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The coiled-coil domain containing protein *CCDC40* is essential for motile cilia function and left-right axis formation

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Abstract

Primary ciliary dyskinesia (PCD) is a genetically heterogeneous autosomal recessive disorder characterized by recurrent infections of the respiratory tract associated with abnormal function of motile cilia. Approximately half of PCD patients also have alterations in the left-right organization of internal organ positioning including situs inversus and situs ambiguus (Kartagener's Syndrome, KS). Here we identify an uncharacterized coiled-coil domain containing protein (*CCDC40*) essential for correct left-right patterning in mouse, zebrafish and humans. *Ccdc40* is expressed in tissues that contain motile cilia and mutation of *Ccdc40* results in cilia with reduced ranges of motility. Importantly, we demonstrate that *CCDC40* deficiency causes a novel PCD variant characterized by misplacement of central pair microtubules and defective axonemal

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assembly of inner dynein arms (IDAs) and dynein regulator complexes (DRCs). CCDC40 localizes to motile cilia and the apical cytoplasm and is responsible for axonemal recruitment of CCDC39, which is also mutated in a similar PCD variant.

Underlying defects in cilia ultrastructure are responsible for altered ciliary beat in PCD patients. The core structure of the cilium is the axoneme: nine peripheral microtubule doublets with or without a central pair of microtubules (9+2 or 9+0), interconnected by outer and inner dynein arms (ODAs and IDAs), radial spokes, nexin links and a central sheath¹. Coordinated activation of the ODAs and IDAs generates the ciliary beat. Most of the characterized PCD variants exhibit mutations in genes that encode dynein arm components such as *DNAI1*, *DNAI2*, *DNAH5*, *DNAH11*, and *TXNDC3*². Mutations in genes encoding cytoplasmic proteins such as C14orf104 (KTU) and LRRC50 also affect assembly of dynein arm complexes in the cytoplasm in a poorly understood process^{3–6}.

In our forward genetic screens to identify genes required for normal development of the mouse embryo^{7–8}, we isolated a mutant which exhibits left-right patterning defects. Greater than one-third of homozygous *links* (*lnks*) mutant embryos (39%, n=172) display laterality defects at E11.5–15.5 (Fig. 1a–d) including situs inversus (8%) or left isomerism (19%) based on lung lobation patterns. The majority of homozygous *lnks* mutant pups die before weaning due to unknown causes. In two homozygous *lnks* mutant pups that were examined, no kidney cysts were detected but hydrocephalus was noted (Supplementary Fig. 1). These observations resemble findings obtained in *Mdnah5* deficient mice, a mouse model for PCD where ependymal cilia motility is important to prevent hydrocephalus⁹. The *lnks* mutation was mapped to a 0.3 MB region of mouse chromosome 11 (Fig. 1e) that included the uncharacterized *Coiled-coil domain containing 40* (*Ccdc40*) gene. Coiled-coil domains typically function in homodimerization and are present in a number of proteins involved in intracellular transport¹⁰. *Ccdc40* is specifically expressed in the embryonic node and midline tissues (Fig. 1f–i), key tissues that control left-right patterning. Upon sequencing the 3378 base pair *Ccdc40* transcript from *lnks* mutant mice, a C to A transversion was identified (Fig. 1j). This nonsense mutation converts Valine⁷⁹² to a stop codon in the middle of the coiled-coil domain, truncating the predicted 1125 amino acid protein (Fig. 1j,k).

In zebrafish embryos, *ccdc40* is expressed in tissues that contain motile cilia including Kupffer's vesicle, floorplate, pronephric tubules and otic vesicle (Fig. 2; and data not shown). To explore the evolutionary conserved role of *ccdc40* in left-right patterning, we designed two different antisense morpholino oligonucleotides (MOs) against zebrafish *ccdc40*. Both MOs disrupt splicing of the *ccdc40* transcript (Supplementary Fig. 2) and produce similar phenotypes upon injection (Fig. 2e,g,i). Injection of MO resulted in a curly-tail down phenotype characteristic of other zebrafish mutants with laterality defects. Uninjected control embryos exhibited predominantly situs solitus (SS) at the 48 hpf stage with normal rightward looping of the heart, liver on the left and pancreas on the right side of the midline. By contrast, injection of either MO resulted in laterality defects: either reversed organ patterning, situs inversus (SI; 15–24%), or randomized organ patterning, heterotaxia (HTX; 13–19%). Both laterality and curly-tail down phenotypes could be rescued by co-injection of *ccdc40* mRNA (Fig. 2h,j).

ccdc40 maps to zebrafish chromosome 6 in a region associated with the zebrafish mutant *locke* (*lok*), previously described as having a strong curly-tail down phenotype, laterality defects and pronephric cysts, without defects in sensory cilia or presence of hydrocephalus^{11–12} (and J. S-B and R.D.B. unpublished). The *locke* phenotype is indistinguishable from knockdown of *Ccdc40* in zebrafish (Fig. 2f,g,i). We sequenced genomic DNA from *lok^{to237b}* mutants and found a C to T transition within the 3370 base pair transcript that changes Glutamine⁷⁷⁸ to a stop codon (Fig. 2d).

The laterality defects observed in mouse zebrafish mutants combined with expression of the transcript in the node/Kupffer's vesicle suggest that *Ccdc40* may act to regulate cilia function (Fig. 3). Indeed, scanning electron microscopy (SEM) revealed that the length of the cilia projecting from the nodal pit cells in *lnks* mutants is drastically reduced (Fig. 3a,b,e,f). Similarly, cilia were shorter in Kupffer's vesicle and the pronephric tubules of *ccdc40* morphants compared to uninjected controls (Fig. 3c,d,g,h). These results indicate that *Ccdc40* is required for proper formation or maintenance of cilia.

Based on the cilia and laterality phenotypes in mouse and zebrafish *ccdc40* mutants, we considered *CCDC40*, a strong candidate gene for human PCD. All coding *CCDC40* exons and the adjacent intron-exon boundaries were amplified by PCR in a cohort of 26 PCD patients displaying a similar axonemal defect (see below). Sequence analyses revealed *CCDC40* loss-of-function mutations in 17 PCD patients (Supplementary Fig. 3 and Table 1). Segregation analyses in all PCD families with *CCDC40* mutations were consistent with autosomal recessive inheritance (Supplementary Fig. 4). Furthermore, in 15 affected individuals originating from 13 families, sequence analyses identified mutations on both *CCDC40* alleles. However, in two families a mutation on the second allele was not identified by this approach. We addressed whether large deletions involving *CCDC40* might be present on the other allele in these patients. Indeed segregation analysis of single nucleotide polymorphisms (SNPs) identified by sequence analysis of PCR products provided evidence for parental non-contribution suggestive of heterozygous *CCDC40* deletion in family OP-43. SNP segregation was consistent with the interpretation that the three affected individuals inherited a large genomic deletion involving at least exon 1 from the mother and the point mutation (c.C1366T; p.R449X) from the father (Supplementary Fig. 3 and 5). In the affected individual of the one remaining family that carried point mutations solely on a single allele, we might have missed larger genomic mutations due to limitations of SNP analyses. Alternatively, mutations may reside in the non-coding regulatory or intronic regions.

The clinical phenotype of PCD patients harboring *CCDC40* mutations is consistent with a severe defect of cilia beating, because patients suffered from recurrent upper and lower airway infections. To examine this directly, high-speed videomicroscopy analyses of respiratory cilia obtained by nasal brushing biopsies revealed a severely altered beating pattern in all analyzed samples. Respiratory cilia from affected patients exhibited markedly reduced beating amplitudes and the cilia appeared rigid with fast flickery movements (Supplementary Fig. 6; Supplementary videos 1–4). These motility defects are similar to those reported for pronephric cilia in *lok* mutants¹¹ and those observed in *ccdc40* morphants (data not shown). No significant difference between cilia length was found in analyses of respiratory cilia from seven PCD patients carrying recessive *CCDC40* loss of function mutations compared with normal controls (Supplementary Fig. 7), implying that ciliary movement can be disrupted in the absence of gross structural defects.

Consistent with a conserved functional role of *CCDC40* for nodal cilia function, five patients displayed situs solitus (32%) and 11 patients situs inversus (68%). Together, these findings provide compelling evidence that recessive loss-of-function mutations within *CCDC40* are responsible for a novel PCD variant characterized by altered mucociliary clearance of the airways and randomization of left/right body asymmetry.

We hypothesize that *CCDC40* affects axonemal assembly of protein complexes leading to abnormal cilia morphology and/or motility. Axonemal structure was examined by Transmission electron microscopy (TEM) of cilia in zebrafish embryos and human cells. Motile cilia display a typical 9+2 microtubule configuration whereas *lok* mutants showed misplaced and/or duplicated central tubules and misplaced peripheral doublets (Fig. 3i–l; see

similar axonemal defects in ¹²). Intriguingly, outer dynein arm morphology appeared normal. Similarly, TEM analyses of *CCDC40*-mutant respiratory cilia from PCD patients revealed defects in several axonemal structures including occasional absent or eccentric central pairs, displacement of outer doublets, reductions in the mean number of inner dynein arms and abnormal radial spokes and nexin links (Fig. 4) yet outer dynein arms appeared normal. Interestingly, in a parallel work Merveille et al. ¹³ show that recessive *CCDC39* mutations cause a PCD variant indistinguishable from that caused by *CCDC40* mutations.

To characterize further the molecular defect in *CCDC40* mutant respiratory cells, we performed high-resolution immunofluorescence analyses on control and *CCDC40* patient samples. In confirmation of the TEM analysis, we found normal composition of axonemal outer dynein arm motor proteins DNAH5, DNAH9 and DNAI2 (data only shown for DNAH5, Fig. 4a). Moreover, we confirmed an absence of the IDA component DNALI1 (Fig. 4c) from respiratory ciliary axonemes in all analyzed samples of affected patients. In most mutant respiratory cells DNALI1 accumulated in the apical cytoplasm (Fig. 4c). The severely reduced beating amplitude of respiratory cilia prompted us to investigate whether the axonemal assembly of the dynein regulatory complex (DRC) is also affected by *CCDC40* deficiency. Thus, we examined expression of the mammalian DRC protein GAS11 (orthologous to *Chlamydomonas* DRC protein PF2 ¹⁴) in all affected patients carrying *CCDC40* mutations (see Table 1). Control respiratory cells showed strong GAS11 localization throughout all ciliary axonemes; however, in *CCDC40* mutant respiratory cells, GAS11 was undetectable in ciliary axonemes (Fig. 4b). Similar to DNALI1, GAS11 accumulated in the apical cytoplasm of most mutant respiratory cells (Fig. 4b). Thus, we provide evidence that *CCDC40* is necessary for correct assembly of at least two distinct axonemal complexes regulating ciliary beat: DNALI1-containing IDAs and GAS11-containing DRC. Furthermore, based on TEM findings, radial spokes are also altered in *CCDC40* deficient respiratory cilia.

We generated polyclonal antibodies to determine the intracellular localization of Ccdc40 in sections of the mouse node (Supplementary Fig. 7). Wildtype embryos at E8.0 showed a punctuate pattern of Ccdc40 localization throughout the cytoplasm of node cells with significant overlap of expression with tubulin in the apical cytoplasmic regions of nodal cells (Fig. 3m–o). Ccdc40 antibody staining in E8.0 *lnks* mutant embryos confirmed antibody specificity and showed that truncation of the coiled-coil domain of Ccdc40 results in markedly decreased antibody staining in the node of *lnks* mutant embryos (Fig. 3p–r). Interestingly, we did not observe Ccdc40 protein localized to the 9+0 cilium in the mouse node (white arrow Fig. 3o); however, we do see axonemal localization of Ccdc40 in 9+2 respiratory (tracheal) cells (white arrow Fig. 3t), which is lost in *lnks*-mutant respiratory cells (3w). Ccdc40 may be at too low a level in the node cilium to detect in this assay, or this may reflect a difference in localization between monociliated 9+0 and 9+2 multiciliated cells. Together, these results suggest that Ccdc40 is required for cilia function by acting in the cytoplasm and possibly in the cilium itself. Because *CCDC39* mutations cause a remarkably similar PCD phenotype ¹³, we analyzed whether *CCDC40* deficiency affects axonemal localization of *CCDC39*. Interestingly, in all analyzed *CCDC40*-mutant respiratory cells, *CCDC39* is absent from the cilium and is instead enriched in the apical cytoplasm at the ciliary base (Fig. 5). Thus, *CCDC40* appears to be responsible for axonemal recruitment of *CCDC39*.

Our findings suggest that *CCDC40* may physically interact with the other axonemal components and serve as a part of the axoneme structural scaffold, possibly as a new DRC component. This conclusion is consistent with findings that mutations in genes encoding DRC components in *Chlamydomonas* cause a similar ultrastructural phenotype in flagella including IDA defects ^{15–17}. Alternatively, it is possible that *CCDC40* is important for

cytoplasmic pre-assembly, axonemal targeting, and/or transport of the axonemal components CCDC39, GAS11 and DNALI1. Mutations in genes responsible for cytoplasmic pre-assembly and/or axonemal targeting of DNALI1-containing IDA complexes, such as KTU and LRRC50, have thus far only been reported when ODA complexes are also affected ^{4,5}. Nothing is yet known of the process of cytoplasmic pre-assembly and axonemal targeting/delivery of DRC complexes. Based on our functional data we propose that CCDC40 belongs to a group of novel evolutionarily conserved coiled-coil domain-containing proteins (including CCDC39) that govern the assembly of DRC and IDA complexes responsible for cilia beat regulation but not ODA complexes. Identification and molecular characterization of this process greatly aids diagnosis of PCD and will help direct research for novel therapeutics.

Methods

Patients and families

Signed and informed consent was obtained from patients fulfilling diagnostic criteria of PCD ¹⁹ and family members using protocols approved by the Institutional Ethics Review Board at the University of Freiburg and collaborating institutions. We studied DNA from a total of 26 PCD patients originating from 24 unrelated families. These patients exhibited axonemal defects documented either by electron microscopy analyses and/or with immunofluorescence analyses as described in ¹³. 22 patients without evidence of *CCDC39* mutations as well as four new patients displaying the same phenotype were analyzed for presence of *CCDC40* mutations.

Transmission electron microscopy

Nasal brush biopsies were taken from the middle turbinate and fixed in 2.5% glutaraldehyde in 0.1M sodium cacodylate buffer at 4°C, washed overnight and postfixed in 1% osmium tetroxide. After dehydration, samples were embedded in a propylene oxide / epoxy resin mixture. After polymerisation several resin sections were cut using an ultra-microtome. The sections were picked up onto copper grids and stained with Reynold's lead citrate. Transmission electron microscopy was performed with a Philips CM10.

Immunofluorescence analysis

Respiratory epithelial cells were obtained by nasal brush biopsy (Engelbrecht Medicine and Laboratory Technology, Germany) and suspended in cell culture medium. Samples were spread onto glass slides, air dried and stored at -80°C until use. Cells were treated with 4% paraformaldehyde, 0.2% Triton-X 100 and 1% skim milk prior to incubation with primary (at least 3 hours at room temperature or over night at 4°C) and secondary (30 minutes at room temperature) antibodies. Appropriate controls were performed omitting the primary antibodies. Monoclonal anti-DNALI1 antibodies, monoclonal anti-Gas11 antibodies and polyclonal anti-DNAH5 were reported previously ^{5,20} (and parallel work Merveille ¹³). Polyclonal rabbit anti- α/β -tubulin was from Cell Signaling Technology (USA); monoclonal mouse anti-acetylated- α -tubulin antibody and polyclonal CCDC39 antibodies were from Sigma (Germany). Highly cross adsorbed secondary antibodies (Alexa Fluor 488, Alexa Fluor 546) were obtained from Molecular Probes (Invitrogen). DNA was stained with Hoechst 33342 (Sigma). Confocal images were taken on a Zeiss LSM 510 i-UV.

High-speed video analyses of ciliary beat in human cells

Ciliary beat was assessed with the SAVA system ²¹. Nasal brush biopsies were rinsed in cell culture medium and immediately viewed with an Olympus IMT-2 inverted phase-contrast microscope equipped with a Redlake ES-310 Turbo monochrome high-speed video camera

(Redlake, San Diego, USA) and a 40× objective. Digital image sampling was performed at 125 frames per second and 640×480 pixel resolution. The ciliary beat pattern was evaluated on slow motion playbacks.

Mouse Strains and Genotyping

The *Inks* mouse line was identified in a screen for recessive ENU-induced mutations that cause laterality defects at E11.5 and E12.5. The *Inks* mutation was generated on a C57BL/6J genetic background and backcrossed to C3H. In a mapping cross of 124 opportunities for recombination, the *Inks* mutation was mapped between the Massachusetts Institute of Technology (MIT) simple sequence length polymorphism (SSLP) markers D11mit48 and D11mit104. For high-resolution mapping, additional polymorphic DNA markers were generated based on nucleotide repeat sequences and include: D11ski2, D11ski10 and D11ski16 (see <http://mouse.ski.mskcc.org/> for sequence of primers). The entire *Inks* transcript was sequenced by RT-PCR (Superscript One-Step RT-PCR, Invitrogen) using RNA isolated from *Inks/Inks* and C57BL/6 control embryos.

Analysis of mutant mouse phenotypes

Whole-mount and section RNA in situ in mouse were performed as described^{22–23}. The expression pattern of *Ccdc40* was determined using an anti-sense RNA probe synthesized from IMAGE clones: 1362516 and 5702143 with identical results. The affinity purified polyclonal anti-Ccdc40 antibody was generated by immunization of rabbits using the peptide AYPPKKAKHRKVRPQAEV (Bio-Synthesis Inc). Antibody specificity was initially tested by immunoblotting. Briefly, protein extracts prepared from wildtype and *Inks* mutant mouse respiratory epithelial cells were resolved on a NuPAGE 4–12% bis-tris gel (Invitrogen, Karlsruhe, Germany) and blotted onto a PVDF membrane (Amersham). The blot was processed for ECL plus (GE Healthcare, UK) detection using anti-Ccdc40 (1:100) and anti-rabbit-HRP (1:3000) antibodies (GE Healthcare, UK). Immunofluorescence experiments were performed as described²⁴ using anti-acetylated tubulin (Sigma; 1:1000) and Hoechst (Sigma; 10 µg/ml). For analysis of *Ccdc40* expression in the mouse node, in addition to examination of staining in 20µm sections, protein expression was examined in whole mount of 4 wildtype embryos and the *Ccdc40* protein was absent in all 56 nodal cilia examined.

Zebrafish injections

Morpholino antisense oligonucleotides targeting *ccdc40* (*ccdc40*^{MO}) were purchased from GENE Tools, LLC (Philomath, OR, USA). MO1 was designed against the splice-donor sites of exon 12 and intron 12 of *ccdc40*; 5'-TGTTAACTGTGGTACATACTC TCTC-3' (e12i12). MO2 was designed against the splice-acceptor sites of intron 10 and exon 11 of *ccdc40*; 5'-GCCTCCTGAAAAATCAAATATACAC-3' (i10E11). Both MOs were conjugated to fluorescein isothiocyanate (FITC). 3ng – 6ng of *ccdc40*MO per 500 pl were injected per embryo and both MOs produced similar results. For mRNA rescue, plasmid lockeT7TS was linearized with XbaI and transcribed using T7 RNA polymerase. 500pg of mRNA per embryo was co-injected with 3 ng per embryo of morpholino (e4i4). To assess splicing defects, total RNA was isolated from morpholino injected embryos or uninjected controls and used to synthesize cDNA libraries for PCR analysis with the SuperScript First-Strand Synthesis System (Invitrogen). Primer sequences used are available upon request.

Analysis of zebrafish phenotypes

The *ccdc40* antisense probe was prepared from EcoRI linearized BL283 using T3 RNA polymerase. RNA in situ hybridization to analyze organ laterality was performed as described²⁵ using standard procedures²⁶. *Ccdc40*^{MO} injected embryos were collected at

11–13 somites, fixed in 4% paraformaldehyde for 45 min. at room temperature, and processed for immunohistochemistry using standard methods. Antibodies used include: anti-acetylated tubulin monoclonal antibody at 1:400 (IgG2b isotype, clone: 6-11B-1; Sigma St. Louis, MO, USA) and goat anti-mouse IgG2b, FITC-conjugated antibody at 1:400 (Southern Biotech, Birmingham, AL, USA). Nuclei were visualized with Hoechst or Draq5 and F-actin was stained with Rhodamine-phalloidin. Embryos were mounted in 50% glycerol/PBS and analyzed using the Zeiss LSM510. Zebrafish TEM samples were prepared as described²⁷ and analyzed on a Zeiss 921AB.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

Acknowledgments

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Figure 1. Mutation of the uncharacterized *Ccdc40* gene in *lnks* mutant mouse embryos results in laterality defects

a–d. Heart, lung and stomach (S) from E13.5 (a,b) and E12.5 (c,d) wildtype (a,c) embryos exhibiting normal situs (NS) and *lnks* mutant viscera exhibiting left isomerism (b, LI), or situs inversus (d, SI). Right ventricle (RV). Heart is outlined in black, left and right lung lobes in blue and red, respectively. e. Genetic map of *lnks* interval on mouse chromosome 11. The number of recombination events over the number of opportunities for recombination is indicated for each polymorphic marker. D11ski16 never separated from the *lnks* phenotype. Within this interval are four transcription units: TBC1 domain family, member 16 (*Tbc1d16*), coiled-coil domain containing 40 (*Ccdc40*), glucosidase, alpha, acid (*Gaa*) and eukaryotic translation initiation factor 4A3 (*Eif4a3*) f–i. *Ccdc40* expression in wildtype E8.0 (f), E8.25 (g), E8.5 (h) and E9.5 (i) embryos as detected by RNA *in situ* hybridization.

Strong staining is detected in the node (arrows in f–h). j. The *lnks* ENU-induced mutation results in a C to A transversion (green arrow) at position 2585 in the *Ccdc40* coding sequence introducing a nonsense mutation changing Valine⁸⁶² to a stop codon. Mouse *Ccdc40* is 63% identical and 78% similar to human *CCDC40*. k. The *lnks* mutation truncates the Ccdc40 protein within the coiled-coil domain (red). Green line in panel k indicates the peptide used to generate the anti-Ccdc40 antibody.

Figure 2. Loss of zebrafish *ccdc40* in *lok* mutants or *ccdc40* morpholino injected embryos produces laterality defects

a–c. Expression of *ccdc40* transcript in wildtype zebrafish embryos at 75% epiboly (a) and 6 somites (b,c). Staining is detected in the dorsal forerunner cells (arrow in a), pronephric tubules (arrows in b) and otic vesicles (arrows in c). d. The predicted domain structure of the 941 amino acid zebrafish Ccdc40 protein. The *lok* mutation introduces a stop codon at position 778 producing a protein with a truncated C-terminal domain. Zebrafish *ccdc40* is 39% and 36% identical and 60% and 58% similar to the human and mouse genes, respectively. e–h. Phenotypes of *lok* mutant and *ccdc40MO* injected embryos at 3dpf. *lok* mutant embryo (f) display the curly-tail phenotype compared to an unaffected sibling (e). Embryo injected with *ccdc40MO1* also displays the curly-tail phenotype (g) which can be rescued by co-injection of *ccdc40* mRNA (h). Insets in g and h indicate the fluorescein labeled MO was injected into both embryos. i. Quantification of left-right organ patterning in *lok* mutant and *ccdc40MO* injected embryos. SS=situs solitus; SI=situs inversus, HTX= heterotaxia, any organ pattern that is not SS or SI. j. Rescue of MO phenotypes by co-injection of *ccdc40* mRNA. Heart looping was scored as an indication of left-right patterning. RLoop = rightward looping of the heart, NLoop = no heart looping (midline) and LLoop = leftward looping of the heart. Curly tail down (CTD) indicates a strong phenotype

such as that pictured in g. $+/++$ indicates tails that were slightly kinked or bent. WT indicates indistinguishable from uninjected embryos (compare tail in h to that in e).

Figure 3. Loss of Ccdc40 results in ciliary defects

a–b. SEM showing morphology of node cilia in E8.0 wildtype (a,e) and *Inks* mutant (b,f) mouse embryos. Panels e and f are higher magnification views of a and b, scale bars are indicated. c,d,g,h. Cilia imaging in zebrafish pronephric tubules (c,d) and Kupffer's vesicle (g,h). Loss of Ccdc40 function in mouse (b,f) and zebrafish embryos (d,h) results in significantly shorter cilia relative to controls. In Kupffer's vesicle, cilia length in uninjected controls averaged 5.2μm (SD 1.486; n=589 cilia) while cilia in MO embryos were consistently shorter, averaging 3.6μm (SD 1.20; n=511 cilia; $p=6 \times 10^{-73}$ by one-tailed student t-test). Shorter cilia are also reported in *lok* mutants^{11–12} i–l. TEM analysis of cilia in the pronephros of *lok* mutant embryos demonstrates defects in central pair positioning (j,k)

or number (l) compared to control (i). Note that outer dynein arms are not affected. m–x. Immunofluorescence analysis showing localization of endogenous Ccdc40 protein (n,q,t,w) and acetylated tubulin (m,p,s,v) in the node of E8.0 wildtype (m–o) and *lnks* mutant (p–r) embryos, and P21 wildtype trachea (s–u) and *lnks* mutant trachea (v–x) (overlay including visualization of nuclei (Hoechst staining) in o, r, u, and x.). Arrow in o points to a node cilium that was not recognized by the anti-Ccdc40 antibody. Note that Ccdc40 is not readily detectable in the 9+0 node cilium, but is present in the axonemes of multiciliated tracheal cells. Motility of node cilia was not evaluated.

Figure 4. Localization of DNAH5, GAS11 and DNALI1 in respiratory epithelial cells from PCD patients carrying *CCDC40* mutations

Immunofluorescence analyses of human respiratory epithelial cells using specific antibodies directed against the outer dynein arm heavy chain DNAH5 (a), the dynein regulating complex component GAS11 (b) and the inner dynein arm component DNALI1 (c). As control, axoneme-specific antibodies against acetylated α -tubulin (a) or α/β -tubulin (b,c) were used. Nuclei were stained with Hoechst 33342 (blue). (a) In respiratory epithelial cells from healthy probands, DNAH5 (red) localizes along the entire length of the axonemes. In respiratory epithelial cells from patient OP-799 carrying compound heterozygous *CCDC40* mutations cilia are shorter but DNAH5 (red) is localized along the entire length of the

axonome as in the healthy control. (b,c) Similarly, GAS11 (b, green) and DNALI1 (c, green) localizes along the entire length of the axonemes in the healthy control, whereas in respiratory epithelial cells from patient OP-712II1 GAS11 (green) and from patient OP-799 DNALI1 (green) is absent from the ciliary axonemes. White scale bars (a–c) are 5µm. (d–i) Transmission electron microscopy of respiratory cilia showing normal axonemal structure in a control (d) and cilia with abnormal tubular organisation in patient OP-712II2 carrying a homozygous loss-of-function mutation in *CCDC40* (e–g) and OP-43II1 carrying compound heterozygous *CCDC40* mutations (h–i). Black scale bars (d–i) are 0.1µm.

Figure 5. Mutations *CCDC40* affect localization of *CCDC39* in respiratory cells

Subcellular localization of *CCDC39* in respiratory epithelial cells from PCD patients carrying *CCDC40* loss-of-function mutations. As control, axoneme-specific antibodies against acetylated α -tubulin (green) were used. Nuclei were stained with Hoechst 33342 (blue). In respiratory epithelial cells from healthy probands (a), *CCDC39* (red) localizes along the entire length of the axonemes and to a weaker degree in the apical cytoplasm. In respiratory epithelial cells from patients carrying *CCDC40* loss-of function mutations OP-799 (b), OP-712 II1 (c), OP-741 (d) and OP-659 (e) *CCDC39* is either markedly reduced or absent in ciliary axonemes and instead accumulates at the ciliary base. White scale bars (a–e) are 5 μ m.

Table 1

CCDC40 Mutations in primary ciliary dyskinesia

Available clinical data do not indicate the presence of sensory hearing deficits, kidney cysts and/or hydrocephalus in affected patients harbouring *CCDC40* mutations. Data on sperm analyses are not available. CP = central pair; del = deletion; fs = frame shift; ins = insertion; IVS = inversion; M = maternal; n.a. = not available; n.d. = not determined; P = paternal; RS = radial spoke

Patients	Origin	Gender	DNA-change	Exons	Protein-change
F-777II1	Germany	female	[c.248delC]	[3] + [3]	[p.A83Vfs82X]
OP-120	Germany	female	[c.248delC]	[3] + [3]	[p.A83Vfs82X]
OP-240II2	Germany	male	[c.C1315T]	[8] + [8]	[p.Q439X]
OP-712III1	Pakistan	male	[c.1527_1558del]	[10] + [10]	[p.D510Sfs22X]
OP-712II2	Pakistan	male	[c.1527_1558del]	[10] + [10]	[p.D510Sfs22X]
OP-57II	Austria	female	[c.C1971T]	[12] + [12]	[p.Q651X]
OP-76II1	Germany	female	[c.3129delC]	[19] + [19]	[p.F1044Sfs35X]
OP-87II2	Germany	male	[c.248delC] + [c.778del]	[3] + [5]	[p.A83Vfs82X] + [p.E260Rfs25X]
OP-799	Denmark	female	[c.248delC] + [IVS11-2A>G]	[3] + [12]	[p.A83Vfs82X] + splicing-mut.
OP-82III1	Germany	male	[c.248delC] + [c.C1810T]	[3] + [12]	[p.A83Vfs82X] + [p.Q604X]
OP-741	Denmark	female	[c.248delC] + [c.2824_2825insTGT]	[3] + [17]	[p.A83Vfs82X] + [p.R942MinsW]
OP-659	Jugoslavia	male	[c.C960T] + [c.C2440T]	[7] + [14]	[p.R321X] + [p.R814X]
OP-43II1	Hungary	male	[c.C1366T] + del	[9] + del	[p.R449X] + del
OP-43II2	Hungary	female	[c.C1366T] + del	[9] + del	[p.R449X] + del
OP-43II3	Hungary	male	[c.C1366T] + del	[9] + del	[p.R449X] + del
OP-277III1	Germany	male	[c.2824_2825insTGT] + [c.3128_3130delC]	[17] + [19]	[p.R942MinsW] + [p.Q1041fs36X]
F-677III1	Germany	male	[c.248delC] + n.d.	[3] + n.d.	[p.A83Vfs82X] + n.d.