CORRECTION

Correction: A novel hypomorphic allele of Spag17 causes primary ciliary dyskinesia phenotypes in mice

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Saima Ali was inadvertently left off the list of authors for this paper. The complete list of authors, their affiliations and contributions are shown above.

The corresponding author would like to apologise for this error. Both the online full text and PDF versions of the paper have been corrected.
A novel hypomorphic allele of Spag17 causes primary ciliary dyskinesia phenotypes in mice

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ABSTRACT

Primary ciliary dyskinesia (PCD) is a human condition of dysfunctional motile cilia characterized by recurrent lung infection, infertility, organ laterality defects and partially penetrant hydrocephalus. We recovered a mouse mutant from a forward genetic screen that developed many of the hallmark phenotypes of PCD. Whole-exome sequencing identified this primary ciliary dyskinesia only (Pcdc) allele to be a nonsense mutation (c.5236A>T) in the Spag17 coding sequence creating a premature stop codon (K1746*). The Pcdc variant abolished several isoforms of Spag17 in the Pcdc mutant testis but not in the brain. Our data indicate differential requirements for Spag17 in different types of motile cilia. Spag17 is essential for proper development of the sperm flagellum and is required for either development or stability of the C1 microtubule structure within the central pair apparatus of the respiratory motile cilia, but not the brain ependymal cilium. We identified changes in ependymal ciliary beating frequency, but these did not appear to alter lateral ventricle but not the brain ependymal cilia. We identified changes in ependymal ciliary beating frequency, but these did not appear to alter lateral ventricle cerebrospinal fluid flow. Aqueductal stenosis resulted in significantly slower and abnormally directed cerebrospinal fluid flow, and we suggest that this is the root cause of the hydrocephalus. The Spag17Pcdc homozygous mutant mice are generally viable to adulthood but have a significantly shortened lifespan, with chronic morbidity. Our data indicate that the c.5236A>T Pcdc variant is a hypomorphic allele of Spag17 that causes phenotypes related to motile, but not primary, cilia. Spag17Pcdc is a useful new model for elucidating the molecular mechanisms underlying central pair PCD pathogenesis in the mouse.

This article has an associated First Person interview with the first author of the paper.

KEY WORDS: Cilia, Hydrocephalus, Infertility, Lung, Primary ciliary dyskinesia, Spag17

INTRODUCTION

Cilia are centriole-derived, microtubule-based membranous extensions that exist in almost every cell (Gilula and Satir, 1972; Pedersen et al., 2008; Satir, 2005). Ciliary structures are highly conserved across the animal kingdom and can be classified broadly into two main types, largely based on the structure of the ciliary axoneme. The nonmotile primary cilia have a ’9+0’ axonemal structure (Nogales et al., 1999; Satir, 2005) because they lack the central pair of microtubules and other molecular motors, such as dynein arms and radial spokes, responsible for ciliary movement. These primary cilia are mainly involved in mechanosensory functions (Goetz and Anderson, 2010; Pazour et al., 2000; Sánchez and Dynlacht, 2016). Nodal cilia are motile cilia that lack the central pair apparatus but do have inner and outer dynein arms and can cause a leftward fluid flow to convey an asymmetric signaling event, resulting in asymmetric expression of laterality genes, such as Pits2 (Brueckner, 2001; Marszalek et al., 1999; Nonaka et al., 1998). The motile cilia develop a central pair of microtubule singlets in the center of the axoneme and are therefore described as having a ’9+2’ arrangement of microtubule doublets (Nogales et al., 1999; Satir, 2005). The well-documented function of motile cilia is the coordinated rhythmic beating to move body fluids in the brain, respiratory tract and male and female genital ducts (Brightman and Palay, 1963; Dirksen, 1971; Jeffery and Reid, 1975). This is consistent with the localized tissue distribution of motile cilia to these organs.

Defects in the assembly or function of motile cilia can cause primary ciliary dyskinesia (PCD). PCD (OMIM: 244400) is a rare and highly heterogeneous condition, with variants in more than 40 causative genes reported to date (Bustamante-Marín et al., 2019; Cindric et al., 2020; Lucas et al., 2019). In spite of this progress, a genetic diagnosis remains elusive in approximately 35% of PCD patients (Kurkowiak et al., 2015; Yang et al., 2018). PCD manifests as chronic respiratory tract infections, infertility, and laterality defects in around 50% of cases (Afzelius, 1981; Leigh et al., 2019). These laterality defects in PCD are thought to develop owing to defective nodal cilia in the developing embryo. Hydrocephalus occurs infrequently in individuals with PCD and might reflect dysfunctional ependymal cilia, although various other mechanisms have also been proposed (Berlucchi et al., 2012; Del Bigio, 2010; Kosaki et al., 2004; Lee, 2013; Vieira et al., 2012; Wessels et al., 2003). Intriguingly, most rodent models of PCD genes develop hydrocephalus, but there is significant evidence for phenotypic variability based on the genetic background (Abdelhamed et al., 2018; Brody et al., 2000; Fernandez-Gonzalez et al., 2009; Finn et al., 2014; Ha et al., 2016; Ibáñez-Tallon et al., 2004; Lechtreek et al., 2008; Lee et al., 2008; Sironen et al., 2011). Ciliary motility is an ATP-dependent process that results in activation of dynein motor proteins between the pairs of microtubule doublets that are the major structural component of the ciliary axoneme (Satir and Sleigh, 1990). A number of studies indicate that signals from the central pair propagate through radial spokes to modulate the dynein activity and, ultimately, affect ciliary beating and flagellar movement (Adams et al., 1981; Smith and Yang, 2004; Wirschell et al., 2009; Yang and Smith, 2009; Zhu et al., 2019). These studies generally conclude that dynein is a downstream
RESULTS

Pcdo is a nonsense allele of Spag17

We identified the Pcdo mutant in a mouse ENU mutagenesis forward genetic screen for recessive alleles disrupting organogenesis. ENU mutagenesis was performed as described previously (Stottmann and Beier, 2014), and we phenotyped the mice to recover mutant alleles at early postnatal stages as part of an experiment to look for mutants with abnormal forebrain development. As described further below, Pcdo mutants were initially identified by an enlarged head and distended lateral ventricles, easily visible upon gross dissection. Further histological examination confirmed the occurrence of hydrocephalus and abnormal accumulation of mucus in the respiratory passages. There was no evidence of heterotaxy or any other abnormal organ laterality.

In order to identify the causal variant in Pcdo mutants, we performed whole-exome sequencing on three phenotypic mutants (Table S1). We filtered for variants that were homozygous for the ‘alternative’ allele in each mutant not present in the dbSNP database (https://www.ncbi.nlm.nih.gov/snp/), to exclude strain polymorphisms predicted to have a ‘high’ or ‘moderate’ impact on protein coding and common to all three sequenced mutants. Only three genes of predicted ‘high’ impact met these criteria: plexin C1 (Plxnc1), Sfi1 homolog, spindle assembly associated (yeast) (Sfi1) and Spag17. A null allele of Plxnc1 exists and does not have the same phenotype as the Pcdo mutants (Pasterkamp et al., 2003). Sfi1 seems to be highly polymorphic, given that this appears routinely in similar analyses in our laboratory; therefore, we initially excluded this as a candidate. The null Spag17 phenotype, however, was was similar to Pcdo to pursue as a candidate (Teves et al., 2013).

The Spag17-204 (ENSMUST00000164539) transcript has 49 exons and encodes for a full-length SPAG17 protein with 2320 amino acids (ENSMUSM00000134066; Fig. 1A). Another smaller splice variant, encoding a 97 kDa SPAG17 isoform, is found in the testis. This is known to be proteolytically processed during the process of spermatogenesis and sperm maturation to generate 72 and 28 kDa SPAG17 fragments (Silina et al., 2011; Zhang et al., 2005). The mouse SPAG17 protein has a small coiled coil domain, followed by two regions of compositional bias with lysine-rich and glycine-rich regions, followed by a PapD-like domain (flagellar-associated PapD-like, Pfam: PF14874). PapD-like domains are known to be highly conserved in mammals (Zhang et al., 2005; Fig. 1A).

Sanger sequencing of Pcdo DNA confirmed the A to T transversion at the start of exon 36, position c.5236, p.1746 (Fig. 1B). This mutation introduces a nonsense stop codon (TAA) instead of the wild-type lysine (K) amino acid at position 1746. The homozygous Spag17<sup>17<sup>5236A>T</sup> mutation segregated completely with the mutant Pcdo phenotype and was confirmed by genotyping assays in more than 50 mutant animals over multiple generations to date. Semi-quantitative RT-PCR analysis of Spag17 RNA using primers spanning exon 33 and 39 indicated high levels of Spag17 transcript in the testis compared with very limited expression in the lungs and brain of 3-month-old animals (Fig. 1C). Quantification of the band intensity and normalization to a corresponding β-actin band were performed. We found that Spag17 transcript was only marginally reduced in the Pcdo/Pcdo mutant tissues compared to wild type, suggesting that the early stop codon did not stimulate a nonsense-mediated decay response (Fig. 1D). We analyzed protein levels using two separate antibodies (Zhang et al., 2005). An antibody against an epitope in the C-terminus (Fig. 1A) reliably identified known isoforms with molecular weights of 97 and 72 kDa. Protein levels in the 1-month-old brain were low and similar in the wild type and mutant. In the testis at 1 month, both isoforms were expressed much more highly than in the brain, and they were completely missing in the mutant. With an antibody
Fig. 1. See next page for legend.
Fig. 1. Pcdo is a nonsense allele of Spag17. (A) The Spag17 gene has 49 exons, and the position of the c.5236A>T ENU variant in exon 36 is indicated with red arrows (K1746* in the protein). Mouse Spag17 has 2320 amino acids, and epitopes for the N-terminal and C-terminal SPAG17 antibodies are indicated. Blue arrows in the transcript indicate positions of the primers used in C. The mouse SPAG17 has coiled coil (blue), lysine-rich (blue dots), glycine-rich (red) and Pfam (gray) domains. (B) Sanger sequencing showing the A>T allele change in the Spag17Pcdo/Pcdo mutants. (C) Semi-quantitative RT-PCR of the Spag17 exon 33-39 and β-actin loading control. (D) Quantification of Spag17 expression in the testis, lung and brain tissues (relative to β-actin loading and normalized to testis wild-type levels: n=3 animals for each genotype; colors show littermates). (E) Western blotting with two different antibodies shows a lack of SPAG17 protein in Pcdo mutant testis compared with wild type and a decrease in the brain. (F) Analysis of the adult testis highlights another form of SPAG17 that is missing in the mutant cells when compared with control cells (Fig. 2H-I; 301 cells from Spag17<sup>+/+</sup> control and 436 cells from Spag17<sup>Pcdo/Pcdo</sup>, four animals of each genotype were included in the analysis). These data indicate the Pcdo mutation does not affect the structure of primary cilia in MEFs or long bone development in the same way as total loss of Spag17.

Spag17<sup>Pcdo</sup> mice show neonatal progressive hydrocephalus

Hydrocephalus is a prevalent phenotype in PCD mouse models. Likewise, Spag17<sup>Pcdo/Pcdo</sup> mutant animals developed a fully penetrant, mildly progressive hydrocephalus. The hydrocephalus was first obvious shortly after birth and before the beginning of the second postnatal week (Fig. 3A-F). Measurement of the lateral ventricle area from mutants compared with wild-type control animals showed no difference at postnatal day (P)1 (Fig. 3A,B; n=3 wild type and 3 mutant). This measurement was significantly increased in the mutants by P7 (Fig. 3C,D; n=3 wild type and 3 mutant) and remained significantly higher at P15 (Fig. 3E-G; n=4 wild type and 3 mutant). No masses of abnormal growths indicative of obstructive hydrocephalus were observed within the ventricular system (Fig. S1). However, we did observe severe overt intracerebroventricular and subarachnoid hemorrhage in a small subset of Spag17<sup>Pcdo/Pcdo</sup> mutant animals (n=3/32). As expected, this led to blood clots and obstruction of the ventricular system at various points and, ultimately, severe obstructive hydrocephalus and fatality (Fig. S2).

The hydrocephalus in the motile cilia models has usually been attributed to perturbed flow of cerebrospinal fluid (CSF) towards the next most caudal opening within the cerebroventricular system. A highly conserved function of the ependymal motile cilia lining the brain ventricles seems essential for directing this CSF flow. Given the Pcdo phenotypes, we hypothesized that ciliary beating might be severely compromised in Spag17<sup>Pcdo/Pcdo</sup> animals (Teves et al., 2013). We examined this using ex vivo high-speed video microscopy at P4 (Movies 1 and 2). We showed previously that the ependymal motile cilia follow a spatiotemporal pattern of development in the developing forebrain and lateral ventricle, and ependymal motile cilogenesis was seen along the medial wall of the lateral ventricle at ~P0, as opposed to the lateral wall, where ependymal cilogenesis starts towards the end of the first postnatal week (Abdelhamed et al., 2018). We recorded the ciliary beating in both the lateral ventricle and the aqueduct. Surprisingly, we did not detect any reduction in the ciliary beat frequency in any of the areas we measured (Fig. 3H-J). On the contrary, we observed that the Pcdo mutant motile cilia of the medial walls of the lateral ventricle were slightly hyperkinetic (Fig. 3J). Detailed analysis of the kymographs of the beating cilia showed altered waveforms, as indicated by a shorter interval between forward strokes in Pcdo/Pcdo mutants (Fig. 3H-I). A quantification of this showed that the mutant cilia were beating at a rate significantly higher than control cilia in the lateral ventricle (P<0.002; Fig. 3J). Interestingly, kymographs of the beating cilia and analysis of the cilia beat frequency did not detect any significant difference in the aqueduct cilia (P=0.189; Fig. 3J). We also measured CSF localized flow with fluorescent microbeads introduced ex vivo into the forebrain slice immediately before the video microscopy recording. This analysis did not show abnormalities in flow speed in isolated forebrain lateral ventricle slices (P=0.30; Fig. 3K; Movies 3 and 4). These data are consistent with our conclusion that the Pcdo K1746* allele of Spag17 is a hypomorph, in comparison to the previously reported Spag17 null allele, which develops paralyzed cilia that affect mucociliary clearance and neonatal survival (Teves et al., 2013).
**Pcdo aqueductal stenosis disrupts bulk CSF flow**

The aqueduct of Sylvius is a narrow channel for CSF flow connecting the third ventricle to the fourth ventricle. This channel is obstructed in mouse models with deficits in primary cilia (Town et al., 2008) and in motile ciliopathy attributable to collapse and fusion of the walls of the ventricle (Ibañez-Tallon et al., 2004). Histological characterization of the aqueduct at P4 from Pcdo mutant animals indicated fusion of the ependymal lining at multiple points of the aqueduct (Fig. 4A-D; n=3 wild type and 3 mutants). We also noted some enlargement of the subcommissural organ (SCO) in the Spag17Pcdo/Pcdo mutant animals (Fig. 4E,F). Later in development, the mutant aqueduct appeared collapsed and shrunken (Fig. 4G-J; n=4 wild type and 4 mutants). The SCO is an area of highly differentiated ependyma located in the dorsalcaudal region of the third ventricle at the entrance to the aqueduct of Sylvius and is well known for secreting high molecular weight glycoproteins necessary for CSF flow and circulation (Rodriguez et al., 1987). Hydrocephalus is a common feature in animal models with loss or defective development of the SCO (Blackshear et al., 2003; Cao and Wu, 2015; Sakakibara et al., 2002; Stoykova et al., 1996). Collectively, these studies and others suggest
that the secretory activity of the SCO is responsible, at least in part, for the maintenance of an open aqueduct. We suspected that aqueductal stenosis could cause localized CSF flow abnormalities and hydrocephalus in the Spag17\textsuperscript{Pcdo/Pcdo} mutants. We tested this with video microscopy and fluorescent microbeads in the intact aqueductal lumen (Movies 5 and 6). We saw that the aqueductal stenosis

Fig. 3. Hydrocephalus was the first obvious phenotype in the Spag17\textsuperscript{Pcdo} mutant line. (A-F) Coronal brain sections from Spag17\textsuperscript{wt/wt} and Spag17\textsuperscript{Pcdo/Pcdo} littermates, showing the dilated lateral ventricles at P7 and older in the mutants. (G) Quantification is shown for multiple sections of three or more animals for each stage and genotype. (H,J) Representative kymographs from Spag17\textsuperscript{control} (H) and Spag17\textsuperscript{Pcdo/Pcdo} (J). (J) Scatter plots of the frequency of the motile cilia beating measurements obtained from lateral ventricle and aqueductal cilia. Lateral ventricle cilia are hyperkinetic and beat with a rhythm significantly faster than in the Spag17 control animals (n=37 Spag17\textsuperscript{wt/wt} lateral ventricle cilia and 69 Spag17\textsuperscript{Pcdo/Pcdo} lateral ventricle mutant cilia obtained from three animals of each genotype; aqueduct, n=19 wild-type and 39 mutant cilia obtained from two wild-type and four mutant animals). (K) Scatter plots showing that there was no significant difference between flow speed of the fluorescent microbeads in lateral ventricle brain slices (n=4 slices from three animals of each genotype). Scale bars: 500 µm in B,D; 1 mm in F; 2 µm in H,J.
significantly affected the directionality and speed of the moving beads in the P4 mutant aqueduct (Fig. 4K; \(P<0.0001\); \(n=2\) control and 4 mutant animals). We conclude from these data that CSF bulk flow is extremely disturbed along with aqueductal stenosis and that this is what ultimately leads to the hydrocephalus in the \(Pcdo\) mutants.

\(Pcdo\) mutants show other PCD-related phenotypes

Gross anatomical assessment of the visceral organs in \(Spag17^{Pcdo/Pcdo}\) mutants showed that organ laterality was unaffected in this model in all animals examined (Fig. S3; \(n=5\) animals each genotype). Histological and gross anatomical examination of the lung did not show any right or left lung isomerism, and gross lung development was not affected in the \(Spag17^{Pcdo/Pcdo}\) mutants (Fig. S3; \(n=18\) control and 15 mutants). However, detailed histological examination of the lungs and trachea from P8 and P14 \(Spag17^{Pcdo/Pcdo}\) mutants showed abnormal accumulation of homogenous eosinophilic material filling the upper respiratory passages, the trachea and main bronchi, indicative of mucus accumulation, most probably attributable to defective mucociliary clearance (Fig. 5A-D; \(n=6\) control and 6/7 mutants).
Another common PCD phenotype is male infertility. All male $Spag17^{Pcdo/Pcdo}$ animals we tested ($n=4$) were infertile and were unable to produce any litters when mated with wild-type control female mice (when paired twice for 5 weeks at each attempt, $n=8$). Conventional histological analysis of the testis was performed at 5 weeks ($n=4$ wild type, 3 mutants), 10 weeks ($n=3$ wild type, 3 mutants) and 4 months of age ($n=3$ wild type, 3 mutants). In all stages of testicular development we examined, the $Spag17^{Pcdo/Pcdo}$ mutant seminiferous tubules were devoid of any sperm, with a clear lumen, whereas all control sections showed evidence of normal spermatogenesis, with mature sperm observed in the center of the seminiferous tubules (Fig. 5E-J). No clear difference was noted at

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**Fig. 5.** $Spag17^{Pcdo/Pcdo}$ mutants are generally viable and develop PCD phenotypes. (A-D) Histological analysis of $Spag17^{wt/wt}$ (A,B) and $Spag17^{Pcdo/Pcdo}$ (C,D) trachea and lung showing mucus accumulation (asterisks) in the trachea and main bronchus in the mutant animals at P8 (A,C) and P14 (B,D). (E-J) Seminiferous tubules of the testis at 5 weeks (E,H), 10 weeks (F,I) and 4 months (G,J) from $Spag17^{wt/wt}$ (E-G) and $Spag17^{Pcdo/Pcdo}$ mutants (H-J). In E-G, arrows in the $Spag17^{wt/wt}$ sections point to a mature sperm flagellum. The $Spag17^{Pcdo/Pcdo}$ mutants lack a sperm flagellum at all stages presented, indicated by asterisks in H-J. Scale bars: 200 µm in A-D; 20 µm in E-J.
the level of the germ cells or the number or morphology of developing spermatocytes (Fig. 5E-I). The round spermatids failed to mature fully and failed to produce flagella (Fig. 5E-I). This indicates that the processes involved in spermiogenesis, including development of the sperm flagellum, require SPAG17.

**Differential requirements of Spag17 for motile ciliogenesis in the brain, lung and sperm flagellum**

Given the functional deficits we observed in the motile cilia, we performed a morphological analysis of the motile ciliary structure. We first performed immunohistochemical analysis with the acetylated α-tubulin antibody to stain the ciliary axoneme. No apparent morphological differences were detected in the Spag17Pcdo/Pcdo mutant ependymal and/or respiratory cilia when compared with wild-type control cilia (Fig. 6A-D). We saw similar results with a more stringent scanning electron microscopy (SEM) analysis. The abundance of cilia and length of cilia appeared comparable in ependymal cilia lining the medial and lateral walls of the forebrain lateral ventricle, indicating that the Spag17Pcdo allele does not perturb production of motile cilia in either the brain ependymal cells or the respiratory epithelial cells (Fig. 6E-H; n=4 wild type and 4 mutants). By contrast, Spag17Pcdo/Pcdo mice showed deficits in formation of the sperm flagellum. Consistent with the histological analysis above, immunohistochemical staining of testicular sections from Spag17Pcdo/Pcdo mutants showed a total lack of acetylated α-tubulin staining at 5 and 10 weeks of age in comparison to the age-matched controls, in which we observed clear and pronounced acetylated α-tubulin staining (Fig. 6I-L; n=3 wild type and 3 mutants for each age).

**Distinct ultrastructural defects in the Spag17Pcdo mutant respiratory and brain ependymal motile cilia**

SPAG17 is a structural protein within cilia essential for development of the C1a process of the C1 microtubule structure of the central pair apparatus (Zhang et al., 2005). We therefore investigated the ultrastructure of the ependymal and respiratory motile cilia using transmission electron microscopy (TEM). Cross-sections of ependymal cilia appeared fairly normal, with no clear defects in either the structure or the orientation of the central pair apparatus or other motility structures, such as inner or outer dynein arms (Fig. 7A-C). This is consistent with the limited functional disturbance in ependymal ciliary beat frequency and the undisturbed localized (intraventricular) CSF flow observed in this model. By contrast, TEM analysis of the cross-sections of respiratory cilia showed predominantly abnormal respiratory tracheal cilia in the Spag17Pcdo mutants. Two types of related deformities can be observed clearly. First, mutant respiratory cilia lack one of the central pair microtubule singlets in a subset of the mutant cilia, with the resulting appearance of ‘9+1’ microtubule organization (Fig. 7D-G). Second, most of the remaining cilia in the mutant trachea have an abnormal central pair apparatus, with one of the central pair singlets appearing smaller or incomplete compared with wild type (Fig. 7H-K). Rotational polarity of the motile cilia axoneme is dependent on the central pair orientation (Kunimoto et al., 2012). Orientation of the axonemal central pair of microtubules was measured by drawing a line through the central pair and measuring the angle of the line with respect to the 0-180° angle arbitrarily set for the first line drawn, as previously described (Hegan et al., 2015; Kunimoto et al., 2012). The deviation from this angle was larger in the Pcdo mutant cilia, and 22.2% of the Spag17Pcdo mutant central pairs (n=42) in the respiratory cilia were not properly aligned (22.2% versus 16.7% in wild type, n=18;
This indicates that rotational planar cell polarity of the central pair was perturbed in the Spag17Pcdo/Pcdo mutant respiratory cilia. SPAG17 is not only essential for central pair development but could also be indispensable for establishing the central pair rotational polarity. The observed degree of central pair disorientation could have a strong negative influence on the highly coordinated ciliary beating and thereby contribute to impaired directional fluid flow and mucociliary clearance in the lung.

The observed central pair orientation defect prompted us also to assay the orientation of the basal body basal feet in the mutant respiratory cilia compared with wild type. The directionality of
ciliary beating is determined by the orientation of the basal feet of the basal bodies, which are usually positioned at the fourth, fifth and sixth circularly arranged triplets of microtubules within the basal bodies. The basal feet associated with the basal bodies are usually oriented in the direction of the effective stroke of the ciliary beating, and this is controlled by the planar cell polarity pathway (Boisvieux-Ulrich et al., 1985; Gibbons, 1961; Kunimoto et al., 2012). However, we found that in the Spag17Pcdo/Pcdo mutant cilia (n=16 cilia), the basal feet of the basal body appeared to be aligned normally when compared with wild-type control cilia (n=52 cilia; Fig. 7N,O). We did not observe further defects in other structures required for ciliary motility, such as inner and outer dynein arms.

Altogether, our data indicate that there is a differential requirement for SPAG17 among the different cell types that produce motile cilia. Based on the phenotypes we observe in the testis, lung and brain, we conclude that SPAG17 is crucial for initiation and formation of the sperm flagellum, whereas it has a more specific role (limited to central pair apparatus and C1a projection development and orientation) during structural development of the respiratory cilia and an even more limited role during differentiation and maturation of cilia of the brain ependymal cells.

**DISCUSSION**

*Pcdo* is an allele of *Spag17* revealing tissue-specific effects on SPAG17 translation

In this study, we present a detailed characterization of the *Pcdo* mutant we recovered from an ENU forward genetic screen and identified as a nonsense allele of *Spag17* (Fig. 1A,B). The *Pcdo* variant is a c.5236A>T nonsense mutation predicted to introduce a premature stop codon at position 1746 of the 2320 amino acid SPAG17 protein. Significantly higher levels of *Spag17* RNA were expressed in the testis in comparison to the lungs and brain tissues of *Spag17Pcdo/Pcdo* mutant animals. The precise mechanism leading to loss of multiple SPAG17 protein isoforms in testis but not in brain, although the ENU-induced variant is common to the full-length isoform of *Spag17* known to be expressed in testis, brain and lung. Our analysis also identified very significantly higher levels of *Spag17* RNA compared to wild-type control cilia (n=52 cilia; Fig. 7N,O). We did not observe other defects in other structures required for ciliary motility, such as inner and outer dynein arms.

Motile and primary ciliopathies can overlap in the same model

Motile and primary ciliopathies have long been considered as two distinct dysfunctions of two related but functionally different organelles, the motile and nonmotile cilia, respectively. However, recent advances and further characterization of ciliopathy mutants have indicated that motile and primary ciliopathies can overlap in the same animal model and/or human patients (Bukowy-Bierylo et al., 2013; Ferkol and Leigh, 2012; Fliegauf et al., 2007; Moore et al., 2006). Characterization of the *Spag17* knockout mice provided new evidence that motile and primary ciliopathies can overlap or develop in the same model organism (Teves et al., 2013, 2015). *SPAG17* mutations are reported to cause human PCD (Andjelkovic et al., 2018), and variants in the gene have been linked to human height (Kim et al., 2010; Takeuchi et al., 2009; Weedon and Frayling, 2008; Weedon et al., 2008; Zhao et al., 2010) and multiple body measurements in goats (Zhang et al., 2019). Interestingly, combined missense changes in *SPAG17* and *WDR35* cause complex neurodevelopmental malformations and skeletal cranioectodermal dysplasia, a primary ciliopathy (Córdova-Fletes et al., 2018). These studies confirm that *Spag17* has a role in the development and function of both motile and immotile primary cilia, but the effects differ based on the precise allele in question.

Similar to other mutants in central pair genes, *Pcdo* mutants have no laterality defects

A hallmark of a central pair PCD ciliopathy is the absence of laterality defects, subtle ciliary beating abnormalities, and unequivocal ultrastructural defects of the ciliary axoneme. Vertebrate organ asymmetry is well known to require the nodal cilia that generate leftward flow across the node. These cilia are motile because they express inner and outer dynein arms but do not have a central pair apparatus. It is therefore intriguing to see that almost no central pair protein mutants, including this allele of *Spag17*, develop laterality defects (Cindrič et al., 2020; Davy and Robinson, 2003; Lechtreak et al., 2008). However, mutants of the...
dynein complexes consistently develop various degrees of laterality defects, including dextrocardia and heterotaxy (Abdelhamed et al., 2018; Bartoloni et al., 2002; Dougherty et al., 2016; Loges et al., 2018; Ta-Shma et al., 2018). The lack of laterality phenotypes in the \textit{Spag17}^{\text{Pcdo/Pcdo}} mutants further supports the model that central pair proteins, including \textit{Spag17}, are dispensable for the development of organ asymmetry and body laterality.

### Ependymal cilia show normal ultrastructure and minimal beating defects

The main function of the ependymal cilia is to beat and generate CSF flow. The forebrain ependymal cilia in the \textit{Spag17}^{\text{Pcdo}} mutant have no apparent ultrastructural abnormalities, and the ciliary beating frequency was unaffected in the ependymal cilia of the aqueduct (Fig. 3J). Surprisingly, a subset of the ependymal cilia on the medial wall of the lateral ventricle in the \textit{Spag17}^{\text{Pcdo/Pcdo}} mutants are hyperkinetic and beat with a rate significantly higher than that of the control cilia (Fig. 3J). Our data suggest, however, that this hyperkinetic beating does not disrupt the local cilia-mediated flow in the acute \textit{ex vivo} isolated medial wall forebrain slice (Fig. 3K). It is possible that this could have a more prominent effect \textit{in vivo}, where there are more limiting space constraints. Alternatively, the lack of an effect on the structural development and function of the ependymal cilia could be attributable to the expression of near-normal levels of \textit{SPAG17} proteins in brain ependymal cells (Fig. 1). Interestingly, the hyperkinetic cilia phenotype in the \textit{Spag17}^{\text{Pcdo/Pcdo}} mutants is consistent with other motile cilia mutant phenotypes that have normal motile cilia ultrastructure but hyperkinetic cilia, such as \textit{DNAH11} PCD patients (Bartoloni et al., 2002; Dougherty et al., 2016; Knowles et al., 2012; Pifferi et al., 2010; Schwabe et al., 2008) and a mouse model (Handel and Kennedy, 1984). Assessment of this beating pattern of the CSF flow \textit{in vivo} would be a better indicator of the effect of the hyperkinetic cilia on the fluid flow and development of hydrocephalus. This would also be helpful to explain whether the hyperkinetic cilia phenotype is causing the hydrocephalus in this model directly or whether other mechanisms are responsible.

### The hydrocephalus in the \textit{Spag17}^{\text{Pcdo/Pcdo}} mutants is attributable to aqueductal stenosis

The aqueduct of Sylvius in the \textit{Pcdo} mutants appeared stenotic, with collapsed walls, and was associated with a slight enlargement of the SCO (Fig. 4). In other models, patency of the aqueduct is compromised in the presence of a dysfunctional SCO or SCO ependymal cilia (Pérez-Figares et al., 2001; Swiderski et al., 2012), leading to aqueductal stenosis or occlusion and noncommunicating hydrocephalus. Ciliary beating function and directionality were defective when assayed in the intact aqueduct lumen. The stenosis could have led to a mechanical constraint on the ciliary beating and thus to significantly reduced flow of CSF into the fourth ventricle. We concluded that \textit{Pcdo} mutants had communicating hydrocephalus because the aqueduct was stenotic and not completely occluded, indicative of a potentially milder dysfunction of the SCO.

### Tissue-specific effects of reduced \textit{Spag17} function

Surprisingly, we noted severely defective structure of the respiratory motile cilia consistent with a central pair protein insult. The \textit{Spag17}^{\text{Pcdo}} mutant cilia either lack one of the central microtubule pair or have an abnormal central pair structure. The orientation of the central pair also appeared not to align in one direction, indicative of disorganization of ciliary central pair polarity (Fig. 7L,M). This is expected to interfere with a directional fluid flow in the respiratory passages and lead to mucus accumulation and recurrent infection. The axis of polarity is controlled by signaling and mechanical cues. The mechanical cue is a cilia-generated fluid flow (Mitchell et al., 2007). The intense mucus accumulation observed in the \textit{Spag17}^{\text{Pcdo/Pcdo}} mutants indicates that ciliary motility in the lung is defective. This is consistent with the structural defects and is likely to contribute significantly to increased morbidity and the shortened lifespan in these mutants. We should note a limitation to our ultrastructural studies. We were able to analyze multiple cilia, but these were from a relatively limited set of animals. Current worldwide experimental conditions are challenging our ability to collaborate in order to obtain more robust sample sizes for this portion of the study. Moreover, development of the sperm flagellum was completely inhibited in the \textit{Pcdo} mutants, and no mature sperm with a flagellum can be detected at any postnatal stage of testicular development (Figs 5E-J and 6I-L).

Altogether, our data indicate that \textit{SPAG17} is necessary for sperm flagellum development and important for proper assembly of the respiratory motile cilia central pair apparatus but is not required in a similar manner in the development of ependymal cilia. \textit{Spag17} is highly expressed in the testis, with moderate expression levels in the lungs, and the lowest levels observed in the brain (Fig. 1C,D). We note a correlation between the basal \textit{Spag17} expression levels and the downstream results of \textit{Pcdo} mutation in the different motile cilia examined. Spermatocytes and round spermatids seem to have an absolute requirement for \textit{SPAG17}. Although other cell types of motile cilia can be more tolerant of \textit{Pcdo} mutation, the respiratory cilia cannot compensate for the mild reduction in \textit{Spag17}. At the protein level, \textit{SPAG17} is known to interact with other central pair proteins, such as \textit{SPAG16} and \textit{SPAG6}, and to form a complex at the C1a microtubule projections (Fu et al., 2019; Wargo et al., 2005; Zhang et al., 2005; Zhao et al., 2019). One explanation of this finding is that the minimal levels of \textit{SPAG17} produced in the \textit{Pcdo} mutant ependymal cells were sufficient to facilitate the interaction with other proteins and to form the C1a projection, but much more \textit{SPAG17} was needed in the respiratory cilia to form these protein complexes and thereby form the correct central pairs of the motile respiratory cilia. All isoforms of \textit{SPAG17} were completely abolished in the \textit{Pcdo} mutant testes; therefore, it seems that the C1a projection interactome was unable to form, and this led to complete failure of formation of the sperm flagellum. Several testis-specific proteasome/ubiquitin enzymes have been reported in rodents (Mochida et al., 2000; Nishito et al., 2006) and humans (Mitchell et al., 1991; Uechi et al., 2014). It is therefore also possible that the mutant \textit{SPAG17} protein is degraded in the testis by a sperm-specific proteasome-mediated quality control mechanism (Morales et al., 2003; Sutovsky, 2011; Zimmerman and Sutovsky, 2009). Other possibilities include the simple explanation that not all tissues construct axonemes from the same protein set, and \textit{SPAG17} is functionally compensated for in cilia outside the testis. Altogether, these data are consistent with recent reports that effects of specific gene mutations on protein translation and thereby function might differ dramatically between various cell types expressing the same allele (Lucas et al., 2019).

In summary, this study describes a new allele of \textit{Spag17} that encodes the C1a projection \textit{SPAG17} protein. The \textit{Pcdo} allele recapitulated most PCD phenotypes previously observed in other models due to central pair abnormalities. These are often the most difficult to diagnose in humans owing to lack of laterality phenotypes, only subtle beating defects and largely undisturbed ciliary ultrastructure (Edelbusch et al., 2017). We suggest that the \textit{Spag17}^{\text{Pcdo}} allele is a very useful model for studying the pathogenesis and molecular mechanisms of human PCD subsequent to central pair defects.
MATERIALS AND METHODS

ENU mutagenesis and recovery of Pcdo mutants

ENU mutagenesis was performed as described by Herron et al. (2002) and Stottmann and Beier (2014). Briefly, 6- to 8-week-old C57BL/6 Mus musculus males (G0 males) were injected intraperitoneally with three weekly fractioned doses of ENU and then bred to FVB Mus musculus females (The Jackson Laboratory) to generate G1 heterozygous carrier males. These G1 males were then outcrossed to FVB females to generate G2 potentially heterozygous carrier females. These G2 females were backcrossed to their respective G1 male parent to generate G3 embryos or pups, which were screened for organogenesis phenotypes. The Pcdo mutant mice were first identified with diluted brain ventricles and hydrocephalus. All animals were maintained through a protocol approved by the Cincinnati Children’s Hospital Medical Center IACUC committee (IACUC2016-0098), and animal care and use complied with all relevant local animal welfare laws, guidelines and policies. Mice were housed in a vivarium with a 12 h-light-dark cycle, with food and water ad libitum.

Histological analysis

Neonatal pups were sacrificed by decapitation. For histology of the adults, littermate animals underwent cardiac perfusion using cold heparinized PBS and formalin (Sigma-Aldrich) solution. Brains, lungs and testes were dissected and fixed for ≤72 h in formalin at room temperature, followed by immersion in 70% ethanol (for histology). Samples were then paraffin embedded and sectioned at 6 μm thickness. Sections were processed for Hematoxylin and Eosin staining with standard methods. Histological sections were imaged using a Zeiss Discovery V8 Stereoscope or Nikon NIE upright microscope. The area of the lateral ventricles was measured from the coronal brain sections. We limited our analysis to sections that contained the anterior commissure as an anatomical landmark for consistency between wild-type and the Pcdo mutant animals. Analysis was performed using the area measurement tool within the Nikon Element 4.50 software.

Immunohistochemistry

Immunohistochemistry was performed as previously described by Driver et al. (2017). Briefly, fixed paraffin blocks from control and mutant animals were sectioned at a thickness of 6 μm. Sections were deparaffinized and rehydrated in graded ethanol. Antigen retrieval was performed by boiling in citrate buffer pH 6.00 for 1 min in a microwave. Non-specific antigens in the tissue sections were blocked in 4% normal goat serum in PBS-Tween, and washes in PBS-Tween and application of Alexa Fluor 596-conjugated goat anti-mouse secondary antibody (1:500, Invitrogen, cat# A11005) for 1 h, followed by three washes in PBS-Tween. Nuclei were counterstained with 4',6-diamidino-2-phenylindole (DAPI) and mounted in ProLong Gold antifade mounting media (Invitrogen). Images were captured using a Nikon C2 confocal microscope.

Whole-exome sequencing

Brain tissue from three obviously affected, postnatal male mice was used for exome analysis. Whole-exome sequencing was done with BGI Americas, using the TaqMan Sample-to-SNP kit (Applied Biosystems) for a single-nucleotide change at mouse chr1:100088282A>T (assay ID ANWCWCA). TaqMan Sample-to-SNP assays were performed according to the manufacturer’s instructions and run using a QuantStudio 6 Real Time PCR machine (Applied Biosystems).

Semi-quantitative PCR (RT-PCR)

Total RNA was extracted from testes, lungs and whole brains of adult mice using TRIzol reagent (Thermo Fisher Scientific) according to the manufacturer’s instructions. Five micrograms of total RNA was used for reverse transcription into complementary DNA (cDNA) using the SuperScript III First Strand synthesis system (Invitrogen) according to the manufacturer’s directions. Final cDNA products were diluted 1:10, after which 2 μl of the diluted cDNA was used to amplify Spag17 transcripts with primers that span exon 33 and exon 39 (forward, 5′-GATGGAGGCTCAGAAAAGC-3′; reverse, 5′-AATCTGTTAGTTGGCTGCAA-3′). β-Actin was used as loading control (forward, 5′-GTGACGTGGCATCCGAAAAGA-3′; reverse, 5′-GCGGACTCATCGACTCCC-3′). PCR products were run on a 2% agarose gel with ethidium bromide for 45 min at 120 V. PCR bands were imaged using a Bio-Rad Universal Hood II Molecular Imager w/CFW-1312M Camera equipped with Image Lab v.6.0.1 software. The image analysis tool in the Image Lab v.6.0.1 software was used to quantify the band intensity value. Data obtained from the Spag17 exon 33-39 bands was normalized against the β-actin value of the corresponding sample. Finally, wild-type and mutant data obtained for testes, lungs and brain were normalized against the average wild-type testis expression values. One-way ANOVA with multiple comparisons statistical analyses were performed using GraphPad Prism v.8.0.1 software.

Western immunoblotting

Adult mouse brain and testis tissues were lysed in Pierce RIPA buffer (Thermo Fisher Scientific, cat# 89901) containing protease inhibitor cocktail (Roche, cat# 11697498001). The protein concentration in the whole cell extracts was determined with the BCA colorimetric assay (Thermo Fisher Scientific) according to the manufacturer’s instructions. Denatured proteins were separated by electrophoresis on a gradient of 4-12% Tris-glycine gel. The protein was transferred to a polyvinylidene difluoride membrane, blocked in Odyssey blocking buffer and incubated overnight at 4°C with 1:3000 rabbit anti-N-terminus or C-terminus anti-SPAG17 antibodies (Zhang et al., 2005) and 1:2000 mouse anti-tubulin antibodies (Sigma-Aldrich, cat# T6793). Membranes were washed and incubated for 1 h in 1:15,000 goat anti-rabbit IRDye 680CW (LICOR) and 1:15,000 goat anti-mouse IRDye 800Rd (LICOR), and bands were visualized on the LICOR Odyssey imaging system.

Scanning electron microscopy

The medial and lateral walls of the P8 forebrains were processed for SEM as previously described (Abdelhamed et al., 2018). Briefly, samples were fixed in electron microscopy grade 2% paraformaldehyde and 2.5% glutaraldehyde in 0.1 M sodium cacodylate buffer (pH 7.4) at 4°C overnight. Tissues were washed thoroughly in 0.1 M sodium cacodylate buffer (pH 7.4) then post-fixed in 1% osmium oxide (diluted in 0.1 M sodium cacodylate buffer) for 1 hour. Samples were washed thoroughly in 0.1 M sodium cacodylate buffer and dehydrated before critical point drying in 100% ethanol. Brain tissues were then coated with gold palladium using a sputter coater (Leica EM ACE600) and scanned with a Hitachi SU8010 scanning electron microscope.

TEM

Brain ependymal cells and trabecal epithelial cells were fixed in 2.5% glutaraldehyde and processed for TEM analyses by standard protocols as previously reported (Wallmeier et al., 2019). Sections were collected on copper grids, stained with Reynold’s lead citrate and visualized using the Philips CM10 or Jeol 1400+.
High-speed video microscopy of cilia, ciliary beat frequency and CSF flow analysis

High-speed video microscopy of the beating ependymal cilia, ciliary beat frequency and CSF flow analysis were performed as previously described (Abdelhamed et al., 2018). Briefly, brains were dissected out from P4 Spag17<sup>−/−</sup> or wild-type control animals in Dulbecco’s modified Eagle’s medium: Nutrient Mixture F-12 (DMEM/F12) supplemented with L-glutamine (Gibco) and 1% N2 supplement (Gibco) at room temperature, and cut into serial 200-μm-thick coronal sections. Sections were obtained from the forebrain and aqueduct. Areas from the medial wall of the lateral ventricle were microdissected further and subjected to video microscopy recording at ~100 frames/s. These videos were used to measure the ciliary beat frequency. For concurrent imaging of the beating cilia with green fluorescent microbeads (FluoSpheres, Thermo Fisher Scientific), the beads were introduced into slices immediately before video recording, and we used an inverted Nikon Ti-E wide-field microscope fitted with a Nikon ×40 Plan Apo 0.95 N.A. objective and an Andor Zyla 4.2 PLUS sCMOS monochromatic camera. Light was channeled through a custom quad-pass filter, and 300 frames were collected at ~60 frames/s. Bead tracking and ciliary beat frequency analysis were performed using NIS Element software v.4.5. Statistical analysis and pairwise comparisons were performed using GraphPad Prism software.

Skeletal preparations

For skeletal preparations, adult animals were sacrificed by induction of deep anesthesia using isoflurane, followed by trans-diaphragmatic cardiac perfusion with 4% formaldehyde, 0.1 M sodium phosphate buffer, 0.5 M sucrose in 0.1 M phosphate buffer. Spinal or maxillary bones were then dissected free of surrounding tissues and fixed for a minimum of 2 weeks in 4% formaldehyde. After fixation, samples were dehydrated and embedded in paraffin. Sections (10 μm thick) were cut using a microtome and stained with Hematoxylin and Eosin (H&E) for histological evaluation. Images were captured using a Zeiss Axioscope microscope equipped with a Zeiss AxioCam MRm digital camera.

Generation, culture and immunocytochemistry of MEFs

MEFs were generated from embryonic day (E)13.5 embryos. Embryos were dissected in PBS, decapitated and eviscerated. The remaining tissue was incubated in trypsin overnight at 4°C. Tissue fragments were incubated with the trypsin in a 5% CO<sub>2</sub> incubator for 30 min. Cells were then allowed to grow to confluency in complete DMEM containing 10% fetal bovine serum and penicillin/streptomycin. MEFs were stained within three passages of growth to confluency in complete DMEM containing 10% fetal bovine serum to minimize the risk of contamination. MEFs were stained within three passages of growth to confluency in complete DMEM containing 10% fetal bovine serum to minimize the risk of contamination. MEFs were stained within three passages of growth to confluency in complete DMEM containing 10% fetal bovine serum to minimize the risk of contamination.

Reagents, methodology and statistical analysis

MEFs were generated in the laboratory directly from mouse embryos with standard protocols. All antibodies (with the exception of anti-SPAG17) used in this study were commercially available, and our results matched multiple published reports. SPAG17 results were consistent with previous results, and our results matched multiple published reports.

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Supplementary information

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