De novo mutations in *FOXJ1* result in a motile ciliopathy with hydrocephalus and randomization of left / right body asymmetry

Julia Wallmeier1, Diana Frank1, Amelia Shoemark2,3, Tabea Nöthe-Menchen1, Sandra 3 Cindric1, Heike Olbrich1, Niki Tomas Loges1, Isabella Aprea1, Gerard W. Dougherty1, Petra 4 Pennekamp1, Thomas Kaiser1, Hannah M. Mitchison4, Claire Hogg3, Siobhan B. Carr3, Maimoona Zariwala 5, Thomas W. Ferkol 6, Michael R. Knowles7, Holger Thiele8, Janine Altmüller8, Angela Brentrup9, Frank Ahrens10, Christian Vogelberg11, Deborah J. Morris-Rosendahl12,13, Heymut Omran1*

1. Department of General Pediatrics, University Children's Hospital Muenster, 48149 Muenster, Germany. ERN-LUNG
2. Molecular & Clinical Medicine, University of Dundee, Dundee, UK
3. Department of Paediatric Respiratory Medicine, Royal Brompton and Harefield NHS Trust, London, UK. ERN-LUNG
4. Genetics and Genomic Medicine, University College London (UCL) Great Ormond Street Institute of Child Health, 30 Guilford Street, London WC1N 1EH, UK
5. Department of Pathology and Laboratory Medicine, Marsico Lung Institute/UNC CF Research Center, School of Medicine, University of North Carolina at Chapel Hill, Chapel Hill, NC 27599, USA
6. Department of Pediatrics, Washington University School of Medicine, St Louis, MO, USA
7. Department of Medicine, Marsico Lung Institute/UNC CF Research Center, School of Medicine, University of North Carolina at Chapel Hill, Chapel Hill, NC 27599, USA.
8. Cologne Center for Genomics, Center for Molecular Medicine Cologne (CMMC), University of Cologne, Cologne, Germany
9. Department of Neurosurgery, University Children's Hospital Muenster, 48149 Muenster, Germany
10. Children's Hospital "Altona", Hamburg, Germany
11. Paediatric Department, University Hospital Carl Gustav Carus Dresden, TU Dresden, Dresden, Germany
12. Clinical Genetics and Genomics, Royal Brompton and Harefield NHS Foundation Trust, London, UK
13. National Heart and Lung Institute, Imperial College London, London, UK

*Corresponding Author: Heymut Omran; E-Mail: heymut.omran@ukmuenster de.

Abstract:

Hydrocephalus remains the most prevalent forms of developmental central nervous system (CNS) malformation treated by neurosurgeons. C The cerebrospinal fluid (CSF) flow depends on both heartbeat and body movement. Furthermore it has been shown, that CSF flow within and across brain ventricles depends on cilia motility of the ependymal cells lining the brain ventricles, which play a crucial role to maintain patency of the narrow sites of CSF passage during brain formation in mice. Using whole exome and whole genome sequencing we

identified the first autosomal dominant cause of a distinct motile ciliopathy related to defective ciliogenesis of the ependymal cilia. Heterozygous *de novo* mutations in *FOXJ1*, which encodes a well-known member of the forkhead transcription factors important for ciliogenesis of motile cilia, cause a motile ciliopathy that is characterized by *hydrocephalus internus*, chronic destructive airway disease and randomization of left / right body asymmetry. Mutant respiratory epithelial cells are unable to generate a fluid flow and exhibit a reduced number of cilia per cell, as documented by high-speed video microscopy, transmission electron microscopy (TEM) and immunofluorescence analysis (IF). TEM and IF demonstrate mislocalized basal bodies. In line with this finding, the expression of the focal adhesion protein PTK2 is reduced and aberrant in ~~in~~ the cytoplasm of the mutant respiratory epithelial cells.

Commented [H1]: reduced and aberrant, do you need to say both. what kind of aberrant ?

Commented [H2]: should you make more of your conclusion that LRD is independent of the NOTCH1-dependent multiciliogenesis pathway ?nothc

Main Text:

Hydrocephalus remains the most prevalent form of developmental central nervous system (CNS) malformation treated by pediatric neurosurgeons[1]. While trauma, intraventricular hemorrhages~~,~~ and CNS infections account for the majority of cases, heritable genetic mutations in human hydrocephalus are relatively rare[1]. Here, we identify a novel genetic cause related to dysfunction of the CNS ependymal cilia.

Commented [H3]: acronym is already in your abstract above

Multiple motile cilia in the respiratory tract, the ependyma or the female fallopian tubes as well as motile monocilia in the embryonic left / right organizer generate the mechanical force to drive extracellular fluid flow in a continuous and coordinated fashion. While formation of a single cilium is a complex process depending on several hundreds of different factors, ciliogenesis in multiciliated cells additionally requires development of a network of oriented cilia within a short period of time[2]. Defects in ciliary generation or motility lead to a mucociliary clearance disorder associated with laterality defects, male infertility and very rarely hydrocephalus, which is referred to as primary ciliary dyskinesia (PCD). So far recessive mutations in 44 genes have been identified to cause PCD [refs?].

Formatted: Font: Not Italic

Here, to our knowledge, we present the first autosomal dominant cause of a distinct motile ciliopathy. Heterozygous *de novo* mutations in *FOXJ1*, which encodes a well-known member

of the forkhead / winged-helix transcription factor ~~s of~~ DNA binding proteins, cause a motile ciliopathy that is characterized by *hydrocephalus internus*, chronic destructive airway disease and randomization of left / right body asymmetry.

Whole exome sequencing performed as previously described[3]~~,~~ in ~~our~~ an initial cohort of individuals with, both hydrocephalus and a mucociliary clearance disorder, identified heterozygous loss-of-function mutations in *FOXJ1* in two non-related individuals (OP-1743 II1 c.901G>T, p.Glu301*; OP-1933 II1 c.826C>T, p.Gln276*). In an additional cohort of individuals with mucociliary clearance disorder, a proportion of whom had hydrocephalus, w~~W~~hole exome sequencing [ref for how performed] ~~in a cohort of individuals with mucociliary clearance disorder as well as~~and whole genome sequencing as part of the UK 100,000 Genomes Project[4] revealed four further affected individuals with heterozygous variants in *FOXJ1* (OP-2950 II1 c.868_871dup, p.Thr271Lysfs*12~~,~~; US-1 II1 c.826C>T, p.Gln276*; US-2 II1 c.939delC p.Ile314Serfs*19~~,~~; RBH II1 c.967delG, p.Glu323Serfs*10) (**Figure 1, Supplemental Figure S1**). These *FOXJ1* variants were not identified in any of the parents or the non-affected siblings in the families and were therefore considered to have arisen *de novo*. For affected individual~~In~~ US-2 II1~~–~~, parental DNA was not available. No mutations in other motile ciliopathy-related genes were identified (**Figure 1, Supplemental Figure 1**). ~~We S~~systematic genetic screening of a total additional ~~ally examined~~ 354 individuals with mucociliary clearance disorders but without co-occurrence of hydrocephalus ~~and~~ did not identify any *FOXJ1* mutations.

*FOXJ1 (*CCDS32739) ~~is~~ located on chromosome 17q25.1 ~~and~~ comprises two coding exons and one alternative first exon, encoding a 2.641 bp transcript and predicted~~ing a~~ 421 amino acid protein (**Figure 1**). Consistent with a mutational hotspot in the *FOXJ1* C-terminal region (**Figure 1**), all identified mutations localize within a small region of exon 3. Interestingly the variant ~~(~~c.826C>T, p.Gln276*~~)~~ occurred *de novo* in two non-related individuals, respectively ~~(~~OP-1933 II1 and~~,~~ US-1 II1), emphazising~~,~~ that this gene region~~area~~ is especially susceptible to *de novo* variants.

Commented [H4]: would be usual to put how big is the cohort

Formatted: Font: Not Italic

Commented [H5]: by the way all your cases have got hydrocephalus as per clinical table, therefore this currently does not quite make sense. Since the second cohort also has hydroceph so you could tweak it as I have indicated. BUT then maybe you put your German OP-2950 into the first cohort (was its hydrocephalus maybe at first not recognised?), so you just say your first German cohort found 3 cases, the international cohort found 3 cases - i dont think you can distinguish between the two cohorts based upon hydrocephalus, unless you mention what % had hydroceph and then it is clear your first cohort is ALL hydroceph, your second cohort is some with hydroceph. You see what I mean?

Commented [H6]: systematically examined, doesnt actually say what you did in these cases. Also, since cohort 2 also had some without hydroceph, then I think the difference between your cohort 1/2/3 do not make sense yet.

Consistent with haploinsufficiency being the disease cause, all variants reported are predicted to result in a premature termination codon (nonsense or frameshift type allele). In addition, both the~~The~~ GnomAD gene constraint scores for *FOXJ1* ~~both utilized to predict likely haploinsufficient genes~~ (pLI=0.97, oe=0) place it~~also indicate a high LOF intolerance consistent with~~ in the ~~haploinsufficiency~~haploinsufficient gene category i.e. the a high intolerance to loss-of-function alleles.[5] The significance of our genetic findings is also supported by the fact that *de novo* mutations are very rare events in humans. While 45 to 60 *de novo* single nucleotide variants occur on average per individual, only one to two *de novo* mutations affect the coding sequence[6]. Interestingly, haploinsufficiency has also been reported in other genes encoding forkhead transcription factors such as *FOXC1* (*FKHL7*) (pLI=0.95, oe=0) and *FOXC2* ((*FKHL14*), which are associated with aberrant ocular development[7] (pLI=0.13 oe=0.3).

We next performed 3´ mRNA- sequencing in *FOXJ1* mutant respiratory cells (OP-1743 II1 and OP-2950 II1) to analyze the effect of the detected *de novo FOXJ1* mutations on the transcript level. Consistent with haploinsufficiency, *FOXJ1* transcripts are reduced compared to healthy controls (Supplemental Figure S8). Furthermore, ~~also~~ direct FOXJ1 gene targets[8] encoding ~~for~~ ciliary axonemal proteins are also reduced in *FOXJ1* mutant cells.

FOXJ1, also referred to as *hepatocyte nuclear factor-3 / forkhead homologue 4* ~~gene~~ (*HFH-4*), has been studied in detail in the past. Consistent with a distinct functional role for motile cilia, *FOXJ1* expression has been detected in ciliated cells of the ependyma lining the brain ventricles, airways, oviduct, and the embryonic left / right organizer[9–11]. Targeted mutation or knock-down of *Foxj1* in zebrafish, *Xenopus laevis* and mice[10,12,13] resulted in a motile ciliopathy characterized by a reduced number of multiple motile cilia (MMC) and mislocalized basal bodies, which nucleate ciliary axonemes. These findings demonstrate the important functional role of FOXJ1 for the generation of motile cilia.

All six individuals with pathogenic *FOXJ1* variants exhibited *hydrocephalus internus* (**Figure 2, Supplemental Figure S2**). In five affected individuals (OP-1743 II1, OP-1933 II1, RBH II1,

Commented [H7]: I know what you mean but not all haploinsufficiency is because of premature termination so this just sounds a bit weird

Commented [H8]: What do you mean here by ‚both' - is ‚oe' a different score ?

Commented [H9]: to be more clear, do you mean this disorder is caused by their haploinsufficiency?

Formatted: Font: Italic

US-1 II1 and US-2 II2) obstructive hydrocephalus was detected within the first few weeks of life, which required immediate treatment by insertion of a ventriculo-peritoneal shunt system to relieve elevated intracranial pressure. Interestingly, *hydrocephalus internus* in OP-2950 II1 was detected at age 54 years, when clinical examination revealed macrocephaly, gait ataxia and optic atrophy consistent with a long-standing, increased intracranial pressure. Cranial magnetic resonance imaging (MRI) confirmed obstructive *hydrocephalus internus*.

The flow of cerebrospinal fluid (CSF) depends on heart-beat as well as body movement. In zebrafish, it has been proposed that the distribution of CSF within and across brain ventricles depends on ciliary motility[14]. CSF is produced by the choroid plexus in both lateral ventricles and the choroid plexus of the third and the fourth ventricle. Before its re-absorption, the CSF traverses several narrow spaces such as the aqueduct of Sylvius (to enter the fourth ventricle), three openings including the foramen of Magendii, and the lateral apertures (to enter the subarachnoid spaces). We have previously reported that motile cilia of the ependymal cells lining the brain ventricles play a crucial role to maintain patency of the narrow sites of CSF passage during brain formation[15]. Consistent with ~~the~~ a role of *FOXJ1* ~~for~~ in generation of motile ependymal cilia, cranial MRI studies demonstrated stenosis of the cerebral aqueduct and / or foramina Magendii and Luschka responsible for the obstructive *hydrocephalus internus* in all six patients (**Figure 2, Supplemental Figure S2**). In agreement with this observation, *Foxj1*-deficient mice develop hydrocephalus[10,16]. Therefore, we assume that hydrocephalus is a characteristic clinical finding in *FOXJ1*-mutant individuals.

While the prevalence of infant hydrocephalus occurs with a frequency of approximately one per 1,000 births[17], most cases are of post-haemorrhagic nature due to prematurity (malabsorption of CSF). About 10% are related to primary causes but so far only a small number of associated genes have been identified[1]. In PCD individuals with severely impaired cilia beating due to altered axonemal motor protein composition of multiciliated cells (MCC), the prevalence of hydrocephalus is only slightly increased (approximately 1/75)[18] . Individuals carrying recessive mutations in *MCIDAS* and *CCNO,* which cause severe multiciliogenesis

defects of MCCs, develop hydrocephalus much more often (10%) [19]. Nevertheless, hydrocephalus is not an obligatory finding in cases arising from these multiciliogenesis defects[20–22] and hydrocephalus has been shown by Behan et al. to not be indicative for overall PCD, because hydrocephalus is very rarely present in PCD individuals[18]. Therefore, aberrant beating or reduced numbers of ependymal cilia alone is not sufficient to explain the occurrence of human hydrocephalus.

Commented [H10]: Lack of ependymal cilia shown in CCNO mice

Besides its function in motile ciliogenesis, FOXJ1 is known to be essential for ependymal cell maturation, which might contribute to the development of hydrocephalus in *FOXJ1*-mutant individuals[10,16]. During early postnatal periods, radial glial cells in various ventricular zones of the brain differentiate into ependymal cells and astrocytes. In mice, it has been shown that *Foxj1* expression in the lateral ventricle is required for the differentiation of radial glial cells into ependymal cells and a small subset of Foxj1(+) astrocytes into a postnatal neural stem cell niche[16]. A chemical screen for modulators of ependymal cell differentiation found that the mature multiciliated ependyma needs constant *FOXJ1* expression to prevent cellular dedifferentiation back to a glial-like morphology[23]. Interestingly, Abdi *et al.* reported that ependymal FOXJ1 has a short half-life, requiring non-canonical IκB kinase activity to prevent rapid degradation via the ubiquitin proteasome system. Thus, constant *Foxj1* expression is crucial to maintain ependymal cell differentiation and prevent hydrocephalus. Interestingly, autosomal dominant mutations in *FOXG1,* another member of the forkhead gene family, cause a neurodevelopmental disorder associated with brain malformations including corpus callosal dysgenesis, indicating that forkhead transcription factors play a crucial role in neurodevelopment[24].

Consistent with a mucociliary clearance disorder also affecting the generation of MMC of the airways, all *FOXJ1*-mutant individuals suffered from recurrent infections of upper and lower airways, chronic productive cough, bronchiectasis as well as chronic rhinitis and sinusitis (**Figure 2, Supplemental Figure S2**). Postnatal respiratory distress syndrome was present in four individuals. This respiratory phenotype resembles that of PCD. Interestingly, the nasal

nitric oxide (NO) production rate is usually markedly reduced in PCD individuals[25]. However, nasal NO production rates of the four tested *FOXJ1*-mutant individuals OP-1933 II1 (141 nl/min), OP-2950 II1 (122 nl/min), RBH II1 (215 nl/min) and US-2 II1 (328nl/min) were within normal ranges. Thus, nasal NO-measurement cannot be used to screen for individuals with *FOXJ1* mutations, and affected individuals will not be identified by the nasal NO testing implemented early within the current diagnostic workup for PCD[26,27].

Affected OP-2950 II1, US-1 II1 and US-2 II1 exhibited *situs inversus,* consistent with previous observations in mice, *Xenopus laevis* and zebrafish, indicating that *FOXJ1* has an important functional role in left / right body asymmetry determination during early embryogenesis[10,12,28–30]. Interestingly, OP-1933 II1 presented with a ventricular septal cardiac defect, which might reflect the increased incidence of congenital heart defects in motile ciliopathies associated with randomization of left / right body asymmetry compared to the healthy population[31,32].

~~The~~ One female patient, RBH II1, was diagnosed on imaging with hydrosalpinx following presentation with abdominal pain at 15 years old (**Supplemental Figure S2**). OP-2950 II1 was unable to become pregnant even after *in-vitro* fertilization. MMC also line the fallopian tube, and there are several reports of individuals with PCD where fallopian tube cilia dysmotility defects mirror those in the respiratory tract, possibly causing subfertility and ectopic pregnancy[33–35].

Consistent with a defect also affecting sperm flagella, the male affected individual US-2 II1 w~~W~~as not able to father a child. Fertility evaluation revealed an adequate sperm count but with severe reduction in the motilty and number of spermatozoa as well as a change in sperm morphology (severe oligoasthenoteratospermia). Futher studies in f~~However, f~~uture ~~studies~~ will reveal whether male infertility is indeed a constant finding in *FOXJ1* mutant males.

To study the effects of *FOXJ1* haploinsufficiency at the cellular level, we analyzed respiratory epithelial cells from *FOXJ1*-mutant individuals and healthy controls by high-speed video microscopy analysis (HVMA). HVMA in ~~(~~OP-1933 II1, OP-2950 II1 and RBH II1~~)~~ showed a

reduced number of motile cilia per MCC. The number of cilia per MCC varied between zero to almost normal numbers, ~~whereas~~ however most cells were lined with very few cilia per cell **(Supplemental Figure S3)**. To corroborate these findings, we also investigated the amount of cells with i) normal (>100 cilia per MCC), ii) slightly reduced (4-100 cilia per MCC) and ~~–~~iii) severely reduced (0-4 cilia per MCC) numbers of cilia, in *FOXJ1*-mutant individuals ~~(~~OP-1743 II1, OP-1933 II1 and OP-2950 II1~~)~~ and healthy controls. In all *FOXJ1* mutant individuals the number of cells with normal amounts of cilia was reduced compared to healthy controls Supplemental Figure S3). The residual cilia of their MCCs exhibited a stiff beating pattern with reduced beating amplitude (**Supplemental Video files S1-S4**). To distinguish between reduced generation and secondary loss of MMC, we cultured primary respiratory epithelial cells from OP-1743 II1, OP-1933 II1 and OP-2950 II1 and performed *in-vitro* ciliogenesis experiments in spheroid and air-liquid interface (ALI-) cultures. HVMA after *in-vitro* ciliogenesis of spheroid cultures confirmed reduced numbers of cilia per MCC, as well as the abnormal beating pattern, in samples from OP-1933 II1 and OP-2950 II1. This is consistent with a primary defect of ciliogenesis, as previously reported in various *FOXJ1*-deficient model organisms[10,12,30] (**Supplemental Video files S5-S7**). Next, we tested whether the residual motile cilia of *FOXJ1*-mutant cells are still able to generate a directed fluid flow. To mimic the process of particulate lung clearance *in-vitro,* we added fluorescent particles to the apical compartment of the ALI-Transwell® inserts from OP-1743 II1 and OP-2950 II1 as well as healthy controls and performed particle-tracking experiments. Consistent with aberrant mucociliary clearance, *FOXJ1*-mutant cilia were not able to propel mucous along the surface of the differentiated epithelium (**Figure 3, Supplemental Video files S8-S13**).

To further characterize the ciliogenesis defect at~~on~~ the cellular level, we performed transmission electron microscopy (TEM) as previously described[22] on native respiratory epithelial cells after nasal brushing (OP-1743 II1, OP-1933 II1, OP-2950 II1, RBH II1, US-1, US-2) as well as after spheroid cultures (OP-1933 II1; OP-2950 II1). Consistent with a defect in ciliogenesis, the number of cilia per MCC was markedly reduced in most cells across all the different samples (**Figure 4**). The apical cell regions showed a severe decrease of basal

bodies. Whereas the overall number of basal bodies per MCC did not seem to be altered, basal bodies were mislocalized within the cytoplasm, indicating a defect in apical docking that is consistent with previous reports on MCCs in *Foxj1*-deficient mice[10,36] (**Figure 4**).

To corroborate these findings, we performed high-resolution immunofluorescence microscopy analyses (IF~~s~~) with antibodies targeting acetylated α-tubulin (marker of the ciliary axonemes) and the centrosomal protein 164 / CEP164 (marker of basal bodies), as previously described[22]. As expected, the number of cilia per MCC in samples of *FOXJ1*-mutant individuals varied, but was severely reduced in most cells, consistent with our findings obtained by HVMA and TEM. The number of CEP164-positive basal bodies per MCC was not altered but basal bodies were mislocalized within the cytoplasm, consistent with a basal body docking defect also documented by TEM (**Figure 4**).

Because focal adhesion components have been shown to be responsible for anchoring basal bodies to the actin network of multiciliated cells[37], we analyzed PTK2 / protein tyrosine kinase 2 (also known as focal adhesion kinase / FAK) localization in respiratory epithelial, utilizing anti-PTK2 antibodies. Consistent with previous findings in *Xenopus*[37], -PTK2 localizes in the basal body area in respiratory epithelial cells of healthy controls **(Figure 5)**. In line with a basal body docking defect, PTK2 localization was markedly reduced in *FOXJ1*-mutant respiratory epithelial cells (**Figure 5**).

Because FOXJ1 is not only known to be involved in ciliogenesis in multiciliated cells but also for expression of axonemal proteins related to ciliary motility[12,13], we thoroughly examined the axonemal structure and composition of~~in~~ *FOXJ1*-mutant respiratory cilia by TEM and IF. Interestingly, ciliary cross sections often exhibited various ultrastructural abnormalities. Whereas some cross sections did not show any abnormalities of the 9+2 axonemal architecture, all affected individuals exhibited defects of tubular organization, or missing central tubules (**Supplemental Figure S6**). Thus, we found heterogeneous ultrastructural defects in *FOXJ1*-mutant cilia indicating that TEM is not a sufficient method in *FOXJ1* mutant individuals.

In PCD, ciliary defects are typically restricted to specific structures depending on the underlying genetic defect (e.g. ODA defects in *DNAH5* or *DNAI2* mutations)[38,39]. However, cilia in *FOXJ1*-mutant cells showed various abnormalities. To further elaborate these findings, we next studied respiratory cells from *FOXJ1* affected individuals using antibodies targeting distinct axonemal structural components such as i) the ODA intermediate chain DNAI2 and heavy chain DNAH5 (absent in PCD subjects with ODA defects), ii) the nexin-dynein regulatory complex protein GAS8 (absent in PCD subjects with defects of microtubular organization e.g. due to pathogenic *CCDC40* or *CCDC39* variant)[40,41], iii) the radial spoke head protein RSPH4A~~a~~ (absent in PCD subjects with pathogenic *RSPH4A~~a~~* variants)[42]. However, subtle reductions of protein content cannot be ~~i be~~ detected by IF. Thus, normal IF analyses of the residual cilia did not show any gross abnormality but this does not rule out subtle axonemal defects as detected by TEM- (**Supplemental Figure S7**). We previously reported mutations in *CCNO* or *MCIDAS* causing a mucociliary clearance disorder referred to as reduced generation of multiple motile cilia (RGMC), due to a defect of mother centriole generation and migration at the late stage of MMC generation[22,43]. FOXJ1 probably acts downstream of MCIDAS and in parallel to CCNO in the NOTCH1-dependent pathway of multiciliogenesis[43] (**Figure 6**). While *FOXJ1* deficiency can be classified as RGMC, the cellular disease phenotype is distinct from *CCNO* or *MCIDAS* defects. *FOXJ1* haploinsufficient MCC in the respiratory tract exhibit a normal number of basal bodies, which are mislocalized within the cytoplasm due to a basal body docking defect. OP-2950 II1 exhibits *situs inversus,* which is consistent with previous reports in mice, zebrafish and *Xenopus laevis* that FOXJ1 deficiency causes randomization of the left / right body asymmetry[10,12,13]. This implies that determination of left / right asymmetry is independent of the NOTCH1-dependent multiciliogenesis pathway. NOTO is a homeodomain transcription factor specifically expressed at the left / right organizer of mouse and other vertebrate embryos; NOTO transcriptionally activates FOXJ1 and thus also regulates ciliogenesis[44]. This NOTO-dependent activation of FOXJ1 at the left / right organizer is crucial for proper determination of the left / right asymmetry, probably explaining situs anomalies in some *FOXJ1*-mutant individuals (**Figure 6**).

This study emphasizes the pathophysiological link between the development of hydrocephalus and a severe mucociliary clearance disorder, which should be considered in clinical care of affected individuals with hydrocephalus and respiratory symptoms. Early clinical and genetic diagnosis will aid implementation of appropriate neurological as well as respiratory care in *FOXJ1*-mutant individuals.

Supplemental Data:

Supplemental Figure S1: Pedigrees of the families OP-1743, OP-1933, OP-2950, RBH, US-1 and US-2.

Supplemental Figure S2: *FOXJ1*-mutant individuals showing, bronchiectasis, randomization of the left / right body asymmetry obstructive hydrocephalus and hydrosalpinx.

Supplemental Figure S3: *FOXJ1*-mutant respiratory epithelial cells show variable number of cilia per cell.

Supplemental Figure S4: PTK2 localizing in the basal body region is mislocalized in *FOXJ1* mutant cells.

Supplemental Figure S5: Western blot analysis with the monoclonal antibodie directed against PTK2.

Supplemental Figure S6: Ciliary cross sections of *FOXJ1*-mutant respiratory epithelial cells show variable structural defects by TEM.

Supplemental Figure S7: Analysis of ciliary axonemal components in multiple motile cilia of *FOXJ1*-mutant respiratory epithelial by IF.

Supplemental Figure S8: Air-liquid interface (ALI-) cultured *FOXJ1*-mutant respiratory epithelial cells show reduced transcript levels for *FOXJ1* and cilia axonemal components.

High-speed video microscopy of native respiratory epithelial cells:

Video S1_Control-respiratory epithelial cells-native

Video S2_OP-1933 II1-respiratory epithelial cells-native

Video S3_OP-2950 II1-respiratory epithelial cells-native

Video S4_RBH II1- respiratory epithelial cells -native

High-speed video microscopy of cultured respiratory epithelial cells:

Video S5_Control-respiratory epithelial cells-culture

Video S6_OP-1933 II1- respiratory epithelial cells-culture

Video S7_OP-2950 II1- respiratory epithelial cells-culture

Particle tracking (exemplary for day 44)

Video S8_Control_Particle tracking_day44_DIC

Video S9_Control_Particle tracking_day44_tracking

Video S10_OP-1743 II1_Particle tracking_day44_DIC

Video S11_OP-1743 II1_Particle tracking_day44_tracking

Video S12_OP-2950 II1_Particle tracking_day44_DIC

Video S13_OP-2950 II1_Particle tracking_day44_tracking

Declaration of Interest: The authors declare no competing interests.

Acknowledgements: We thank the PCD affected individuals and their families for their participation and acknowledge the German PCD support group 'Kartagener Syndrom und Primaere Ciliaere Dyskinesie e.V and the UK PCD Family Support Group. We would like to thank Karl Peter Schlingmann for the discussion. We thank A. Borgscheiper, A. Dorißen, D. Ernst, S. Helms, M. Herting, A. Robbers, L. Schwiddessen, F.J. Seesing, S. Sivalingam, M. Tekaat, K. Wohlgemuth, C. Westermann and S. Wilkinson for excellent technical work. This work was supported by the Deutsche Forschungsgemeinschaft WA 4283/1-1 to J.W., OM6/7, OM6/8, OM6/10 and OM6-11/ DFG KFO 326 to H.O., OL450/1 (H.Ol.), by the IZKF Muenster to H.O. (Om2/009/12 and Om/015/16), the "Innovative Medical Research" of the University of Muenster Medical School to N.T.L. (I-LO121517) and WA 1 2 14 18 (J.W.)) and "Dekanat der Medizinischen Fakultät der WWU" to J.W. The authors participate in the COST action BEAT-PCD: Better Evidence to Advance Therapeutic options for PCD network (BM11407). H.M.M. acknowledges Great Ormond Street Children's Charity (V1299, V2217) and the NIHR Great Ormond Street Hospital Biomedical Research Centre (GOSH BRC). We thank Sayyid Hasan (RBHT) as WL GMC Validation Coordinator for results from the UK 100K Project. We acknowledge Dr. Anne Schmidt for the clinical workup of patient RBH II-1 as well as Mitali P. Patel for the IF-stainings. The findings in patient RBH II1 were made possible through access to the data and findings generated by the 100,000 Genomes Project. The 100,000 Genomes Project is managed by Genomics England Limited (a wholly owned company of the Department of Health). The 100,000 Genomes Project is funded by the National Institute for

Health Research and NHS England. The Wellcome Trust, Cancer Research UK and the Medical Research Council have also funded research infrastructure. The 100,000 Genomes Project uses data provided by patients and collected by the National Health Service as part of their care and support.

Authors contributions: H. Omran and J.W. designed the study. J.W., H. Omran and D. F. wrote the paper. H.T. and J.A. performed WES sequencing. J.W., H.O., H.M.M. -and D.M-R. performed mutation analyses. A.S., I.A. and T.N-M. performed TEM analyses. J.W., H.Omran, A.S. analysed TEM pictures. S.C., N.T.L. and D.F. performed IF analysis. J.W., H.Omran, C.H., S.C. performed clinical diagnostic analysis including nNO, HVMA and interpretation of imaging. G.W. D. and D.F. performed immunoblotting analysis. Particle tracking was performed by D.F., S.C. and P.P., F.A., C.V. and S.C. provided clinical data. D.F. and J.W. prepared the figures.

Commented [H11]: Missing other contribs from T.K., M.Z., T.W.F., M.R.K., H.T., J.A. A.B.

Commented [H12]: S.B.C. the clinician UK versus S.C. Germany?

Formatted: Highlight

Formatted: Highlight

Websites:

https://gnomad.broadinstitute.org/

https://nmdprediction.shinyapps.io/nmdescpredictor/

References:

1. Kahle, K.T., Kulkarni, A. V, Limbrick, D.D., and Warf, B.C. (2016). Hydrocephalus in children. Lancet *387*, 788–799.

2. Fliegauf, M., Benzing, T., and Omran, H. (2007). When cilia go bad: cilia defects and ciliopathies. Nat. Rev. Mol. Cell Biol. *8*, 880–893.

3. Altmüller, J., Motameny, S., Becker, C., Thiele, H., Chatterjee, S., Wollnik, B., and Nürnberg, P. (2016). A systematic comparison of two new releases of exome sequencing products: the aim of use determines the choice of product. Biol. Chem. *397*, 791–801.

376 4. The 100,000 Genomes Project Protocol.

377 5. Lek, M., Karczewski, K.J., Minikel, E. V, Samocha, K.E., Banks, E., Fennell, T., O'Donnell-
378 Luria, A.H., Ware, J.S., Hill, A.J., Cummings, B.B., et al. (2016). Analysis of protein-coding
379 genetic variation in 60,706 humans. Nature *536*, 285–291.

380 6. Goldmann, J.M., Wong, W.S.W., Pinelli, M., Farrah, T., Bodian, D., Stittrich, A.B.,
381 Glusman, G., Vissers, L.E.L.M., Hoischen, A., Roach, J.C., et al. (2016). Parent-of-origin-
382 specific signatures of de novo mutations. Nat. Genet. *48*, 935–939.

383 7. Smith, R.S., Zabaleta, A., Kume, T., Savinova, O. V, Kidson, S.H., Martin, J.E., Nishimura,
384 D.Y., Alward, W.L., Hogan, B.L., and John, S.W. (2000). Haploinsufficiency of the
385 transcription factors FOXC1 and FOXC2 results in aberrant ocular development. Hum. Mol.
386 Genet. *9*, 1021–1032.

387 8. Mukherjee, I., Roy, S., and Chakrabarti, S. (2019). Identification of Important Effector
388 Proteins in the FOXJ1 Transcriptional Network Associated With Ciliogenesis and Ciliary
389 Function. Front. Genet. *10*,.

390 9. Blatt, E.N., Yan, X.H., Wuerffel, M.K., Hamilos, D.L., and Brody, S.L. (1999). Forkhead
391 transcription factor HFH-4 expression is temporally related to ciliogenesis. Am. J. Respir. Cell
392 Mol. Biol. *21*, 168–176.

393 10. Brody, S.L., Yan, X.H., Wuerffel, M.K., Song, S.K., and Shapiro, S.D. (2000).
394 Ciliogenesis and left-right axis defects in forkhead factor HFH-4-null mice. Am. J. Respir. Cell
395 Mol. Biol. *23*, 45–51.

396 11. Hackett, B.P., Brody, S.L., Liang, M., Zeitz, I.D., Bruns, L.A., and Gitlin, J.D. (1995).
397 Primary structure of hepatocyte nuclear factor/forkhead homologue 4 and characterization of
398 gene expression in the developing respiratory and reproductive epithelium. Proc. Natl. Acad.
399 Sci. U. S. A. *92*, 4249–4253.

12. Stubbs, J.L., Oishi, I., Izpisúa Belmonte, J.C., and Kintner, C. (2008). The forkhead protein Foxj1 specifies node-like cilia in Xenopus and zebrafish embryos. Nat. Genet. *40*, 1454–1460.

13. Choksi, S.P., Lauter, G., Swoboda, P., and Roy, S. (2014). Switching on cilia: transcriptional networks regulating ciliogenesis. Development *141*, 1427–1441.

14. Olstad, E.W., Ringers, C., Hansen, J.N., Wens, A., Brandt, C., Wachten, D., Yaksi, E., and Jurisch-Yaksi, N. (2019). Ciliary Beating Compartmentalizes Cerebrospinal Fluid Flow in the Brain and Regulates Ventricular Development. Curr. Biol. *29*, 229–241.e6.

15. Ibañez-Tallon, I., Pagenstecher, A., Fliegauf, M., Olbrich, H., Kispert, A., Ketelsen, U.-P., North, A., Heintz, N., and Omran, H. (2004). Dysfunction of axonemal dynein heavy chain Mdnah5 inhibits ependymal flow and reveals a novel mechanism for hydrocephalus formation. Hum. Mol. Genet. *13*, 2133–2141.

16. Jacquet, B. V, Salinas-Mondragon, R., Liang, H., Therit, B., Buie, J.D., Dykstra, M., Campbell, K., Ostrowski, L.E., Brody, S.L., and Ghashghaei, H.T. (2009). FoxJ1-dependent gene expression is required for differentiation of radial glia into ependymal cells and a subset of astrocytes in the postnatal brain. Development *136*, 4021–4031.

17. Munch, T.N., Rostgaard, K., Rasmussen, M.-L.H., Wohlfahrt, J., Juhler, M., and Melbye, M. (2012). Familial aggregation of congenital hydrocephalus in a nationwide cohort. Brain *135*, 2409–2415.

18. Behan, L., Dimitrov, B.D., Kuehni, C.E., Hogg, C., Carroll, M., Evans, H.J., Goutaki, M., Harris, A., Packham, S., Walker, W.T., et al. (2016). PICADAR: a diagnostic predictive tool for primary ciliary dyskinesia. Eur. Respir. J. *47*, 1103–1112.

19. Amirav, I., Wallmeier, J., Loges, N.T., Menchen, T., Pennekamp, P., Mussaffi, H., Abitbul, R., Avital, A., Bentur, L., Dougherty, G.W., et al. (2016). Systematic Analysis of CCNO Variants in a Defined Population: Implications for Clinical Phenotype and Differential

425 Diagnosis. Hum. Mutat.

426 20. Amirav, I., Wallmeier, J., Loges, N.T.N.T., Menchen, T., Pennekamp, P., Mussaffi, H.,
427 Abitbul, R., Avital, A., Bentur, L., Dougherty, G.W.G.W., et al. (2016). Systematic Analysis of
428 CCNO Variants in a Defined Population: Implications for Clinical Phenotype and Differential
429 Diagnosis. Hum. Mutat. *37*,.

430 21. Boon, M., Wallmeier, J., Ma, L., Loges, N.T.N.T., Jaspers, M., Olbrich, H., Dougherty,
431 G.W.G.W., Raidt, J., Werner, C., Amirav, I., et al. (2014). MCIDAS mutations result in a
432 mucociliary clearance disorder with reduced generation of multiple motile cilia. Nat.
433 Commun. *5*, 4418.

434 22. Wallmeier, J., Al-Mutairi, D.A.D.A., Chen, C.-T.C.-T., Loges, N.T.N.T., Pennekamp, P.,
435 Menchen, T., Ma, L., Shamseldin, H.E.H.E., Olbrich, H., Dougherty, G.W.G.W., et al. (2014).
436 Mutations in CCNO result in congenital mucociliary clearance disorder with reduced
437 generation of multiple motile cilia. Nat. Genet. *46*, 646–651.

438 23. Abdi, K., Lai, C.-H., Paez-Gonzalez, P., Lay, M., Pyun, J., and Kuo, C.T. (2018).
439 Uncovering inherent cellular plasticity of multiciliated ependyma leading to ventricular wall
440 transformation and hydrocephalus. Nat. Commun. *9*, 1655.

441 24. Ariani, F., Hayek, G., Rondinella, D., Artuso, R., Mencarelli, M.A., Spanhol-Rosseto, A.,
442 Pollazzon, M., Buoni, S., Spiga, O., Ricciardi, S., et al. (2008). FOXG1 Is Responsible for the
443 Congenital Variant of Rett Syndrome. Am. J. Hum. Genet. *83*, 89–93.

444 25. Beydon, N., Chambellan, A., Alberti, C., de Blic, J., Clément, A., Escudier, E., and Le
445 Bourgeois, M. (2015). Technical and practical issues for tidal breathing measurements of
446 nasal nitric oxide in children. Pediatr. Pulmonol. *50*, 1374–1382.

447 26. Lucas, J.S., Barbato, A., Collins, S.A., Goutaki, M., Behan, L., Caudri, D., Dell, S., Eber,
448 E., Escudier, E., Hirst, R.A., et al. (2017). European Respiratory Society guidelines for the
449 diagnosis of primary ciliary dyskinesia. Eur. Respir. J. *49*, 1601090.

27. Shapiro, A.J., Zariwala, M.A., Ferkol, T., Davis, S.D., Sagel, S.D., Dell, S.D., Rosenfeld, M., Olivier, K.N., Milla, C., Daniel, S.J., et al. (2016). Diagnosis, monitoring, and treatment of primary ciliary dyskinesia: PCD foundation consensus recommendations based on state of the art review. Pediatr. Pulmonol. *51*, 115–132.

28. Chen, J., Knowles, H.J., Hebert, J.L., and Hackett, B.P. (1998). Mutation of the mouse hepatocyte nuclear factor/forkhead homologue 4 gene results in an absence of cilia and random left-right asymmetry. J. Clin. Invest. *102*, 1077–1082.

29. Zhang, M., Bolfing, M.F., Knowles, H.J., Karnes, H., and Hackett, B.P. (2004). Foxj1 regulates asymmetric gene expression during left-right axis patterning in mice. Biochem. Biophys. Res. Commun. *324*, 1413–1420.

30. Yu, X., Ng, C.P., Habacher, H., and Roy, S. (2008). Foxj1 transcription factors are master regulators of the motile ciliogenic program. Nat. Genet. *40*, 1445–1453.

31. Kennedy, M.P., Omran, H., Leigh, M.W., Dell, S., Morgan, L., Molina, P.L., Robinson, B. V, Minnix, S.L., Olbrich, H., Severin, T., et al. (2007). Congenital heart disease and other heterotaxic defects in a large cohort of patients with primary ciliary dyskinesia. Circulation *115*, 2814–2821.

32. Harrison, M.J., Shapiro, A.J., and Kennedy, M.P. (2016). Congenital Heart Disease and Primary Ciliary Dyskinesia. Paediatr. Respir. Rev. *18*, 25–32.

33. Vanaken, G.J., Bassinet, L., Boon, M., Mani, R., Honoré, I., Papon, J.-F., Cuppens, H., Jaspers, M., Lorent, N., Coste, A., et al. (2017). Infertility in an adult cohort with primary ciliary dyskinesia: phenotype–gene association. Eur. Respir. J. *50*, 1700314.

34. Raidt, J., Werner, C., Menchen, T., Dougherty, G.W., Olbrich, H., Loges, N.T., Schmitz, R., Pennekamp, P., and Omran, H. (2015). Ciliary function and motor protein composition of human fallopian tubes. Hum. Reprod. *30*, 2871–2880.

474 35. Antony, D., Becker-Heck, A., Zariwala, M.A., Schmidts, M., Onoufriadis, A., Forouhan,
475 M., Wilson, R., Taylor-Cox, T., Dewar, A., Jackson, C., et al. (2013). Mutations in *CCDC 39*
476 and *CCDC 40* are the Major Cause of Primary Ciliary Dyskinesia with Axonemal
477 Disorganization and Absent Inner Dynein Arms. Hum. Mutat. *34*, 462–472.

478 36. Gomperts, B.N., Gong-Cooper, X., and Hackett, B.P. (2004). Foxj1 regulates basal body
479 anchoring to the cytoskeleton of ciliated pulmonary epithelial cells. J. Cell Sci. *117*, 1329–
480 1337.

481 37. Antoniades, I., Stylianou, P., and Skourides, P.A. (2014). Making the Connection: Ciliary
482 Adhesion Complexes Anchor Basal Bodies to the Actin Cytoskeleton. Dev. Cell *28*, 70–80.

483 38. Loges, N.T., Olbrich, H., Fenske, L., Mussaffi, H., Horvath, J., Fliegauf, M., Kuhl, H.,
484 Baktai, G., Peterffy, E., Chodhari, R., et al. (2008). DNAI2 mutations cause primary ciliary
485 dyskinesia with defects in the outer dynein arm. Am. J. Hum. Genet. *83*, 547–558.

486 39. Olbrich, H., Häffner, K., Kispert, A., Völkel, A., Volz, A., Sasmaz, G., Reinhardt, R.,
487 Hennig, S., Lehrach, H., Konietzko, N., et al. (2002). Mutations in DNAH5 cause primary
488 ciliary dyskinesia and randomization of left-right asymmetry. Nat. Genet. *30*, 143–144.

489 40. Becker-Heck, A., Zohn, I.E., Okabe, N., Pollock, A., Lenhart, K.B., Sullivan-Brown, J.,
490 McSheene, J., Loges, N.T., Olbrich, H., Haeffner, K., et al. (2011). The coiled-coil domain
491 containing protein CCDC40 is essential for motile cilia function and left-right axis formation.
492 Nat. Genet. *43*, 79–84.

493 41. Merveille, A.-C., Davis, E.E., Becker-Heck, A., Legendre, M., Amirav, I., Bataille, G.,
494 Belmont, J., Beydon, N., Billen, F., Cl?ment, A., et al. (2011). CCDC39 is required for
495 assembly of inner dynein arms and the dynein regulatory complex and for normal ciliary
496 motility in humans and dogs. Nat. Genet. *43*, 72–78.

497 42. Frommer, A., Hjeij, R., Loges, N.T.N.T., Edelbusch, C., Jahnke, C., Raidt, J., Werner, C.,
498 Wallmeier, J., Große-Onnebrink, J., Olbrich, H., et al. (2015). Immunofluorescence Analysis

and Diagnosis of Primary Ciliary Dyskinesia with Radial Spoke Defects. Am. J. Respir. Cell Mol. Biol. *53*, 563–573.

43. Boon, M., Wallmeier, J., Ma, L., Loges, N.T., Jaspers, M., Olbrich, H., Dougherty, G.W., Raidt, J., Werner, C., Amirav, I., et al. (2014). MCIDAS mutations result in a mucociliary clearance disorder with reduced generation of multiple motile cilia. Nat. Commun. *5*,.

44. Beckers, A., Alten, L., Viebahn, C., Andre, P., and Gossler, A. (2007). The mouse homeobox gene Noto regulates node morphogenesis, notochordal ciliogenesis, and left right patterning. Proc. Natl. Acad. Sci. *104*, 15765–15770.

Figure 1:

***De novo* pathogenic variants in *FOXJ1* found to be heterozygous in affected with hydrocephalus and chronic destructive airway disease.**

(**A**) Schematic overview of chromosome 17. *FOXJ1 (*CCDS32739) is located on chromosome 17q25.1 (red mark). (**B**) *FOXJ1* consists of two coding exons and one alternative first exon encoding a 2641 bp transcript and 421 amino-acid protein. (**C**) Electrophotographs of Sanger sequencing results for family OP-1933, OP-1743, OP-2950, RBH, and US-1. Consistent with *de novo* mutations, none of the variants were identified in either the parents or non-affected siblings. In US-2 no parental DNA was available.

Figure 2:

Affected with pathogenic *FOXJ1* variants display obstructive hydrocephalus, randomization of left / right body asymmetry and a chronic destructive airway disease.

(**A**) Cranial magnetic resonance imaging of OP-1933 II1 was performed after shunt insertion (right lateral ventricle) to relieve raised intracranial pressure. The left lateral ventricle and the third ventricle are dilated. The lateral view documents stenosis of the aqueduct of Sylvius and a small fourth ventricle. OP-2950 II1 shows massively dilated brain ventricles. Lateral view indicates a patent aqueduct and a dilated fourth ventricle due to closure of the foramen of Magendii and the lateral apertures. (**B**) Chest X-ray of OP-2950 II1 shows *situs inversus totalis*. The computed tomography scan of OP-2950 II1 and RBH II1 exhibit atelectasis and bronchiectasis of the middle lobe. (**C**) Summary of clinical findings in the affected individuals.

Figure 3:

Air liquid interface (ALI-) cultures of *FOXJ1*-mutant respiratory epithelial cells are unable to generate a directed fluid flow.

(**A**) Schematic depicts the experimental set-up of particle tracking analyses performed on ALI-cultured respiratory epithelial cells. (**B**) Respiratory epithelial cells from *FOXJ1*-mutants (OP-1743 II1, OP-2950 II1) as well as healthy controls were cultured under ALI-conditions. After complete differentiation, (30 days, 37 days and 44 days after airlift) 0.5 µm fluorescent particles were added to the apical compartments of the cells. Tracking videos are represented as z-stack projections, while the transport direction of each particle is summarized in polar graphs. Under healthy conditions, the fluorescent particles were transported in a linear direction along the cell layer, whereas the particle transport in *FOXJ1*-mutant cells (OP-1743 II1, OP-2950 II1) was highly reduced (**C**) and non-oriented (**D**). For statistical evaluation 15 videos per person were analyzed. Thereby, 253 particles were tracked per video on average.Scale bars represent 20 µm.

Figure 4:

***FOXJ1*-mutant respiratory epithelial cells show a reduced number of cilia and mislocalized basal bodies by TEM and IF.**

(**A**) Respiratory epithelial cells from a control and OP-1933 II1 are cultured as spheroids. Cilia are stained with antibodies targeting acetylated α-tubulin (acet. Tub.; green) after complete differentiation. Cells of OP-1933 II1 demonstrate a variable reduction of cilia in comparison to the control. (**B**) TEM photographs of MCC (first row) from a healthy control show basal bodies attached to the apical membrane and nucleating multiple motile cilia. Respiratory epithelial cells from mutant individuals with pathogenic *FOXJ1* variants (OP-1743 II1, OP-1933 II1, OP-2950 II1, RBH II1) exhibit mislocalized basal bodies (representative examples shown by red arrows) within the cytoplasm, consistent with a basal body docking defect. Respiratory epithelial cells are stained with antibodies targeting acetylated α-tubulin (acet. Tub.; green) and antibodies targeting mother centrioles (CEP164, red). In control cells, basal bodies (red) are aligned at the apical cell region, whereas in *FOXJ1*-mutant cells they are mainly mislocalized within the cytoplasm, consistent with TEM findings. Right row shows higher magnification images of regions of CEP164-positive basal bodies. Nuclei were stained with Hoechst33342 (blue).

Figure 5:

PTK2, a member of the subapical protein network, shows abnormal localization in *FOXJ1*-mutant cells by IF.

Respiratory epithelial cells from control and *FOXJ1*-mutant individuals (OP-1743 II1, OP-1933 II1, OP-2950 II1) are stained with antibodies targeting PTK2 (green). PTK2, which forms complexes named ciliary adhesions that are associated with basal bodies and striated rootlets, shows reduced localization in *FOXJ1*-mutant cells compared to the control. Regions around the subapical cell membrane showing PTK2 at higher magnification (right row). Nuclei are stained with Hoechst33342.

Figure 6:

FOXJ1 is an essential component in signaling pathways for the generation of motile cilia.

Schematics illustrating the function of FOXJ1 in the generation of motile cilia in the NOTCH1- and NOTO-dependent pathway in (**A**) multiciliated cells and (**B**) the ciliated cells of the embryonic node[44], respectively. Pathogenic variants in *MCIDAS* and *CCNO* (marked in green) are known to cause a ciliogenesis defect in multiple motile cilia causing a mucociliary clearance disorder referred to as reduced generation of multiple motile cilia[21,22] (RGMC).