Mutations in radial spoke head protein genes *RSPH9* and *RSPH4A* cause primary ciliary dyskinesia with central microtubular pair abnormalities

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Abstract

Primary Ciliary Dyskinesia is a genetically heterogeneous inherited disorder arising from dysmotility of motile cilia and sperm. This is associated with a variety of ultrastructural defects of the cilia/sperm axoneme which affect their movement, leading to clinical consequences on respiratory tract mucociliary clearance and lung function, fertility, and left-right body axis determination. We performed whole genome SNP-based linkage analysis in seven consanguineous families with Primary Ciliary Dyskinesia and central microtubular pair abnormalities. This identified two loci, in two families with intermittent absence of the central pair structure (chromosome 6p21.1, Zmax = 6.7), and five families with complete absence of the central pair (chromosome 6q22.1, Zmax 7.0). Mutations were subsequently identified in two positional candidate genes, RSPH9 on chromosome 6p21.1 and RSPH4A on chromosome 6q22.1. Haplotype analysis identified a common ancestral founder effect RSPH4A mutation present in UK-Pakistani pedigrees. RSPH9 and RSPH4A both encode protein components of the axonemal radial spoke head. In situ hybridisation of murine Rsph9 shows gene expression restricted to regions containing motile cilia. Investigation of the effect of knockdown or mutations of RSPH9 orthologs in zebrafish and Chlamydomonas indicate that radial spoke head proteins are important in maintaining normal movement in 9+2 motile cilia and flagella. This effect is rescued by re-introduction of gene expression to restore a normal beat pattern in zebrafish. Disturbance in function of these genes was not associated with defects in left-right axis determination in humans or zebrafish.
Introduction

Primary Ciliary Dyskinesia (PCD; MIM 242650) refers to a heterogeneous group of genetic ciliopathies characterised by ultrastructural defects in the axonemal structure of ‘9+2’ motile cilia and sperm flagella. The incidence is estimated at 1:15-30,000, with higher incidence in certain consanguineous and isolated populations. Clinical features reflect the distribution of dysmotile cilia in the body and include neonatal respiratory distress, chronic respiratory infections, sinusitis and bronchiectasis, due to deficient cilia function in the upper and lower airways. Male and female subfertility occurs due to defective sperm flagella and oviduct cilia, respectively. There is occasional hydrocephalus due to deficient ependymal cilia. In most families there is apparent randomisation of left-right axis development with about half of patients manifesting as situs inversus or more severe laterality defects such as cardiovascular abnormalities, proposed to result from defective function of embryonic nodal cilia.

PCD is usually recessively inherited and five PCD genes have been identified: DNAI1 (MIM 604366), DNAH5 (MIM 603335), DNAH11 (MIM 603339), DNAI2 (MIM 605483) and TXNDC3 (MIM 607421) (reviewed in 10). These all encode axonemal dyneins and are associated with reduction or loss of axonemal outer dynein arms, which are the multisubunit axonemal ATPase complexes that generate the force for cilia motility and govern beat frequency. DNAH5 and DNAI1 are a relatively more common cause of disease, underlying an estimated 28% (DNAH5) and 2-10% (DNAI1) of total cases. The other genes are so far only associated with single or rare PCD cases, and therefore the genetic basis of at least 60% of PCD cases is not yet known. Several additional PCD loci have been mapped, including on chromosome 19q13, 16p12.3,
15q13-q15 \(^3\) and 15q24-q25 \(^{15}\), but these genes remain to be isolated (reviewed in \(^{16}\)).

The possibility of involvement of PCD genes in sensory and retinal cilia functions is suggested by reports of syndromic forms similar to PCD which have motile cilia dysfunction and additional features including retinitis pigmentosa \(^{17}\), polycystic kidney disease \(^{18}\) and mental retardation \(^{19}\).

Patients with ultrastructural abnormalities affecting the central microtubular pair represent a well recognised sub-group in whom laterality defects have not been observed \(^{20}\). This is assumed to reflect the 9+0 ultrastructure of nodal cilia which may be unchanged by mutations affecting a structural element which they do not possess (in most species). Using two distinct family groups affected by PCD associated with central microtubular pair defects, we performed SNP-based whole genome linkage analysis to identify the disease-causing genes.
Subjects and Methods

Families, clinical information and controls

Informed consent was obtained from patients and family members in accordance with protocols approved by the University College London Hospital NHS Trust ethical committee and collaborating institutions. The diagnosis of PCD was based on exclusion of CF, immunodeficiency and TB and presentation of classic clinical features. These included some or all of reduced exercise tolerance, chronic wet cough, recurrent respiratory infections, nasal symptoms with rhinorrhea, rhinitis, nasal blockage, sinusitis, in addition to glue ear and consequent hearing problems. Bronchiectasis also occurred. Low weight and short stature were noted in the Bedouin and Pakistani families.

The pedigrees are presented in Figure 1A. Family UCL146 from the United Arab Emirates (UAE) was previously reported, showing one patient with a collapsed lower left pulmonary lobe. They originate from the Bedouin Bani Tameem tribe and the parents are first cousins. There is an unusual intermittent loss of the central pair in this family which has been confirmed by longitudinal section electron microscopy in all three affected children, such that cilia cross sections show a proportion with 9+0 structure. Family UCL152 from Israel are also Bedouin, with multiple consanguineous unions within the extended pedigree. Transmission electron microscopy (TEM) performed on one affected showed a normal axoneme ultrastructure. Family UCL152 were included in the study despite an apparently normal ultrastructure because of respiratory symptoms consistent with a diagnosis of PCD as described above, and dysmotility of
the respiratory cilia as described below. Furthermore, dysmotile sperm in two males was also reported, and one female required IVF treatment due to sub fertility.

Family UCL105 is a non-consanguineous UK-Northern European family. Families UCL131, UCL132, UCL138 and UCL170 are all of Pakistani origin, now resident in the North of England. The parents in all except UCL132 are first cousins, any consanguinity in family UCL132 was not known to the family. TEM in affected children from all these five families show a classic ciliary transposition defect indicative of complete central pair loss, as described in 20.

Where available, cilia motility studies on nasal biopsy samples collected from affected patients in all these seven families showed an abnormal circular movement but with a close to normal beat velocity, except for UCL152 which was recorded as having ‘abnormal motility’. Visualising a circular pattern is dependent on viewing at the correct orientation, and this can account for some variability in recording.

Control DNAs consisted of UK-Northern European samples from panels 1-2 of the ECACC Human Random Control Collection; anonymized UK-West Midlands Pakistani individuals (UK-Pakistani); Bedouin samples purchased from the National Laboratory for the Genetics of Israeli populations; unrelated UAE males previously reported 21; and additionally unrelated members of other pedigrees (13 Pakistani and 26 Arabic) collected for mapping/polymorphism studies.

**Linkage analysis and autozygosity mapping**
Genome wide linkage was performed in all families using the 0.64 cM density Illumina Linkage IVb 6008 SNP panel and additionally just in family 152, using the 8 cM density deCODE 500 microsatellite panel. SNP genotyping was performed at the Turku Centre for Biotechnology, Finland or at deCODE Genetics, Reykjavik, Iceland. Multipoint linkage analysis using MERLIN 1.0.1 (within easyLINKAGE) or GENEHUNTER 2.1r5 was performed under the assumption of autosomal recessive inheritance, 0.9 penetrance, 0.007 disease allele frequency, 0.00001 phenocopy rate, with allele frequencies set according to Illumina and deCODE map information.

Candidate gene identification and sequence and protein analysis

All known and predicted genes were identified at the RSPH9 locus using the UCSC, NCBI and Ensembl genome browsers, then prioritised for sequencing on the assumption that PCD genes should be conserved in distantly related ciliated organisms but absent in non-ciliated organisms. To identify this class of gene, a protocol of serial BLAST searches was developed to interrogate draft genome sequences of twelve organisms, three non-ciliated (A.thaliana, S.pombe, S.cerevisiae) and nine ciliated (C.reinhardtii genome, proteome and deflagellation dataset, T. brucei proteome, T. brucei/L.major combined genomes, C. intestinalis genome, T. thermophilia genome, C. elegans proteome (sensory cilia only), plus a Foxj1 mouse ciliogenesis microarray of genes down-regulated in this cilia-less mouse (Dr. S Brody, Washington University, unpublished). The process of database formatting, local BLAST searching and data parsing was automated using the custom perl script, blast2matrix (available from R.D. Emes on request, r.d.emes@hfac.keele.ac.uk). For this experiment significant alignment results were determined using an E value of $\leq 10^{-5}$ cut-off. The
C6orf206/RSPH9 protein was conserved in 8 ciliate databases but not *C.elegans* (sensory cilia only) nor the 3 non-ciliate databases.

Three prioritized positional candidate genes on chromosome 6p21.1 *TBCC* (MIM 602971), *KNSL8* and *C6orf206* (initially annotated as a mitochondrial ribosomal gene), in addition to the *RSPH4A* gene on chromosome 6q22.1, were sequenced using genomic DNA from linked patients and primers designed to span the coding exons and splice sites. Primer sequences for *RSPH9* and *RSPH4A* are shown in Supplementary Table 1 and all other primer sequences are available on request. Sequence alignments to analyse conservation of mutated residues were generated using ClustalW and Boxshade. Protein domain analysis was conducted using SMART, PFAM, Genthreader and Scansite prediction tools.

Restriction digest tests (*RSPH9* p.Lys268del, MboII (shown in Supplementary Figure 3); *RSPH4A* p.Gln154X, BsmAI; p.Pro87Ser, SacII; p.Gln109X, BspCNI) or sequencing (*RSPH4A* p.Arg490X) confirmed the inheritance pattern of all mutations in all extended kindreds.

**In situ hybridization**

The *Rsp9* probe corresponded to nucleotide 317 in exon 2 to 837 in exon 5, amplified from mouse Riken cDNA clone 1700027N10 (RZPD German Resource Centre) using primers 5’-TGGTGAGTGGCCGTTTCAT-3’ and 5’-CCATGTTCTTCTCTCTGTGC-3’, and the product was cloned into pCR4 by TOPO-TA cloning (Invitrogen). The mouse *Dnah5* probe in pBluescript SKII+ was kindly provided by Prof. H. Omran, University
Hospital Freiburg. Sense and antisense digoxygenin–labelled riboprobes were prepared using a digoxigenin RNA labelling kit (Roche), and purified on Chroma spin-100 columns (BD, Bioscience), prior to in situ hybridization on whole mount embryos or 7μm paraffin wax sections. Random-bred CD1 mice served as a source of mouse embryos and fetuses. Litters were generated by timed matings and the day of finding a copulation plug was designated embryonic day 0.5 (E0.5). Embryos were collected at E7.5, E18.5 and E19.5. Whole mount in situ hybridization was performed as described previously 31. In situ hybridization on sections was performed as described 32 and photographed using an MZFLIII, DC500 imaging system (Leica). Sense-strand controls yielded no specific hybridisation signal.

**Zebrafish morpholino injections**

Morpholinos were designed against the splice donor sites of zebrafish rsph9 exon 2 (MOex2: 5’- GGTGTAAGGCTTTTACCCTGACCTC -3’) and exon 3 (MOex3: 5’- GCTGTAAGTATACCTCCAAAGCTTC -3’) (GeneTools). 1-2 cell stage embryos were injected with 6.25 ng of morpholino in Danieau buffer (5mM HEPES pH7.6, 58mM NaCl, 0.7mM KCl, 0.4mM MgSO4, 0.6mM Ca(NO3)2). Green Fluorescent Protein (GFP) mRNA was co-injected to determine correct morpholino distribution. Siblings from the same pool as the MO-injected embryos served as negative controls. Effective doses were determined for each morpholino using a concentration where MO-injected embryos developed normally. Higher doses causing detectable detrimental effects on development were not pursued further.
Primers 5'-AGCGAATAGATCGAGATGGAC-3' and 5'-TGGAGATTGTGTCGCTGAAG-3' were used to confirm mis-splicing by RT-PCR of RNA isolated using TRIzol (Invitrogen) from 10 control or morpholino-injected embryos at 24 and 72 hours post-fertilisation (hpf). PCR products were gel purified (Invitrogen) and sequenced to reveal mis-splicing events.

For motility studies, 7-9 control and MO-injected embryos at 72 hours post-fertilisation (hpf) were analysed individually under glass coverslips, confirmed as alive by heart beat beforehand. Olfactory pit cilia were viewed under a water immersion objective lens (x50) on an inverted Nikon Diaphot microscope in a humidified (80 %) and temperature (28°C) controlled chamber. High-speed (500 frames per second (fps)) video sequences of the zebrafish olfactory pits were captured (Trouble-shooter 500, Lake Image systems, UK). The stored sequences were then replayed in slow motion (Midas 2.0 player, Xcitex) and the cilia beat frequency (CBF) was calculated as previously described \(^{33}\) using the following formula: frames per second (500)/ number of frames elapsed for 5 beat cycles X beat cycles counted (5) = CBF (Hz). The immotility (II) and dysmotility (DI) index were determined by counting the number of static (II) or dysmotile (abnormal movement and static together, DI) cilia as a percentage of the total number present in the video sequences. The means ± SEM were calculated for 7-9 embryos and data tested by one-way ANOVA with individual datasets compared using an unpaired Student’s t-test with Bonferroni correction for repeated measures.

Zebrafish phenotype rescue
Full-length mouse Rsph9 mRNA was subcloned into pβUT3 vector and mRNA was transcribed using the mMessage machine kit (Ambion) and T3 polymerase, then titrated to determine the maximum effective injection dose. One cell stage embryos were co-injected with 150 pg mRNA and 6.25 ng of morpholino using methods described above, and olfactory cilia movement was assessed at 72 hpf as described above. The controls were wildtype and morpholino-only treated embryos.

*Chlamydomonas* strains and maintenance

Wild-type (CC-1732 and cw15), *pf17* parental (CC-1035) and progeny (CC-1332, CC-1143, CC-2645 and CC-262) strains were obtained from the *Chlamydomonas* Genetics Center (http://www.chlamy.org/). Cells were grown and expanded using standard protocols in Tris–acetate phosphate (TAP) medium {Harris, 1989 #20} under light (~45 µE/m²/s) at 25°C.

Identification of *Chlamydomonas pf17* strain mutation

The precise genetic lesion in *Chlamydomonas* mutant strain *pf17* had not previously been identified. The five coding exons, intron-exon boundaries and 5’ and 3’ UTRs of *RSP9* were sequenced in genomic DNA isolated using a method adapted from {Goldschmidt-Clermont, 1990 #37} from wild-type Chlamydomonas and mutant *pf17*. This revealed a single base pair deletion in exon 2 (c.131delG) present in *pf17* but not wild type, predicting a premature stop codon after Ser45 in the 269 residue protein. This change abolishes a *BspEI* restriction site and we confirmed the mutation causes the paralysed flagella phenotype and had not arisen since original isolation of *pf17*, by
BspEI restriction digest of a 1.1 kb exon 2 PCR product amplified using primers 5’-CGCAGCTCACTTATCTCTTCT-3’ and 5’-AGCACACGCCTCATCCAATAG-3’, from genomic DNA isolated from wild-type, the original pf17 mutant strain CC-1035, and its four pf17 progeny strains CC-1332, CC-1143, CC-2645 and CC-262. The c.131delG mutation identified in mutant pf17 was present in the original pf17 and all progeny strains but not the wild-type control (data not shown).

Chlamydomonas RSP9 vectors

Vectors for transformation to create the pf17-T and pf17-Tmut strains were made as follows. The wild-type genomic RSP9 gene was amplified from genomic DNA using pFusion Taq (Invitrogen) and PCR primers P1 (5’-AGATTCCACACCTCACGGATAC-3’) and P2 (5’-ACCAGTCAAACTTTCGAACCAG-3’) to include the 5’ and 3’ UTR and approximately 400 bp of the upstream sequence incorporating the tub box sequence motif known to enhance transcription after deflagellation {Davies, 1994 #21}, and cloned into pBluescript SKII-. A mutated RSP9 was created by introducing a 3 bp deletion into this wild-type DNA construct to mimic the human c.801_803delGAA; p.Lys268del mutation. The resulting mutation in Chlamydomonas RSP9, a deletion of the homologous residue, is c.780-783delCGC; p.Arg261del (Supplementary Figure 2A). This was done using a primer with the relevant three bp. missing and a BmgBI restriction site at the 5’ end (P3: 5’-CGAGCTGACGTGGGGCGACGGCGGACGGGCTGAACACACC-3’), and a reverse primer (P4: 5’-GTATGTTGTGGTGAATTGAGCGG-3’) complementary to pBluescript, downstream of a unique NotI site. The pBluescript-wildtype RSP9 construct was amplified with P3-P4 and the product sub-cloned into
pCR4 by TOPO-TA cloning (Invitrogen) then excised by BmgBI and NotI double digest.

pBluescript-wildtype RSP9 was separately BmgBI-NotI double digested to eliminate the wild-type RSP9 insert, then these DNAs were ligated to create a pBluescript plasmid containing mutated RSP9.

**Chlamydomonas transformation**

Immotile *pf17* (CC-1035) mutant *Chlamydomonas* were transformed with the wild-type or mutated RSP9 vectors, using a modified protocol from {Kindle, 1990 #22}. *pf17* was first back-crossed with cell wall-less mutant *cw15*, for ease of transformation. Back-crossed *pf17* (and cell wall-less *cw15* control) cultures were then used. Cells were resuspended to 2x10⁸ cells/ml, 300 µl was transferred to 5 ml tubes containing ~0.3 g of 0.4 mm diameter washed glass beads (BDH). 1 µg plasmid DNA and 4 µg plasmid pSI103, which confers paramomycin antibiotic resistance for selection of transformant colonies, were added together, the mixture vortexed for 15 seconds and grown overnight with slow shaking to allow for recovery and gene expression. Cells were plated on TAP 2% agar supplemented with 20µg/ml paramomycin and inverted in the light at 22°C. Transformed cell wall-less *pf17* colonies were visible after 7 -10 days and then selected by viewing 100 colonies of each and monitoring for rescued motility. The *pf17* strain transformed with wild-type RSP9 was called *pf17*-T, and *pf17* transformed with mutant RSP9 was called *pf17*-Tmut.

The correct incorporation of wild-type and mutant RSP9 constructs into the genetic material of these transformed strains was confirmed in genomic DNA by restriction digest making use of the *BspEI* site destroyed by the original *pf17 RSP9* c.131delG
mutation. In addition, the c.780_783delCGC mutation mimicking that of human RSPH9 patients destroys an FspI site. BspEI and FspI digests showed control cw15 strain carried wild-type RSP9, pf17-T carried both wild-type and c.131delG RSP9. pf17-Tmut strain carried no wild-type RSP9, but carried c.131delG in addition to c.780_783delCGC mutated RSP9. Thus, the wild type and mutant RSP9 constructs had incorporated correctly into the genetic material of pf17–T and pf17–Tmut.

**Chlamydomonas motility analysis**

Cell wall-less cw15, pf17, pf17-T and pf17-Tmut were picked into TAP medium, grown over night and analysed under a water immersion objective lens (x50) on an inverted Nikon Diaphot microscope in a humidified (80 %) and temperature (30°C) controlled chamber. High-speed (500 fps) video sequences were captured (Trouble-shooter 500, Lake Image systems, UK) and the stored sequences replayed in slow motion (Midas 2.0 player, Xcitex) to measure flagella beat frequency (FBF) using the published formula \( \frac{\text{frames per second (500)}}{\text{number of frames elapsed for 5 beat cycles}} \times 5 = \text{FBF (Hz)} \). *Chlamydomonas* flagella do not beat with a planar motion as cilia do, but can change the beat direction to facilitate directional movement, and motility was too variable to calculate a meaningful dysmotility index as done for zebrafish, therefore only an immotility index was calculated, when the beat pattern was symmetrical. This was determined by counting the number of static flagella as a percentage of the total number present in the video sequences. The means ± SEM were calculated for 21 (cw15), 26 (pf17), 20 (pf17-T), 21 (pf17-Tmut) embryos for FBF and for 10 (cw15), 5 (pf17), 7 (pf17-T), 10 (pf17-Tmut) embryos for immotility index. Data were tested by one-way ANOVA and individual
datasets were compared using an unpaired Student’s t-test with Bonferroni correction for repeated measures.
Results

We undertook genome-wide linkage analysis and subsequent positional candidate gene analysis in seven PCD families with central microtubular pair defects, five of which were consanguineous: two Bedouin, four UK-Pakistani and one UK-Northern European (Figure 1A). No patients displayed laterality defects. For those in whom ciliary movement was studied, the ciliary beat frequency was within the normal range. However, the beat pattern was recorded as being circular rather than the normal forward and backward planar motion \(^ {33}\) for all seven families except one Bedouin family recorded as having ‘abnormal motility’ (detailed in Methods). One of the two Bedouin families UCL146 had an unusual intermittent absence of the central pair resulting in cilia cross sections with both a 9+2 and a 9+0 ultrastructure (\(^ {20}\) and Figure 1B), and the second family UCL152 had apparently normal ultrastructure. The other five families UCL105, UCL131, UCL132, UCL138 and UCL170 had a classic ‘transposition’ defect consisting of complete absence of the central pair, with ciliary transposition. As previously described \(^ {20}\), in this defect a proportion of cilia cross sections have an absent central pair (9+0) and a proportion have an 8+1 arrangement where the central pair is absent and one peripheral microtubule doublet with attached dynein arms is transposed to the centre (Figure 1B).

Regions of homozygosity identical-by-descent (IBD) shared amongst the affected individuals were identified in the two family groups. The full genome linkage scan results are shown in Supplementary Figure 1. For the two Bedouin families, a 4.8 Mb region in UCL146 between \(rs1738240\) and \(rs945131\) overlapped with a 10.8 Mb region in UCL152, between \(D6S291\) and \(D6S452\) (not shown). Higher resolution genotyping
using known or in-house designed microsatellites defined a common 1.9 Mb critical region of IBD between markers D6S400 and rs3734693 on chromosome 6p21.1, with a peak multipoint LOD score of 6.7 across D6S1604-D6S451 (Figure 1C). Across this 1.9 Mb IBD region the two families shared alleles at only two microsatellite markers located either side of RSPH9, such that any linkage disequilibrium between the families is small (0.6 Mb maximum) (not shown).

All 5 transposition families linked, using just Illumina data without additional genotyping, to a single 6.7 Mb region between rs2030926-rs937091 on chromosome 6q22.1, defined by recombination events in family UCL138 (Figure 1C, 1D). There was a peak multipoint LOD score of 7.0 across rs873460-rs941815. No other genomic region was significant for linkage. The four UK-Pakistani families shared an IBD region and a common 5.2 Mb haplotype across markers rs1158747-rs2243379, suggesting a founder effect. This was shared amongst all ten affected individuals including those of UCL132, a family which appears to have previously unknown consanguinity and ancestral sharing with the other three UK-Pakistani families (Figure 1D). The UK-Northern European family (UCL105) was consistent for linkage across 57 Mb (rs1014976-rs1385732) spanning this region, but had no significant marker homozygosity or allele sharing with the Pakistani families (not shown).

The linked region in the two Bedouin families harboured the positional candidate gene RSPH9. A homozygous 3 bp deletion (c.801_803delGAA) was identified in all seven affected Bedouin individuals, predicting in-frame loss of the C-terminal Lys268 (p.Lys268del) (Figure 2A, B). This residue is largely conserved across distant phyla (Supplementary Figure 2A). Screening of population-matched controls revealed that
this change was not carried on 126 Bedouin and Arabic control chromosomes. However, screening of chromosomes from a collection of 160 unrelated control UAE males \(^{21}\) showed that three individuals were heterozygous for this amino acid deletion, a result that was not regarded as surprising given the high incidence of consanguineous unions in the culturally isolated UAE population \(^{38}\). The overall frequency of this change in all the control chromosomes screened was 0.7%. These findings therefore suggest that RSPH9 mutations probably cause PCD.

The linked region in the five transposition defect families harboured the positional candidate gene RSPH4A, located also within the region spanned by the ancestral UK-Pakistani haplotype (Figure 1D). Four C to T transition sequence variants were identified (Figure 2A, B). All ten UK-Pakistani affected individuals were homozygous for a nonsense mutation, p.Gln154X (c.460C>T), accompanied by a second upstream missense variant, p.Pro87Ser (c.259C>T). Patients in the Northern European family UCL105 were compound heterozygotes for nonsense mutations p.Gln109X (c.325C>T) and p.Arg490X (c.1468C>T). Co-segregation of all mutations with the disease status in all extended kindreds was confirmed and was found to be in accordance with haplotypes (Supplementary Figure 3 and Methods). Screening of population-matched controls showed that they did not carry any of the RSPH4A mutations identified. These controls comprised 154 UK-Pakistani chromosomes screened for the p.Pro87Ser and p.Gln154X mutations; 170 and 354 UK-Northern European controls chromosomes respectively were also screened for these changes. In addition, 348 and 368 UK-Northern European chromosomes were screened for the p.Gln109X and p.Arg490X mutations, respectively.
RSPH9 and RSPH4A are both predicted to encode radial spoke head proteins (Figure 2C), based on homology with proteins of known function in the biflagellate alga Chlamydomonas reinhardtii and other ciliates. The RSPH9 protein was identified as 28% identical to biflagellate alga Chlamydomonas reinhardtii protein RSP9, radial spoke head 9. The RSPH4A protein was identified as 31% and 30% identical, respectively, to two similar Chlamydomonas radial spoke proteins, RSP4 and RSP6 (Supplementary Figure 2B).

Radial spokes are regularly spaced along cilia, sperm and flagella axonemes and have a multisubunit ‘stalk’ and ‘head’ which form a signal transduction scaffold between the central pair and dynein arms (Figure 2C). Available evidence suggests they regulate dynein-induced movement and govern cilia/flagella waveform. Central pair-radial spoke interactions determine bend direction and shape (waveform), while radial spoke-inner dynein arm interactions determine velocity.

Further investigations of orthologs of RSPH9 were undertaken in three model organisms: the mouse, zebrafish (Danio rerio) and Chlamydomonas, to further determine the pathogenic potential of the in-frame Lys268del change. We determined the tissue distribution of Rsph9 gene expression in mouse. In situ hybridisation showed specific expression in nasal, lung, trachea and brain ventricle epithelium at E18.5-19.5 and at the embryonic node at E7.5. This was a similar pattern to Dnah5, restricted to regions where motile cilia are located (Figure 3).

We investigated rsph9 knockdown in zebrafish. Two different rsph9 splice site morpholinos directed against the exon 2 (MOex2) and exon 3 (MOex3) splice donor
sites were used to disrupt gene expression (Supplementary Figure 4). Both gave a
dose-dependent phenotype of dysmotile olfactory pit cilia with a normal beat frequency
but an ineffective circular beat pattern (66% cilia dysmotile) (Figure 4A-C and
Supplementary movies S1-3). Statistical comparison between datasets indicated the
immotility and dysmotility indexes for both sets of MO-injected embryos were
significantly higher than observed for controls (p<0.001). Morphants had a more static
fluid flow than wild-types and this allowed debris to accumulate in the pits (Figure 4B
and Supplementary movies S1-3). Their beat pattern resembled that of cilia in RSPH
patients, confirming that ablating gene expression causes similar cilia dysmotility
defects. This effect was rescued by coinjection of mouse Rsph9 mRNA, which restored
the normal beat pattern (Figure 4A, B).

Comparison at 24 and 48 hours post-fertilisation between 163 exon 2 and 163 exon 3
morphant zebrafish and 270 wild-type zebrafish showed that laterality was unaffected.
Three of the exon 3 morphants, none of the exon 2 morphants and one wild-type fish
displayed situs inversus which was a non-significant difference. Situs inversus
presents occasionally as a well recognised ‘background’ zebrafish phenotype (L.R and
S.W.W., unpublished data).

The Chlamydomonas mutant strain pf17 has a mutation in RSP9, the ortholog of
human RSPH9, resulting in immotile flagella. The entire radial spoke head complex is
absent, and there is central pair displacement rather than loss. We first determined
that pf17 carries a single base pair deletion c.131delG in RSP9, predicting an early
premature stop codon (p.Ser45AlafsX3). We then used the presumed Rsp9-null
background of pf17 strain to investigate the effects of the human RSPH9 p.Lys268del
mutation. We stably transformed *pf17* with the wildtype *RSP9* gene to create strain *pf17-T*. *pf17-T* regained a normal beat velocity and pattern, indicating complete phenotype rescue (Figure 4C and Supplementary movies 4-7). We then stably transformed *pf17* with a mutated version of *RSP9* carrying the equivalent of the 3 base-pair p.Lys268del deletion to create strain *pf17-Tmut*. Statistical comparison between datasets indicated the FBF for *pf17-Tmut* was significantly reduced and the immotility index significantly increased compared to controls (p<0.001). Therefore *pf17-Tmut* showed only partial rescue, with a beat velocity at half the wild-type level and flagella either immotile (73% immotile) or displaying a slowed, disorganised beat ineffective for normal movement (Figure 4C and Supplementary movies 4-7). Flagella-cilia movement differences preclude rigorous comparisons of beat pattern. Thus, recreation of the human *RSPH9* mutation in *Chlamydomonas* provides direct evidence of its pathogenic effect on motility of cilia and flagella.
Discussion

We have identified mutations in two genes encoding radial spoke head proteins, *RSPH9* and *RSPH4A*, in PCD families that have defects of the central microtubular pair. This is the first report of PCD genes that cause disease associated with cilia axoneme defects other than a loss or reduction of the outer dynein arms. Using model organisms we have also shown that *Rsph9* is expressed in ciliated epithelia, and that gene knockdown and mimicking of the human *RSPH9* p.Lys268del mutation recapitulates its detrimental effect on cilia motility.

Analysis of *Chlamydomonas RSP9* mutant strains indicates that the human *RSPH9* p.Lys268del mutation is likely to be hypomorphic since some flagella function is retained in these mutants, in contrast to those with a null allele. *RSPH9* residue Lys268 is conserved in all mammals but not in some of the non-vertebrates and ciliates tested. This could reflect a functional distinction amongst the cilia of different species, or it may be that the loss rather than specific chemical properties of this amino acid are what confer the disease-causing effect. Although the p.Lys268del mutation was detected on 0.7% of control chromosomes it was never present in homozygous state in the control individuals, whereas sequencing of the entire open reading frame in affected patients showed they were all homozygous for this single mutation. The population frequency of the mutant allele is not significantly different from what is expected of a pathogenic recessive mutation. Additionally, the majority of control chromosomes were sampled from the highly consanguineous UAE population which one family carrying the mutation (UCL146) originates from. These observations support that the p.Lys268del mutation is disease-causing.
We expect that the three premature nonsense mutations we have identified in \textit{RSPH4A} are more likely to be null alleles since they are predicted to result in premature protein truncation, but this requires further functional work for confirmation. These \textit{RSPH4A} nonsense mutations predict a disruption of the ‘radial spoke domain’ (Supplementary Figure 2B). No other functional domains could be identified by computer modelling in either of the two radial spoke head proteins, although \textit{RSPH4A} is noted a proline-rich \cite{44}, thereby preventing significant genotype-phenotype predictions.

Determination of ultrastructural changes in the axoneme is limited by methodological constraints. In particular, observations reflect a sampling of total tissue such that local changes in ciliary structure may be missed. The structural changes observed appear to vary between species, organelle type, protein involved and specific mutation. Truncation mutations in \textit{RSPH4A} in human motile cilia are associated with a loss of the central pair, yet for an \textit{RSP9} truncation mutation in \textit{Chlamydomonas pf17} the central pair is retained, but displaced. The different contribution of \textit{RSPH4A} and \textit{RSPH9} proteins to the spoke head structure is not clear. Furthermore it is not yet known whether differences in central pair structural constraints and waveform between cilia and flagella could explain the disparity, or whether the human patients may have more intact and functionally preserved radial spoke heads than the \textit{Chlamydomonas} mutants. In humans with a single amino-acid deletion in \textit{RSPH9} the structural consequences are distinct with only a localised loss of the central pair microtubules, perhaps reflecting the milder mutation. Of the two families with the \textit{RSPH9} p.Lys268del mutation, in family UCL152 a normal central pair ultrastructure was in fact recorded, but in view of the shared mutation in common with UCL146, it seems likely that the minor
central pair defect observed in UCL146 could have missed detection in UCL152 without
the more detailed sampling undertaken in UCL146 which included generation of
longitudinal sections

The functional consequences we have observed to arise from RSPH9 and RSPH4A
defects in patients and model organisms support a more significant role for radial
spokes and central pair microtubules in determining cilia beat waveform rather than
velocity, although velocity may also be affected. This is consistent with the retention of
the force-generating dynein arms that govern velocity that is observed in the cilia
axonemes of central pair defect patients. The natural movement of 9+2 motile cilia and
9+0 nodal cilia differs: the 9+0 cilium has a circular motion, rather than the planar
‘whiplash’ movement with effective and recovery strokes of 9+2 cilia. In zebrafish
and RSPH9 and RSPH4A patients, altered function of the radial spoke heads caused
9+2 cilia motility to resemble this simpler rotary 9+0 cilia movement. This is consistent
with previous evidence that a disconnection of radial spoke head-central pair
interactions in 9+2 cilia would lead to a change from the normal planar motion, to
abnormal movement. For example, an antibody to sea urchin RSPH4A was
previously shown to affect sperm flagella beat pattern but not velocity, changing the
movement from planar to circular.

The normal laterality observed in RSPH9 and RSPH4A patients and rsph9 zebrafish
morphants is consistent with the notion that radial spoke proteins are not essential for
nodal ciliary function. Their role in waveform appears more important in central pair-
containing 9+2 cilia. Our observation from in situ hybridisation that Rsph9 is expressed
in mouse nodal cilia (which lack a central pair) suggests either redundancy or an
alternative, perhaps structural, role. The precise function of the radial spoke head proteins at the embryonic node is likely to be relevant to the molecular basis of the difference in 9+0 node cilia and 9+2 motile cilia waveforms and remains of considerable interest. However many unresolved questions remain concerning the correlation of structure and function in different cilia types.

In summary, our observations provide new insights into the role of radial spoke head proteins in the structure and function of cilia and flagella, and the molecular genetic basis of primary ciliary dyskinesia. RSPH9 and RSPH4A represent good candidate disease-causing genes for cases of PCD with central pair defects, and also cases where the axonemal dynein arms are retained and the patients do not display laterality defects. Characterisation of RSPH9 and RSPH4A and continuing elucidation of the molecular basis of PCD provides new opportunities for non-invasive diagnosis and the possibility of new therapies in what we have shown is, at least in some model organisms, a reversible (rescuable) molecular defect.
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Web resources

References


**Figure 1. Central pair agenesis in PCD patients and linkage analysis**

(A) PCD central pair defect pedigrees. Black indicates affected, double line indicates consanguineous union with a dashed upper line if the exact degree of relatedness is unknown, arrow indicates proband.

(B) Transmission electron micrographs of nasal ciliary epithelium from individuals with a central pair defect. Left panel, Bedouin UCL146 IV:1 longitudinal section with intermittent central pair loss, confirming the previous report of 9+2 (normal) and 9+0 cross-sections. Right panels, cross-sections from UK-Pakistani UCL170 IV:1 indicative of complete central pair loss (transposition defect), showing 9+2 (upper panel), 9+0 (middle panel) or 8+1 (lower panel) ultrastructure.

(C) Linkage mapping of central pair defect families to two loci on chromosome 6p21.1 and 6q22.1. Upper panel, multipoint MERLIN linkage analysis of UCL146 and UCL152 on 6p21.1 across $D6S291-D6S1638$, using information from Illumina and deCODE scans and in-house microsatellite genotyping. The overlapping shared region of IBD between $D6S400-rs3734693$ is reflected by a significant multipoint lodscore $>3$, which rises to a peak of 6.7 across $D6S1604-D6S451$. The location of $RSPH9$ is shown. Lower panel, multipoint GENEHUNTER linkage analysis of transposition defect families on 6q22.1 across chromosome 6, using Illumina scan information. The location of $RSPH4A$ is shown, located centromeric to the peak HLOD score of 7.0 which was generated across markers $rs873460-rs941815$.

(D) Homozygosity identical-by-descent and founder effect in $RSPH4A$ families. Chromosome 6q22.1 disease chromosome haplotypes for each of the four UK-Pakistani families are shown, displaying extended homozygosity across the $RSPH4A$
locus. Bold black lines and boxes indicate the linked region in each pedigree, defined by recombination events or loss-of-homozygosity. The minimal critical region containing \textit{RSPH4A} is \textit{rs2030926-rs937091} (defined by recombinations in UCL138). Homozygous allele sharing occurs amongst affecteds (grey shading), with a putative minimal common ancestral haplotype spanning the \textit{RSPH4A} gene, across markers \textit{rs1158747-rs2243379} (light grey).

**Figure 2. RSPH9 and RSPH4A mutations**

(A) Chromosome 6 location of \textit{RSPH9} (left) and \textit{RSPH4A} (right), their derived proteins and mutations.

(B) Electropherograms indicate the normal sequence (top traces) and mutations (bottom traces).

(C) Cilia axoneme model with putative RSPH9 and RSPH4A location, based on \textit{Chlamydomonas} homology. CP, central microtubular pair; RS, radial spoke; IDA, inner dynein arm; MT, microtubule.

**Figure 3. Expression of Rsph9 in the node and ciliated epithelia**

Whole mount \textit{in situ} hybridisation of \textit{Rsph9} reveals specific expression in the node (No) of E7.5 mouse embryos (arrow); side (A) and anterior (B) views. Expression of \textit{Rsph9} (C, E, G, I, K) and \textit{Dnah5} (D, F, H, J, L) detected by \textit{in situ} hybridisation on sagittal (C-F and I-L) and coronal (G-H) sections at E18.5 (G-H) and E19.5 (C-F and I-L). Similar expression of both genes was detected in the epithelia lining the trachea (T) (arrowheads in C-D), bronchi (asterisks in E-F) and the nasopharynx (Np) and neuroepithelium of the lateral ventricles (VN) (G, H). Expression was also prominent in
the olfactory epithelium (I-J, magnified in K-L). L, lung; LV, lateral ventricle; NE, nasal epithelium; 3V, third ventricle. Scale bar, 500μm.

Figure 4. Loss of zebrafish and *Chlamydomonas RSPH9* gene function causing cilia/flagella dysmotility

(A) Olfactory pit cilia movement in wild-type (WT) zebrafish and rsph9 morphants. Top panel, 18.2% (Moex2), 22.2% (MOex3) and 0.8% (MOex2 + rsph9 mRNA) of cilia in morphants were immotile (1.4% in wild-type). Middle panel, 65.9% (MOex2), 65.6% (MOex3) and 7% (MOex2 + rsph9 mRNA) of cilia were dysmotile (3.1% in wild-type), this number including immotile in addition to dysmotile i.e. displaying an ineffective circular beat pattern. Bottom panel, cilia beat frequency was unaffected at 42.3 (MOex2), 40.0 (MOex3) and 39.8 (MOex2 + rsph9 mRNA) Hz (wild-type 43.6 Hz). Means±SEM from 7 wild-type, 8 MOex2, 9 MOex3 and 5 MOex2 plus co-injected rsph9 mRNA embryos shown, *** p<0.001.

(B) Accumulation of debris in rsph9 zebrafish morphant olfactory pit. Top panel, 72 hpf zebrafish indicating nasal pit in relation to the eye (arrow head). Bottom panel left, representative example of 72 hpf rsph9 MOex3 morphant zebrafish with debris accumulation evident in the nasal pit (arrows). Bottom panel right, wild-type zebrafish showing normal debris clearance due to fluid vortex created by cilia beating. MOex2 showed the same defect (not shown).

(C) *Chlamydomonas* flagella movement in wild-type (*cw15*), *pf17*, *pf17-T* and *pf17-Tmut*. Top, flagella motility was within the normal range at 4.6% in *pf17-T*, but in *pf17-Tmut* 73.0% of flagella were immotile, compared to 1.0% in wild-type and 99.8% in *pf17*. Bottom, flagella beat frequency was within the normal range at 53.5 Hz in *pf17-T*,
but reduced to 24.9 Hz in *pf17*-Tmut (wild-type 47.9 Hz, *pf17* 0.0 Hz). Means±SEM from 5-10 (immotility) or 20-26 cells (FBF) shown, *** p<0.001.