CCDC103 mutations cause primary ciliary dyskinesia by disrupting assembly of ciliary dynein arms

Jennifer R Panizzi,1,2,17, Anita Becker-Heck,3,4,17, Victoria H Castleman,5 Dalal A Al-Mutairi,6,7, Yan Liu,1, Niki T Loges,4, Narendra Pathak,1,2, Christina Austin-Tse,6, Eamonn Sheridan,6, Miriam Schmidts,3, Heike Olbrich,4, Claudius Werner,4, Karsten Häfner,3, Nathan Hellman,1,2, Rahul Chodhari,9, Amar Gupta,1, Albrecht Kramer-Zucker,10, Felix Olale,11, Rebecca D Burdine,12, Alexander F Schier,13, Christopher O’Callaghan,14, Eddie M K Chung,9, Richard Reinhardt,15, Hannah M Mitchison,5, Stephen M King,16,18, Heymut Omran,18 & Iain A Drummond1,2,8,18

Cilia are essential for fertilization, respiratory clearance, cerebrospinal fluid circulation and establishing laterality.1 Cilia motility defects cause primary ciliary dyskinesia (PCD, MIM244400), a disorder affecting 1:15,000–30,000 births. Cilia motility requires the assembly of multisubunit dynein arms that drive ciliary bending.2 Despite progress in understanding the genetic basis of PCD, mutations remain to be identified for several PCD-linked loci3. Here we show that the zebrafish cilia paralysis mutant schmalhans (s mh/h222) encodes the coiled-coil domain containing 103 protein (Ccdc103), a fox1a-regulated gene product. Screening 146 unrelated PCD families identified individuals in six families with reduced outer dynein arms who carried mutations in Ccdc103. Dynein arm assembly in s mh mutant zebrafish was rescued by wild-type but not mutant human CCDC103. Chlamydomonas Ccdc103/Pr46b functions as a tightly bound, axoneme-associated protein. These results identify Ccdc103 as a dynein arm attachment factor that causes primary ciliary dyskinesia when mutated.

Zebrafish schmalhans (s mh/h222 hereafter referred to as s mh/h−/−) mutants2 have a curved body axis, randomized left-right asymmetry and pronephric kidney cysts (Fig. 1a,b and Supplementary Table 1), which are characteristic features of ciliopathies6. Electron microscopy of s mh/h−/+ cilia showed that, compared to wild-type cilia (Fig. 1c), s mh mutant cilia completely lacked both inner dynein arms (IDAs) and outer dynein arms (ODAs) (Fig. 1d and Supplementary Fig. 1), whereas cilia length in these mutants was not altered (Supplementary Fig. 2). High-speed microvideo and linescan analyses of wild-type zebrafish pronephric cilia (Fig. 1e and Supplementary Video 1) or olfactory placode multicilia (Supplementary Fig. 3 and Supplementary Video 2) revealed rhythmic cilia motility. However, consistent with dynein arm defects, s mh mutant cilia were completely paralyzed in the pronephros (Fig. 1f and Supplementary Video 3), the olfactory placode (Supplementary Fig. 3b,d and Supplementary Video 4) and the spinal canal (Supplementary Videos 5 and 6). We mapped the s mh/h−/− mutation to a ~2-Mb region of chromosome 3 and subsequently fine-mapped it to a 0.43-Mb region containing eight genes (Supplementary Fig. 4 and Supplementary Tables 2 and 3). Sequencing candidate genes in this interval identified a mutation of cytosine to thymine at nucleotide 79 in exon 1 of ccdc103 (previously called zgc:100838), generating a predicted glycine to stop alteration at amino acid 27 of the 247-amino-acid protein (Fig. 1g,h). Injection of embryos with Myc-tagged wild-type ccdc103 mRNA rescued the axis curvature, left-right asymmetry defects and kidney cyst phenotypes and also restored cilia motility in s mh mutants (Fig. 1i,j, Supplementary Video 7 and data not shown), confirming that ccdc103 was the s mh mutant gene. Mutant ccdc103 mRNA carrying the p.Gln27Stop Smh alteration not only failed to rescue but also increased the frequency of axis curvature defects, suggesting it may act as a dominant-negative alteration (Fig. 1k). Antisense morpholino knockdown of ccdc103 induced curved body axes, left-right asymmetry defects, hydrocephalus and kidney cysts (Supplementary Fig. 5a,b and Supplementary Table 4) and caused cilia paralysis (Supplementary Video 8), phenocopying

1Nephrology Division, Massachusetts General Hospital, Charlestown, Massachusetts, USA. 2Department of Medicine, Harvard Medical School, Boston, Massachusetts, USA. 3Department of Pediatrics, University Hospital Freiburg, Freiburg, Germany. 4Klinik und Poliklinik fuer Kinder und Jugendsmedizin Allgemeine Paediatric, Universitaetsklinikum Muenster, Muenster, Germany. 5Molecular Medicine Unit, University College London, Institute of Child Health, London, UK. 6Leeds Institute of Molecular Medicine, Wellcome Trust Brenner Building, St James’s University Hospital, Leeds, UK. 7Department of Pathology, Kuwait University, Faculty of Medicine, Kuwait University, Kuwait. 8Department of Genetics, Harvard Medical School, Boston, Massachusetts, USA. 9General and Adolescent Paediatrics Unit, University College London, Institute of Child Health, London, UK. 10Renal Division, University Hospital Freiburg, Freiburg, Germany. 11Skirball Institute of Biomolecular Medicine, New York University School of Medicine, New York, New York, USA. 12Department of Molecular Biology, Princeton University, Princeton, New Jersey, USA. 13Department of Molecular and Cellular Biology, Harvard University, Cambridge, Massachusetts, USA. 14Department of Infection, Immunity and Inflammation, University of Leicester, Leicester, UK. 15Genome Centre Cologne at Max Planck Institute for Plant Breeding Research, Kοln, Germany. 16Department of Molecular, Microbial and Structural Biology, University of Connecticut Health Center, Farmington, Connecticut, USA. 17These authors contributed equally to this work. 18These authors jointly directed this work. Correspondence should be addressed to I.A.D. (idrummon@receptor.mgh.harvard.edu), H. Omran (heymut.omran@ukmuenster.de) or S.M.K. (king@neuron.uochc.edu).

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the smh mutation. Together, these data establish ccdc103 as an essential gene for dynein arm assembly and cilia motility.

Whole-genome linkage analysis in a group of four consanguineous Pakistani PCD families from a UK-based genetic isolate\(^6\) with absent IDAs and ODAs identified a new PCD locus on chromosome 17q12-q22 containing the human ccdc103 ortholog, CCDC103. The maximum heterogeneity log\(_{10}\) odds score among these families was 4.8, with an \(\alpha\) value (proportion of linked families) of 1. The critical genetic interval spanned 14 cM (16 Mb) between the SNP markers rs1859212 and rs2045418 (Supplementary Fig. 6). Screening the affected individuals in the Pakistani PCD families as well as nine other affected Pakistani individuals with identified linkage to CCDC103 and 132 unrelated PCD families (54% of which had ODA defects and 47% of which had functional or other structural PCD defects) for mutations in CCDC103 identified ten individuals with PCD carrying DNA alterations in the CCDC103 (NM_213607) coding sequence (Fig. 2 and Supplementary Figs. 7 and 8). We found homozygous loss-of-function mutations in three Pakistani PCD families that showed linkage to chromosome 17 (UCL-120, OP-1192 and OP-1193) with six total affected individuals (c.383_384insG) that predicted a frame shift and premature termination of translation (p.Gly128fs25\(^*\)) (Fig. 2 and Supplementary Fig. 7). The three families originated from nearby regions in Pakistan, making it very probable that the mutations resulted from a common founder (ancestor). The consanguineous parents of families UCL-120 and OP-1193 were heterozygous for this truncating mutation (Fig. 2), which is consistent with autosomal recessive inheritance and homozygosity by descent. In two other chromosome 17–linked Pakistani families (Supplementary Fig. 8; UCL-143 and OP-1194) and a German family (Supplementary Fig. 8; OP-32), we identified in all four affected individuals (OP-31I1, OP-31I2, UCL-143I1 and OP-1194I1) a homozygous transversion (c.A461C) that converts a threonine to a proline at amino acid 154 (p.His154Pro) that cosegregated with the disease phenotype (Supplementary Fig. 8). Multiple sequence alignment (Supplementary Fig. 9) showed that the p.His154Pro exchange occurred in a region flanking a highly conserved domain of CCDC103. A Polyphen-2 software analysis generated a protein damage score of 0.972 for this mutation, which is highly suggestive of pathogenic relevance. The c.A461C variant was absent in 180 controls of European ancestry as well as in the dbSNP, 100 Genomes Project and National Heart, Lung, and Blood Institute (NHBLI)–Exome...
Sequencing Project (ESP) human polymorphism databases. Eight other UK-Pakistani families compatible with linkage to the CCDC103 locus (see Supplementary Fig. 6 for two of these families, UCL-130 and UCL-145) did not carry mutations in their CCDC103 coding sequences. In one other family, an affected child carried a heterozygous transversion (c.G31C), predicting an alanine to proline change (p.Ala11Pro), with no mutation on the other allele.

All six affected individuals carrying recessive truncating CCDC103 mutations had typical clinical findings for PCD (Supplementary Table 5) comprising recurrent upper and lower airway infections, sinusitis and documented lung damage (bronchiectasis), and two affected individuals had dextrocardia and one had situs inversus. In four other individuals with PCD, we identified the homozygous amino-acid exchange p.His154Pro. Two of these individuals had situs inversus totalis, one had situs inversus abdominalis, one had dextrocardia and one had normal organ situs. Our findings indicate that CCDC103 mutations cause PCD and the randomization of left-right body asymmetry, resembling findings in smh mutant zebrafish.

To test the pathogenic relevance of the identified CCDC103 variants (Fig. 3a), we assayed their function in zebrafish smh mRNA rescue experiments. Injection of Myc-tagged wild-type human CCDC103 mRNA completely rescued the smh mutant phenotype (Fig. 3b,c). mRNA encoding the CCDC103 p.Ala11Pro variant also completely rescued the smh mutant phenotype (Fig. 3b,c), confirming that the heterozygous p.Ala11Pro variant that we observed in one individual with PCD is a rare, nonpathogenic polymorphism. mRNAs encoding the p.His154Pro and p.Gly128fs25* variants did not fully rescue the smh mRNA encoding the rescuing CCDC103 p.Ala11Pro protein (M2; Fig. 3d), even when injected at tenfold higher doses than the initial dose used (350 pg; data not shown). Consistent with its ability to rescue smh mutant phenotypes, wild-type human CCDC103 mRNA also restored dynein arms to genotypically mutant axonomes (Fig. 3e).

We analyzed defects in ODA composition in human axonemes by immunofluorescence microscopy of respiratory cells. Cells of patient OP-1192I11 carrying the homozygous p.Gly128fs25* loss-of-function alteration showed a partial loss of ODA complexes along the ciliary axonomes (Fig. 4a–c), with some proximally localized type 1 ODA complex components, namely DNAH5 and DNAI2, still present in the ciliary axonomes. In contrast, DNAH5 and DNAI2 were absent from the distal ciliary axonomes, with DNAH5 accumulating in the perinuclear and apical cytoplasm (Fig. 4a). This is consistent with an absence of distally localized type 2 ODA complexes, which we confirmed by an absence of DNAH9 from the ciliary axonomes in mutant cilia (Fig. 4c). This defect is very similar to findings obtained in patients carrying mutations in the gene encoding the dynein arm assembly factor KTU. In addition, we analyzed respiratory cells from patient OP-1194II1 carrying the homozygous hypomorphic p.His154Pro variant. The mutant cells had normal localization of DNAH5, DNA12 and DNAH9 (data not shown). Notably, both individuals showed normal localization of the IDA component DNAI1 (data not shown); thus, at least parts of IDA components can be assembled in the ciliary axoneme. A transmission electron microscopy (TEM) analysis of all three affected individuals from the UCL-120 family harboring the homozygous CCDC103 p.Gly128fs25* loss-of-function alteration showed either a loss of or a strong reduction in ODAs (shown for UCL-120II2 and UCL-120II3; Fig. 4d), which is similar to the smh mutant phenotype (Fig. 1d). Variation in the degree of the ODA defects in the three affected siblings from family UCL-120 (Fig. 4d) may reflect the position (proximal compared to distal) of the cilia cross section, as some proximal ODA components can persist in CCDC103 p.Gly128fs25* mutant cilia (Fig. 4a,b).

We further analyzed the effects of CCDC103 mutations using high-speed videomicroscopy of respiratory cells obtained by nasal brushing biopsy. Normal respiratory cell cilia beat vigorously and coordinately in vivo and did not generate substantial amounts of protein (p.His154Pro (M1) and p.Gly128fs25*(M3); Fig. 3d), even when injected at tenfold higher doses than the initial dose used. (50% Mendelian ratio of affected homozygous mutants (yellow bar). Supplementary Video 9). Consistent with the severe outer dynein arm defects observed by electron microscopy (Fig. 4d), individuals carrying the homozygous p.Gly128fs25* alteration (for example,
The images on the right are differential interference contrast images corresponding to the antibody-labeled cells. As a control, we used axoneme-specific antibodies against acetylated α-tubulin (ace. α-tubulin) (a,c) or β-tubulin (b). Nuclei were stained with Hoechst 33342 (blue). (a) In respiratory epithelial cells from healthy probands (WT), DNAH5 (red) localizes along the entire length of the axonemae. In respiratory epithelial cells from patient OP-1192 carrying the CCDC103 loss-of-function mutation, DNAH5 (red) localization is restricted to the proximal part of the axoneme and shows mislocalization to the subapical cytoplasm and the perinuclear region (white arrowheads). (b) DNAI2 (green) is localized along the entire length of the axonemae of healthy probands (WT). In contrast, in respiratory cells of patient OP-1192, DNAI2 (green) localizes solely to the proximal ciliary axonemae (the white arrowhead denotes the cilia tips, which are devoid of DNA12 green fluorescence). (c) DNAH9 (red) localization is restricted to the distal ciliary axonemae of respiratory epithelial cells from healthy probands (WT) because it is only present in type 2 ODA complexes. In patient OP-1192, DNAH9 is completely missing (white arrowhead), which is consistent with the variable reduction of DNAH9 expression observed in all zebrafish cells bearing motile cilia (Supplementary Fig. 11) and was not expressed in foxj1a-deficient zebrafish embryos (Supplementary Fig. 11), indicating that ccdc103 expression is controlled coordinately with foxj1 motile cilia target genes6. Ccdc103 was expressed both in the cytoplasm and axonemae of zebrafish olfactory placode cells (Fig. 5a); we did not observe Ccdc103 staining in smh morpholino knockdown embryos (Supplementary Fig. 12). To gain insight into the function of Cdc103 in dynein arm assembly, we examined the Chlamydomonas ccdc103 ortholog, PR46b (Supplementary Fig. 9). Like zebrafish Cdc103, Pr46b (hereafter referred to as Ccdc103/Pr46b) was present in both flagella and cytoplasmic extracts and migrated as apparent monomers and dimers on SDS gels (Fig. 5b,c), similar to the CCDC103 protein.

OP-1193II1) had complete cilia paralysis (Supplementary Video 10). Individuals carrying the homozygous p.His154Pro alteration had either a reduced cilia beat amplitude (patient OP-32II1; Supplementary Video 11) or a loss of beat coordination and cilia paralysis (patient OP-32II2; compare with the controls shown in Supplementary Video 13), which is consistent with the variable reduction in electron microscopy ODA arm structure in these individuals, which ranged from apparently normal to complete ODA and IDA loss (patient UCL-143II1; Supplementary Fig. 10). The less severe phenotype seen in the affected children of family OP-32 is consistent with our functional assays in zebrafish that identified the p.His154Pro exchange as a hypomorphic mutation. The variable phenotype observed within the UCL-120 family and among the affected children of different families harboring identical mutations (UCL-143 and OP-32) may suggest a modulation of the phenotype by genetic modifiers, as is frequently observed in ciliopathies8.

ccdc103 mRNA was highly expressed in all zebrafish cells bearing motile cilia (Supplementary Fig. 11) and was not expressed in foxj1a-deficient zebrafish embryos (Supplementary Fig. 11), indicating that ccdc103 expression is controlled coordinately with foxj1 motile cilia target genes6. Cdc103 was expressed both in the cytoplasm and axonemae of zebrafish olfactory placode cells (Fig. 5a); we did not observe Ccdc103 staining in smh morpholino knockdown embryos (Supplementary Fig. 12). To gain insight into the function of Cdc103 in dynein arm assembly, we examined the Chlamydomonas ccdc103 ortholog, PR46b (Supplementary Fig. 9). Like zebrafish Cdc103, Pr46b (hereafter referred to as Ccdc103/Pr46b) was present in both flagella and cytoplasmic extracts and migrated as apparent monomers and dimers on SDS gels (Fig. 5b,c), similar to the CCDC103 protein.
expressed in zebrafish embryos (Fig. 3d). In isolated flagella, Ccdc103/Pr46b was tightly associated with axonemes even after an extraction with 0.6 M NaCl (Fig. 5b). Fractionation of *Chlamydomonas* flagella shows Ccdc103/Pr46b is present in flagella and remains tightly associated with axonemes after extraction with 0.6 M NaCl (asterisk). Ccdc103/Pr46b migrates both as a monomer (m) and a dimer (d). (c) Ccdc103/Pr46b monomers (m) and dimers (d) co-purify with dynein light chain 2 (LC2) in a high–molecular-weight fraction (440,000–2,000,000 Da, left) of *Chlamydomonas* cytoplasm and are also present in a lower–molecular-weight cytoplasmic fraction (<440,000 Da, right). (d) A circular dichroism (CD) spectroscopic analysis of recombinant Ccdc103/Pr46b reveals strong α-helical content and robust resistance to heat denaturation (T° C, temperature in °C). (e) Gel filtration of recombinant Ccdc103/Pr46b shows a mixture of dimer and monomer peaks. A single-gel lane (left) shows Ni²⁺ column eluate containing recombinant His10-tagged Ccdc103/Pr46b (His10-Ccdc103/Pr46b) in total eluate protein. Pooled fractions from a Superose 6 column (left chromatogram; black bar) fractionated on a Superdex 200 column (main chromatogram) revealed a mixture of monomer- and dimer-sized protein peaks. Western blotting for Ccdc103 confirmed Ccdc103 monomers and dimers in the Superdex 200 fractions (Supplementary Fig. 16). A280, absorbance at 280 nm. (f) Mutant Smh protein disrupts Ccdc103 dimer formation in vivo. Western blotting using e-Myc antisera to detect the expression of Myc-tagged full-length zebrafish ccdc103 mRNA (18 pg) when co-injected into wild-type embryos at increasing amounts (with the gradient indicated) of truncated Smh p.Gln27Stop protein. This analysis revealed Ccdc103 dimers when it was coexpressed with low amounts of Smh truncated protein (18 pg mRNA) but revealed primarily monomers when it was coexpressed with high amounts of Smh truncated protein (74 pg mRNA). Anti-α-tubulin was used as a loading control.

Figure 5 Ccdc103 homodimers assemble with dynein light chain 2 in the cytoplasm and bind tightly to cilia axonemes. (a) Ccdc103 (red) is expressed both in the cytoplasm (red arrowhead) and in cilia (labeled with anti-acetylated α-tubulin; green) of a 52 hpf zebrafish olfactory placode. Co-localization of Ccdc103 in cilia is marked with a yellow arrowhead. The dashed white line indicates the dimensions of a single olfactory placode multiciliated cell. Scale bar, 10 μm. See Supplementary Video 7 for a comparison to the live image of the olfactory placode cilia. Ccdc103 staining was not observed in *smh* morpholino knockdown embryos (Supplementary Fig. 11). (b) Fractionation of *Chlamydomonas* flagella shows Ccdc103/Pr46b is present in flagella and remains tightly associated with axonemes after extraction with 0.6 M NaCl (asterisk). Ccdc103/Pr46b migrates both as a monomer (m) and a dimer (d). (c) Ccdc103/Pr46b monomers (m) and dimers (d) co-purify with dynein light chain 2 (LC2) in a high–molecular-weight fraction (440,000–2,000,000 Da, left) of *Chlamydomonas* cytoplasm and are also present in a lower–molecular-weight cytoplasmic fraction (<440,000 Da, right). (d) A circular dichroism (CD) spectroscopic analysis of recombinant Ccdc103/Pr46b reveals strong α-helical content and robust resistance to heat denaturation (T° C, temperature in °C). (e) Gel filtration of recombinant Ccdc103/Pr46b shows a mixture of dimer and monomer peaks. A single-gel lane (left) shows Ni²⁺ column eluate containing recombinant His10-tagged Ccdc103/Pr46b (His10-Ccdc103/Pr46b) in total eluate protein. Pooled fractions from a Superose 6 column (left chromatogram; black bar) fractionated on a Superdex 200 column (main chromatogram) revealed a mixture of monomer- and dimer-sized protein peaks. Western blotting for Ccdc103 confirmed Ccdc103 monomers and dimers in the Superdex 200 fractions (Supplementary Fig. 16). A280, absorbance at 280 nm. (f) Mutant Smh protein disrupts Ccdc103 dimer formation in vivo. Western blotting using e-Myc antisera to detect the expression of Myc-tagged full-length zebrafish ccdc103 mRNA (18 pg) when co-injected into wild-type embryos at increasing amounts (with the gradient indicated) of truncated Smh p.Gln27Stop protein. This analysis revealed Ccdc103 dimers when it was coexpressed with low amounts of Smh truncated protein (18 pg mRNA) but revealed primarily monomers when it was coexpressed with high amounts of Smh truncated protein (74 pg mRNA). Anti-α-tubulin was used as a loading control.

An analysis of human CCDC103 expression in respiratory epithelial cells confirmed that CCDC103 is present as apparent monomers and dimers (Supplementary Fig. 13). The remarkable stability of the putative dimeric forms of Ccdc103 protein in heat-denatured SDS gel samples prompted us to further examine the properties of recombinant Ccdc103/Pr46b protein. A circular dichroism spectroscopic analysis of Ccdc103/Pr46b revealed a high α-helical content, and thermal titration (with a temperature range of 4–85 °C) showed that Ccdc103/Pr46b was remarkably thermo stable, as it was not fully denatured even at 85 °C (Fig. 5d). Furthermore, the helical content of Ccdc103/Pr46b was not diminished by addition of 1% SDS and heating at 100 °C for 2.5 min (Supplementary Fig. 14). A gel filtration of purified Ccdc103/Pr46b protein revealed peaks corresponding to a mixture of the monomeric and dimeric forms of Ccdc103/Pr46b (Fig. 5e and Supplementary Fig. 15), confirming that Ccdc103/Pr46b can homodimerize. Thus, the stability of Ccdc103 dimers during SDS gel electrophoresis is the result of the extraordinary thermal tolerance of the protein. We noted previously that overexpression of Smh mutant p.Gln27Stop Ccdc103 protein containing part of the N-terminal coiled-coil domain could have dominant effects in zebrafish embryos and induce ciliopathy phenotypes (Fig. 1k). To test whether Smh mutant Ccdc103 protein might exert these effects by interfering with wild-type Ccdc103 dimer formation, we coexpressed full-length Myc-tagged Ccdc103 and Smh N-terminal p.Gln27Stop mRNAs in zebrafish embryos. Increasing amounts of N-terminal p.Gln27Stop Ccdc103 reduced the Ccdc103 dimer-to-monomer ratio (Fig. 5f), linking Ccdc103 function to homodimer formation. Finally, Ccdc103/Pr46b protein remained stably associated with axonemes in mutants lacking other dynein arm components, including ODA docking complex proteins (Supplementary Fig. 16). This, along with its stable association with axonemes in a high salt environment (Fig. 5b), distinguishes Ccdc103 from the docking complex proteins and indicates that it is a core factor in the binding of dynein arms to cilia microtubules. Taken together, our findings show an essential role for Ccdc103 as a newly discovered coiled-coil–containing, *foxi1a*-regulated dynein arm anchoring factor that is required for cilia motility. Although the families examined in this work showed typical recessive PCD, the ciliopathy phenotypes induced by overexpression of the mutant protein encoded by the *ccdc103 schmallians* allele raise the possibility that dominant mutations could also contribute to PCD. Future studies should reveal how Ccdc103 assembles with other ODA proteins to ultimately anchor dynein motor complexes to cilia microtubules.

**URLs.** Polyphen-2, http://genetics.bwh.harvard.edu/pph2/; dbSNP, http://www.ncbi.nlm.nih.gov/projects/SNP/; 1000 Genomes Project human polymorphism database, http://www.1000genomes.org/; NHLBI-ESP human polymorphism database, http://evs.gs.washington.edu/EVS/.
METHODS
Methods and any associated references are available in the online version of the paper.

Accession codes. The zebrafish ccdc103 full-length wild-type gene coding sequence corresponds to GenBank record NM_001002753. Human CCDC103 corresponds to GenBank record NM_213607.

Note: Supplementary information is available in the online version of the paper.

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AUTHOR CONTRIBUTIONS
J.R.P., Y.L., C.A.-T., A.G., N.H., A.K.-Z., R.D.B., F.O., A.F.S., N.P. and I.A.D. performed genetic mapping and Ccdc103 characterization in zebrafish. A.B.-H., V.H.C., D.A.A.-M., N.T.L., E.S., M.S., H. Olbrich, C.W., K.H., R.C., C.O., E.M.K.C., R.R., H.M.M. and H. Omran conducted studies with human patient samples. S.M.K. performed Chlamydomonas studies. J.R.P., A.B.-H., S.M.K., H. Omran and I.A.D. prepared the manuscript.

COMPETING FINANCIAL INTERESTS
The authors declare no competing financial interests.

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ONLINE METHODS

Zebrafish maintenance, embryo generation and staging. Wild-type zebrafish of from the AB, AB/TU, TL and hybrid strains were maintained as previously described. Embryos were obtained from natural matings, kept at 28.5 °C in E3 solution with or without 0.003% 1-phenyl-2-thiourea (PTU, Sigma) to suppress pigmentation and staged according to morphology and/or age, as indicated.

Approval for animal studies was obtained from the Massachusetts General Hospital Subcommittee on Research Animal Care (OLAW Assurance number A3596-01). The protocol as submitted and reviewed conforms to the US Department of Agriculture Animal Welfare Act, PHS Policy on Humane Care and Use of Laboratory Animals, the ILAR Guide for the Care and Use of Laboratory Animals and other applicable laws and regulations.

Positional cloning of the mutant locus. A genomic DNA panel was isolated from 1,200 homozygous mutant zebrafish embryos of a heterozygous mating, along with genomic DNA from siblings, using lysis buffer (10 mM Tris-Cl, pH 8.3, 50 mM KCl, 0.3% Tween20, 0.3% Nonidet P40 and 0.5 µg/µl Proteinase K). A simple sequence repeat polymorphism analysis was conducted by PCR amplification of markers, followed by 6% polyacrylamide gel electrophoresis and staining with SYBR gold in 0.5× Tris/borate/EDTA. Zebrafish SNPs were identified using the Ensembl browser and were then PCR amplified and sequenced (Massachusetts General Hospital DNA Core).

Cloning of cdc3103. Total RNA was isolated from 48-h-old wild-type zebrafish embryos with TRIzol Reagent (Invitrogen) and used to produce complementary DNA with Superscript First-Strand Synthesis (Invitrogen). Using zf-cdc3103 reverse transcriptase primers (Supplementary Table 2), zebrafish cdc3103 was PCR amplified with Platinum Pfx DNA Polymerase (Invitrogen) to incorporate Gateway cloning sites. The complete coding sequence was cloned into the plasmid pDONR221 (Invitrogen) and was then pCS2+ vector-modified for Gateway cloning and verified by sequencing. N-terminal–tagged cdc3103 constructs were made by subcloning into Gateway-Myc-pCS2+ (gift from N. Lawson, University of Massachusetts, Worcester). Human CCDC103 was cloned in the same manner, except it was PCR amplified from Open Biosystems clone 5455678 (GenBank BC041060) using human CCDC103 reverse transcriptase primers (Supplementary Table 2). The zebrafish cdc3103 full-length wild-type gene coding sequence reported in this paper corresponds to the existing GenBank record NM_001002753.

For genotyping embryos, genomic DNA was isolated as described above with lysis buffer, and then a 436-bp fragment spanning the mutation point was PCR amplified with smsn genotyping primers (Supplementary Table 2). The fragment was digested with Rsal (New England Biolabs), a restriction site introduced by the smsn mutation. Mutant DNA was cut into fragments of 351 bp and 85 bp, which were separated by agarose gel electrophoresis. When necessary, these results were verified by DNA sequencing.

In situ hybridization. Sense and antisense probes for cdc3103 were made from a 1,329-bp pCRII construct containing three 5′ untranslated region sequence made by TOPO TA cloning (Invitrogen) after amplification from complementary DNA with zf-cdc3103 in situ primers (Supplementary Table 2). Probes were synthesized with digoxigenin NTPs (Roche) after template linearization with BamHI (sense) or XbaI (antisense) before RNA synthesis with T7 or SP6 RNA polymerases, respectively. Embryos were fixed at the indicated stages and processed essentially as previously described. Antisense probes for cardiac myosin light chain 2 (myl2)17, preproinsulin (insl)18, foxa3 (ref. 19) and ptx2 (ref. 20) were prepared as described previously.

RNA and morpholino injections. Capped sense RNA encoding Cdc3103 was synthesized with SP6 RNA polymerase (Ambion mMessage mMachine) after linearization of pCS2-cdc3103 with NotI. RNA was purified using G-50 Sephadex Quick Spin Columns (Roche) and diluted to the indicated concentrations with tissue-culture-grade distilled water. Microinjections were performed on one to four cell embryos using a NanoInet 2000 (World Precision Instruments, Inc.)

A translation-blocking morpholino oligonucleotide, MOASUR (Supplementary Table 2; Gene-Tools, LLC), targeted to the 5′ untranslated region, and a splice-blocking morpholino oligonucleotide, MOAUG (Supplementary Table 2; Gene-Tools, LLC), targeted to the exon 2–intron 2 boundary, were resuspended in diethylpyrocarbonate-treated water to stock concentrations of 20 µg/ml and then further diluted to the given concentrations with diethylpyrocarbonate-treated water before injection. foxa1a morpholino injections were performed using a cocktail of splice- and translation-blocking oligonucleotides, as previously described.

Protein expression and antibody production. The full-length zebrafish Cdc3103 coding region was subcloned and expressed using the Champion pET160 Gateway Expression System (Invitrogen). The protein was purified from cell-insoluble fractions after resuspension in 3 M NaSCN and then purified using HisTrap HP affinity columns (GE Healthcare) with imidazole gradient elution. Purified protein was dialyzed overnight into PBS and sent to Rockland Immunoclonicals for guinea pig polyclonal antibody production. Antibody was purified separately from the serum of two guinea pigs by affinity purification on a Cdc3103 column prepared using an AminoLink Plus Immobilization Kit (Thermo Scientific).

The residue 70–267 region of Chlamydomonas Cdc3103/Pr46b was obtained by PCR from the expressed sequence tag clone AVE626473 and subcloned into the pMAL-c2 vector (New England Biolabs). The complete fusion protein was used for the production of the rabbit antibody CT285 (Covance Immunology Services, Denver, PA). Specific antibody was obtained by blot purification against recombinant Cdc3103/Pr46b (residues 70–267).

Western blotting. Western blotting on embryonic extracts was performed as previously described using rabbit anti–c-Myc (RDI-CMYC1; RDI Division of Fitzgerald Industries) at 1:10,000 and goat anti–rabbit-HRP (111-035-144; Jackson ImmunoResearch) at 1:5,000, or using 1:2,000 mouse anti–α-tubulin (T5168; Sigma) and 1:4,000 goat anti–mouse-HRP (111-035-146; Jackson ImmunoResearch).

Immunofluorescence analyses. Zebrafish embryos were fixed with Dent’s fixative (20% DMSO and 80% methanol) overnight and then washed in PBS plus 0.1% Tween20 (PBST) before incubation in blocking solution (5% goat serum, 5 mg/ml bovine serum albumin and 2% DMSO). Before blocking the embryos for anti-zebrafish Cdc3103 staining, they were incubated in 6 M urea and 0.1 M glycine at 4 °C for 10 min. After blocking, primary antibodies were added in block solution overnight at 4 °C. Guinea pig anti-zebrafish Cdc3103 was used at 1:100 dilution, and mouse antibody to acetylated α-tubulin (T6793; Sigma) was used at 1:1,000. Embryos were washed with PBST, and then secondary antibodies were added in block solution for 2 h at room temperature. CY3-conjugated anti–guinea pig secondary antibodies (706-165-148; Jackson ImmunoResearch Laboratories, Inc.) were used at 1:200, and Alexa488 or Alexa546 anti-mouse antibodies (A-11003, A21121; Invitrogen) were used at 1:1,000. After washing in PBST, embryos were analyzed by confocal microscopy (Zeiss LSM 5 Pascal) using a 63× oil immersion lens. Confocal stacks were deconvolved using Huygens Universal software and rotated to the desired position before acquiring single-frame images.

Human 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 milk before incubation with primary (at least 3 h at room temperature or overnight at 4 °C) and secondary antibodies (30 min at room temperature). Controls were produced by omitting the primary antibodies. Monoclonal anti-DNAL1 and monoclonal anti-DNAH5 were reported previously. Polyclonal rabbit anti–α-β-tubulin was from Cell Signaling Technology (USA). Highly cross-absorbed secondary antibodies (Alexa Fluor 488 and 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.

Subjects and families. Signed and informed consent was obtained from individuals fulfilling the diagnostic criteria of PCD and their family members using protocols approved by the Institutional Ethics Review Board at the University of Freiburg, University College London Hospital National Health
The coiled-coil domain containing protein CCDC40 is mislocalization of DNAH5 and DNAH9 in respiratory cells fractionation.

Cells were harvested by centrifugation and deflagellated using dibucaine, and flagella were isolated by standard methods. Cells were disrupted using an Emusiflex-C3 cell disruptor, clarified by centrifugation and concentrated using an Amicon Ultra 4 ultrafiltration unit (with a 10,000 Da cutoff). Extract was fractionated in a Superose 6 10/300 GL column equilibrated with 20 mM Tris.Cl, pH 8.0, 0.5 mM EDTA, 5 mM MgSO4 and 150 mM NaCl. The column was calibrated with blue dextran (>2 × 106 Da) and ferritin (440,000 Da), and 0.5-ml fractions were collected. Samples were electrophoresed in 10% SDS polyacrylamide gels and either stained with Coomassie blue or blotted to nitrocellulose and probed with antibody CT285. Immune reactive bands were detected by chemiluminescence using an ImageQuant LAS4000 digital imaging system.

Recombinant protein analysis. Soluble recombinant His10-tagged Ccdc103/Pr46b was purified by nickel chromatography. After removal of the tag by cleavage with Factor Xa, the protein was analyzed by gel filtration using Superose 6 and Superdex 200 columns. Circular dichroism spectroscopy was performed using a Jasco J-715 spectropolarimeter.

Human linkage analysis. For the whole-genome linkage analysis, family samples were analyzed using the Illumina SNP-based Linkage IVb Panel consisting of 6,008 evenly spaced SNPs with an average density of 0.64 cM. The standard parametric multipoint linkage analysis was carried out using MERLIN 1.0.1 software assuming autosomal recessive inheritance, a disease allele frequency of 0.007 and a disease penetrance of 0.9.

TEM. Zebrafish embryos were prepared for TEM using previously published protocols. Human biopsies were prepared for TEM as previously reported.

High-speed video analyses of ciliary beat. For human cells, ciliary beat was assessed with the Sisson-Ammons Video Analysis (SAVA) system. Transnasal brush biopsies were rinsed in cell culture medium and immediately viewed on a Nikon Eclipse E800 with DragonFly Express high-speed camera (Point Grey Research, Inc.) and processed as described for viewing at 15 fps.

Chlamydomonas strains. Strains used in this study were obtained from the Chlamydomonas Center and grown as described.

Chlamydomonas fractionation. Cells were harvested by centrifugation and deflagellated using dibucaine, and flagella were isolated by standard methods. Cell bodies were disrupted using an Emusiflex-C3 cell disruptor, clarified by centrifugation and concentrated using an Amicon Ultra 4 ultrafiltration unit (with a 10,000 Da cutoff). Extract was fractionated in a Superose 6 10/300 GL column equilibrated with 20 mM Tris.Cl, pH 8.0, 0.5 mM EDTA, 5 mM MgSO4 and 150 mM NaCl. The column was calibrated with blue dextran (>2 × 106 Da) and ferritin (440,000 Da), and 0.5-ml fractions were collected. Samples were electrophoresed in 10% SDS polyacrylamide gels and either stained with Coomassie blue or blotted to nitrocellulose and probed with antibody CT285. Immune reactive bands were detected by chemiluminescence using an ImageQuant LAS4000 digital imaging system.