164 localization (9), but not distal appendage formation itself, and this is supported by evidence from human patient KIAA0586/− cells which show Cep164 within the distal centriole (46). We also find evidence for a vesicle docking defect, demonstrated by an increase in centriolar satellites. However, we propose that KIAA0586 loss primarily causes abnormal distal appendages and impaired Cep164 localization, similar to what can be observed in OFD1 mutants (60). In addition, determination of Cep164 expression in cells of highly polarized tissue demonstrates a further centriolar defect not easily distinguished in in vitro assays – the loss of centriole orientation to the apical membrane of the cell. Whether this defect is due to the depletion of KIAA0586...
from the centriole or impairment of another function of KIAA0586 in pericentriolar regions or cytoskeleton remains to be elucidated.

We have identified \textit{KIAA0586} as a novel gene for JBTS, and we propose that it is not only required for ciliogenesis, but also to establish cell and thus tissue polarity. \textit{JBTS23}, and possibly other JBTS subtypes, may result from impairment of both functions.

\textbf{ACKNOWLEDGMENTS}

We are indebted to the families who participated in our study. We thank Prof. Chi-chung Hui, Department of Molecular Genetics, University of Toronto, for the kind gift of the \textit{Kif7} mouse line, Prof. Andrew Forge for help with basal papilla dissection, John James, CHIPs, University of Dundee, UK, Maurits Jansen of Edinburgh Preclinical Imaging, University/BHF Centre for Cardiovascular Science, University of Edinburgh for technical help, ESRIC for support with advanced imaging, Dr Denis Headon for the kind gift of the β-catenin probe and Dr Jeremy Reiter for the kind gift of the Azi1 antibody. We thank Elisabeth Sehn, and Gabi Stern-Schneider (both JGU Mainz) for their skillful technical assistance.

\textbf{Conflict of Interest Statement:} H.J.B. is employee of Bioscientia which is part of a publicly traded diagnostic company. The work described in this study is unrelated to this employment. The other authors have no competing interests.

\textbf{Funding}

MGD, LAS, GMD and LM are supported by BBSRC Career Track Fellowship funding to MGD (BB/F024347/1) and through funding to The Roslin Institute via Institute Strategic Grant funding from the BBSRC. AMF is funded by a BBSRC DTG EastBio Studentship. UW was supported by European Community FP7/2009/241955 (SYSCILIA), FAUN-
Stiftung, Nuremberg, Foundation Fighting Blindness (FFB), and the BMBF “HOPE2” (01GM1108D). HJB was supported by funding from the Imhoff-Stiftung, Köln Fortune (University Hospital of Cologne), the Deutsche Heredo-Ataxie-Gesellschaft e.V., DHAG and Stiftung Auge (Deutsche Ophthalmologische Gesellschaft). RAJ was supported by the DFG (AB393/2-2).
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LEGENDS TO THE FIGURES

FIGURE 1

Patients with Joubert syndrome (JBTS) and KIAA0586 mutations (A – C). (WT, wildtype; M, mutation). The "molar tooth sign" in cranial axial MRI is indicated by arrows. A Family 1. Homozygosity mapping yielded eight homozygous chromosomal candidate regions (not shown), including the JBTS23 locus comprising KIAA0586. Patients MR026-01 and MR026-04 carry a homozygous splice site mutation, c.2414-1G>C. B Patient MD1 of Family 2 is compound heterozygous for two truncating mutations, including the prevalent c.428delG (p.Arg143Lysfs*4) allele. C Family 3: Patient G2 is double heterozygous for c.428delG in KIAA0586, and a frameshift mutation in KIF7 (JBTS12; c.811delG, p.Glu271Argfs*51). He also carries three potentially pathogenic variants in the ciliopathy genes CEP41, KIF14 and WDPCP (blue). D Genomic structure of KIAA0586 with mutations in exons 5 and in/adjacent to exon 18 indicated. The gel electrophoresis shows the aberrant transcripts due to c.2414-1G>C. E Scheme of human KIAA0586 protein and predicted consequences of JBTS-associated mutations. Orange colour: unrelated residues included due to frameshift mutations. The 3rd coiled coil domain is the counterpart of the functionally essential 4th coiled coil domain in chicken (framed in red). F Chicken KIAA0586 is highly similar to the human protein. The talpid3 mutation results in an early frameshift and loss of three coiled coil domains, including the 4th one. The in-frame deletion of exons 11 and 12 of mouse KIAA0586 (270049A03Rik) is depicted above the scheme of the chicken ortholog.

FIGURE 2

Localization of KIAA0586 in primary cilia and in photoreceptor cilia of mammalian retinas. A Triple labeling of a ciliated IMCD3 cell demonstrates localization of Talpid3 (green) in the basal body (BB) and the adjacent centriole (Ce) at the base of the primary cilium co-stained by antibodies against Pericentrin-2 (PCNT2, red) and anti-acetylated tubulin (acTub, cyan), a biomarker of the axoneme (Ax). B Longitudinal cryosections through a mouse retina stained for Talpid3 (green) and
counterstained for the ciliary marker Centrin-3 (Cen3, red) and for the nuclear DNA marker DAPI reveal Talpid3 localization in the ciliary region (CR) at the joint between the inner (IS) and the outer segment (OS) of the photoreceptor layer, the outer (OPL) and inner inner plexiform layer (IPL). Overlay of DIC (differential interference contrast) image with DAPI (blue) nuclear stain in the outer (ONL) and the inner nuclear layer (INL) and in the ganglion cell layer (GC). C-F Immunostaining of cryosections through the photoreceptor layer of a mouse (C) and a human retina (E) demonstrate co-localization of KIAA0586 and Cen3 in the CR of photoreceptor cells. Higher magnification of double labeled mouse (D) and human (F) photoreceptor cilium reveals substantial localization of Talpid3/KIAA0586 at the centriole (Ce), the BB and between the Ce and BB of the photoreceptor cilium, but not in the connecting cilium (CC). G, H Immunoelectron microscopy analysis of longitudinal section through the cilium of a mouse rod photoreceptor cell and (G) higher magnification of the ciliary base (H) labeled for Talpid3 reveals Talpid3 in the periciliary region namely in the Ce and BB. J Schematic representation of Talpid3/KIAA0586 localization in the photoreceptor cilium. Scale bars: A, 1 μm; B, 10 μm; C, E, 5 μm; D, F, 0.5 μm; G, H, 200 nm.

FIGURE 3

Loss of TALPID3 (KIAA0586) causes abnormal tissue and cell polarity and abnormal intracellular organization. A-B β-catenin expression is localized anteriorly within feather buds of the wildtype chicken at day 9.5. Black circles indicate featherbuds with correct polarity; dashed black circles represent no polarity; blue circles represent abnormal polarity (Schematic C) in the talpid3 chicken (B’’) not seen in the wildtype chicken (A’’). Asterisks represent merged feather buds. D,E SEM of the basilar papilla in wildtype (D) and talpid3 (E) chickens. Arrows indicate cilia. Curved lines represent the base of stereocilia hair bundles. F-K Actin bundles identified by phalloidin (green) and centriolar localization identified by γ tubulin (red). F,G Red circles with line represent orientation of polarized actin bundles in basilar papilla; dashed red circles represent unpolarized actin bundles (Schematic L,M). H-K Dashed white circles represent magnified images (Ji-Kii’). Ji-Kii White arrows indicate aligned centrosomes; blue arrows indicate unaligned centrosomes (Schematic N). O,P
Orientation based on placement of Golgi (TGN46, red) in comparison to actin indicating the leading edge (phalloidin, green) and nucleus (Dapi, blue, schematic in Q) in MEFs. Asterisks represent areas of higher magnification (not all represented at lower magnification). Scale Bars: A,B 5 mm; A’,A’’,B’,B’’ 1 mm; D,E 1 μm; F,G,H,I,J,K 20 μm; F’,G’ 100 nm; Ji,Ji’,Jii’,Ki,Ki’,Kii,Kii’ 10 μm; O,P 100 μm; Oi,Oii,Oiii,Oiv,Ovi,Ovi,Pi,Pii,pii,Pii,Piv,Pv,Pvi 25 μm.

**FIGURE 4**

**Loss of TALPID3 causes abnormal intracellular organization and centriolar orientation**

A,B The chicken choroid plexus at E8 is a highly polarized structure with docked centrioles (asterisk, A) identified within a clear centriolar zone apically (CZ, A) and a mitochondrial zone (MZ; m indicates mitochondria). The talpid\(^3\) choroid plexus (B) lacks these defined zones, with mitochondria identified in the most apical zone (m, B) centrioles identified throughout the cell, failing to dock (asterisk, B). Quantification of distance of mitochondria to cell surface (C). D-G talpid\(^3\) tissue is capable of producing mature centrioles. Wildtype centrioles (D) and talpid\(^3\) centrioles (E,F) exhibited subdistal appendages (SD), and distal appendages (DA), although DA were less frequently observed on talpid\(^3\) centrioles, quantified in (G). CEP164 localizes to the distal mother centriole in wildtype and talpid\(^3\) choroid plexus neuroepithelium (purple arrow indicated distal mother centriole, green arrow proximal centriole; H,I,K,L) and fibroblasts (J,M), but CEP164 puncta are smaller and disorganized in talpid\(^3\) choroid plexus and fail to orientate to the apical surface of the cell (arrows L). Centrioles in wildtype tissue were on average 0.7 μm (red line indicating centriole/basal body; N,R) compared to 0.9 μm in the talpid\(^3\) choroid plexus (O,P,Q,R). Scale bars: A,B=1 μm, D,E,F=100 nm; H,K=10 μm I,J,L,M=5 μm, N,O,P,Q=200 nm.

**FIGURE 5**

**Analysis of centriolar satellites in the talpid\(^3\) choroid plexus.** An area clear of electron-dense condensations was observed around the basal body in wildtype cells (area outlined by dots; A), electron-dense condensations were observed adjacent to talpid\(^3\) centrioles (indicated by arrows, D).
Quantified in (G). Immunostaining for a centriolar satellite marker in the choroid plexus, PCM1 (magnified area outlined by dashed line; PCM1=red, γ tubulin, green B, C, E, F). KIAA0586 protein does not colocalize with AZI1, a satellite protein in human RPE1 cells (KIAA0586=red, AZI1=green H, J). Scale bars: A,D=500 nm; B,E 10=μm; C,F= 2 μm H,I,K 5 μm.

**Figure 1- figure supplement 1**

**Analysis of potential interactions between Talpid3/TALPID3, Kif7/KIF7 and IFT57 in the mouse and in chicken.** Biallelic KIF7 mutations cause JBTS type 12 in human (34). Although both the KIAA0586 mutation c.428delG and the KIF7 mutation c.811delG were paternally inherited in patient G2, we sought to test for subtle abnormalities resulting from this double heterozygosity. In addition, we had previously found through a microarray analysis of talpid3 limb buds that IFT57, a protein associated with ciliopathy phenotypes in mice and zebrafish, is downregulated in talpid3 embryos (10). Using in ovo complementation of the talpid3 neural tube, we could not detect induction of ISLET1 expression or ventralized PAX7 in the wildtype or talpid3 neural tube by overexpression of KIF7 or IFT57 (Figure SF1A,B). We then used siRNA constructs against KIF7 to model a heterozygous loss of KIF7 in the TALPID3+/− neural tube. Knock-down with two siRNA constructs had a weak effect on neural tube patterning compared to the mouse KIF7−/− knockout (22, 61).

Although the NKX2.2 expression domain could be marginally expanded in wildtype embryos (not shown), there was no expansion of ISLET1 positive motorneuron progenitors in wildtype or TALPID3+/− embryos. PAX7, however, was weakly dorsalized in both wildtype and talpid3+/− embryos (Figure SF1C). These results suggested that some KIF7 function may be cilia-independent as has been suggested (61). To more precisely investigate for a possible epistatic relationship between Kif7 and Talpid3, particularly in the organs primarily affected in JBTS, such as the cerebellum, we undertook a Talpid3+/− × Kif7+/− mouse cross in order to determine if double Talpid3+/−/Kif7+/− heterozygous animals had brain patterning malformations. We first dissected embryos at E15.5, 16.5 and 17.5 and found that Talpid3+/−/Kif7+/− embryos were morphologically normal, including size, situs and limb patterning. MEFs derived from E12.5 embryos were normally ciliated, with the percentage of ciliated
cells and cilia length comparable to those seen in wildtype, Talpid3+/+Kif7+/+ and Talpid3+/+Kif7+/+ cells (Figure SF1F,G). MRI and sectioning of the brain also showed no brain patterning abnormalities (Figure SF1D,E). Subsequently Talpid3+/+/Kif7+/− animals were born and grew normally compared to their litter mates and showed no abnormal brain morphology (Figure SF1). We conclude that KIAA0586 (TALPID3) and KIF7 do not act epistatically and hypothesize that additional genetic alterations in ciliopathy genes of patient G2, eventually including those identified in CEP41 (JBTS15), KIF14 and WDPCP, may contribute to a mutational load that is sufficient to elicit a JBTS phenotype on a KIAA0586+/−; KIF7+/− background. A Overexpression of IFT57 does not have an effect on patterning of the neural tube in the talpid3 chicken. B Overexpression of KIF7 does not rescue or alter neural tube patterning in the talpid3 chicken. C siRNA knockdown of KIF7 resulted in a weak dorsalization of PAX7 but no expansion of ISLET1. D Talpid3+−Kif7+/− mice showed no gross anatomical abnormalities, neither were developmental brain defects identified through MRI (D) or histology (E). F, G No abnormalities were identified in either the percentage of ciliated cells (F), nor the length of cilia (G) in MEFs derived from wildtype, Talpid3+/+Kif7+/+, Talpid3+−Kif7+/− or Talpid3+−Kif7+/− mice.
Figure 3
Figure 5