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Myosin Id is required for planar cell polarity in ciliated tracheal and ependymal epithelial cells

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Abstract

In wild type (WT) tracheal epithelial cells, ciliary basal bodies are oriented such that all cilia on the cell surface beat in the same upward direction. This precise alignment of basal bodies and, as a result, the ciliary axoneme, is termed rotational planar cell polarity (PCP). Rotational PCP in the multi-ciliated epithelial cells of the trachea is perturbed in rats lacking myosin Id (Myo1d). Myo1d is localized in the F-actin and basal body rich subapical cortex of the ciliated tracheal epithelial cell. Scanning and transmission electron microscopy of Myo1d knock out (KO) trachea revealed that the unidirectional bending pattern is disrupted. Instead, cilia splay out in a disordered, often radial pattern. Measurement of the alignment axis of the central pair axonemal microtubules was much more variable in the KO, another indicator that rotational PCP is perturbed. The asymmetric localization of the PCP core protein Vangl1 is lost. Both the velocity and linearity of cilia-driven movement of beads above the tracheal mucosal surface was impaired in the Myo1d KO. Multi-ciliated brain ependymal epithelial cells exhibit a second form of PCP termed translational PCP in which basal bodies and attached cilia are clustered at the anterior side of the cell. The precise asymmetric clustering of cilia is disrupted in the ependymal cells of the Myo1d KO rat. While basal body clustering is maintained, left-right positioning of the clusters is lost.

Key terms

rotational planar cell polarity; translational planar cell polarity; cilia; basal body

INTRODUCTION

Myosin Id [Myo1d] is one of eight class one myosins expressed in mammals including humans [Berg et al. 2001]. It is one of six “short-tailed” Myo1s, consisting of an N-terminal head/motor domain, a neck domain containing two calmodulin binding IQ motifs and a short, positively charged C-terminal tail (tail homology 1; TH1) domain [Bahler et al. 1994].

Short-tailed Myo1s lack the proline rich TH-2 and sarc homology 3 -containing TH3 domains of long-tailed Myo1s [Krendel and Mooseker 2005]. Myo1d is expressed in a broad range of tissues with highest levels in brain [Bahler et al. 1994]. Myo1d is expressed in both the central and peripheral nervous system. Myo1d is highly expressed in myelinated axons [Benesh et al. 2012] and it has been identified as an enriched component in proteomic analyses of purified myelin fractions [Ishii et al. 2009; Jahn et al. 2009; Yamaguchi et al. 2008]. Recent studies have shown that Myo1d is also expressed in myelinating oligodendrocytes [Yamazaki et al. 2014]. A potential role for Myo1d in myelination was established with the finding that it interacts with and colocalizes with aspartoacylase [Benesh et al. 2012], an enzyme that plays a critical role in myelination [Mattan et al. 2010]; deficiencies in this enzyme are responsible for the neurodegenerative disease, Canavan disease [Namboodiri et al. 2006]. There is also evidence that Myo1d is involved in endocytic membrane traffic [Huber et al. 2000]. Finally, Myo1D has been identified as a candidate gene involved in autism spectrum disorders (ASD) [Stone et al. 2007].

The homolog of Myo1d in *Drosophila*, myosin IA (Myo1A or Myo31d-f) [Morgan et al. 1995; Morgan et al. 1994], has been shown in an elegant series of studies to play an essential role in determination of left-right visceral asymmetry (reviewed in [Geminard et al. 2014]). Two independent studies discovered this role for Myo1 in screens for mutations that disrupted left-right visceral asymmetry [Hozumi et al. 2006; Speder et al. 2006]. Myo1A mutants revealed *situs inversus* of the dextral looping of the hindgut and male genitalia. These initial studies also provided key insights into molecular bases for the involvement of Myo1A in determining left-right asymmetry. Myo1A was shown to interact with and colocalize with the adherens junction component, β catenin [Speder et al. 2006]. Myo1A is negatively regulated by another *Drosophila* class I myosin with which it is co-expressed, Myo1B (or Myo61f; [Morgan et al. 1995]). Overexpression of Myo1B in a Myo1A mutant background results in partial restoration of visceral left-right asymmetry; conversely knock down of Myo1A in a wild type (WT) background results in defects in left-right patterning [Hozumi et al. 2006]. More recent studies have provided additional insights into the cellular and molecular bases for the involvement of *Drosophila* Myo1A in left-right asymmetry determination. Myo1A also interacts with the adherens junction protein (D) E-cadherin, and this interaction is inhibited by Myo1B [Petzoldt et al. 2012]. In the embryonic gut, epithelial cells become asymmetric along their left/right axis, a pattern termed planar cell chirality (PCC). The distribution of DE cadherin also becomes asymmetric and its localization is dependent on Myo1A; PCC is lost in the Myo1A mutant [Taniguchi et al. 2011]. Phenotypic characterization of flies doubly mutant for Myo1A and Myo1B demonstrated that these myosins have both overlapping as well as tissue specific functions in determination of left-right asymmetry [Okumura et al. 2015]. Most recently it has been shown that Myo1A interacts with the atypical cadherin, Dachshous and acts as a left/right organizer to control cell polarity of adjoining gut progenitor cells. Left/right hindgut looping asymmetry is lost in the absence of Dachshous [Gonzalez-Morales et al. 2015].

In the present study we have conducted an initial phenotypic characterization of a Myo1d knock out (KO) rat generated by transposon insertional mutagenesis [Lu et al. 2007]. Myo1d is not functionally homologous to *Drosophila* Myo1A in that these rats do not exhibit *situs*

inversus. However, these KO rats do exhibit marked disruption of rotational planar cell polarity (PCP) in the multi-ciliated cells of the trachea. Translational PCP, the anterior clustering of cilia in brain ventricular ciliated ependymal cells, is also perturbed.

RESULTS

Verification of loss of Myo1d expression in the Myo1d KO rat

As detailed in Materials and Methods, Myo1D knockout rats were generated in the Fischer (F344) rat background by transposon insertional mutagenesis [Lu et al. 2007]. Briefly, animals transgenic for a mutagenic gene-trap transposon element and *Sleeping Beauty* transposase transgene were bred to generate offspring with random transposon insertion mutations. In one rat, a spontaneous transposon insertion was identified in intron 20 of the *Myo1D* gene by linker-mediated PCR and Sanger sequencing, and confirmed by PCR amplification using one primer in the flanking genomic sequence and one primer inside the transposon (see Materials and Methods). This animal was back crossed and intercrossed to establish a colony homozygous for the gene-trap insertion. When the KO rat line was first made available to us our initial studies focused on effects of loss of Myo1d function on the brush border cytoskeleton of the intestinal epithelial cell. Myo1d is associated with the both the tips and bases of brush border microvilli [Benesh et al. 2010]. Thus to verify loss of Myo1d protein expression, immunoblot analysis of intestinal epithelial cells and isolated brush borders from WT and Myo1d KO rats was performed. This analysis confirmed the absence of Myo1d in the Myo1d KO (Supplementary Figure 1). Unfortunately, although we did observe frequent phenotypic defects the phenotypes observed varied from rat to rat, and thus this analysis was put aside. Ironically, in light of the studies presented here however, the variability we observed might be due to effects on PCP dependent orientation of the mitotic spindle in crypt stem cells [Fleming et al. 2007].

The loss of Myo1d function does not cause visceral *situs inversus*

As noted in the Introduction, based on the phenotype of *Drosophila* Myo1A mutants, and the interaction of Myo1d with aspartoacylase, two potential functions for Myo1d include determination of left-right visceral asymmetry and axon myelination. However, no significant incidence of *situs inversus* has been observed (only 1 rat of ~ 50 examined thus far exhibited reversed organ asymmetry). Moreover, these rats exhibit no obvious motor defects, suggesting that axon myelination is not significantly affected. We also examined Myo1d KO hippocampal synapse morphology and number and found no obvious differences at the ultrastructural level. Kidney and liver morphology also were indistinguishable from WT. With respect to behavioral phenotypes, we have observed nothing overtly obvious, although given the link between Myo1d and ASP, more detailed and controlled assessment of behavior in the KO would certainly be warranted (see Discussion).

Myo1d is expressed in the subapical cortex of ciliated tracheal epithelial cells

Frustrated by the variability we observed in our studies on effects of loss of Myo1d on intestinal epithelial cells, and prompted by the role of its *Drosophila* homolog in determining PCC, we decided to see if there might be a functional connection between Myo1d and PCP. Thus we chose to examine ciliated epithelial cells of the trachea, one of the

best characterized cell types with respect to PCP determination in mammals. To determine if loss of Myo1d might contribute to PCP defects in tracheal epithelial cells, it was critical to first determine if this myosin is expressed in these cells. Expression of Myo1d in trachea was confirmed by immunoblot analysis of homogenates of mechanically isolated airway epithelial cells (Fig. 1D inset). Localization of Myo1d in WT trachea epithelial cells verified that Myo1d is expressed and is localized within the F-actin (Fig. 1A) and basal body rich (Fig. 1C) sub-apical cortex of tracheal epithelial cells. In addition there is a thin zone of Myo1d staining that is apical to both the cortical F-actin (Fig. 1A) and γ -tubulin-stained basal bodies (Fig 1C). However, through-focus imaging of WT tracheal epithelia co-stained for Myo1d and F-actin indicated that in cells expressing Myo1d, Myo1d predominantly co-localizes with F actin and is not asymmetrically distributed. Myo1d is absent from cilia based on co-localization with the axonemal marker, acetylated α -tubulin (Fig. 1B). The intensity of subapical Myo1d staining in non-ciliated cells is substantially less than in ciliated cells (Fig 1B, C). There is no obvious difference in subapical F-actin staining in non-ciliated cells of WT and KO in which cells with both high and low intensity of F actin staining cells are observed (Supplementary Figure 2). Cells with low or no apical F-actin staining are likely to be mucous secreting cells. The diffuse Myo1d staining in the cytoplasm of the WT tracheal epithelial cells (Fig 1A–C) is most likely nonspecific given the similar diffuse staining observed in the KO (Fig. 1D).

Multi-ciliated cells of the tracheal epithelium exhibit defects in rotational PCP

In multi-ciliated cells such as those of the trachea, beat direction is determined by the asymmetric orientation of ciliary basal bodies [Satir 1980]. In the trachea this results in the coordinated upward and unidirectional lung-to-mouth beating of cilia which results in clearance of the airway of particulates and uniform distribution of the protective mucus layer. This asymmetric basal body orientation is termed rotational PCP [Wallingford 2010]. Another key feature of basal body asymmetry is the positioning of the basal foot, a triangular structure that projects from the side of the basal body in the direction of ciliary beat [Kunimoto et al. 2012]. Examination of ciliary organization by immunofluorescence microscopy of tracheal epithelium labeled for the axonemal marker acetylated α -tubulin indicated that the cilia in the Myo1d KO had a disheveled appearance in comparison to WT (Fig. 2). However, the tight cortical organization of basal bodies observed in the WT (Fig. 1) is not perturbed in the Myo1d KO (Supplementary Fig. 3) and the overall tissue morphology of the epithelium in the KO is comparable to WT.

The disrupted orientation of cilia in the Myo1d trachea was confirmed by scanning electron microscopy (SEM). In WT trachea, both densely (Fig. 3A) and more sparsely populated regions of ciliated cells (Fig. 3C), the cilia all bend in the same direction on a given cell and with respect to surrounding cells (the intercellular alignment of cilia is termed tissue-level PCP [Wallingford 2010]). In contrast, in the Myo1d KO, cilia are splayed out in different directions, most commonly in a somewhat radial pattern in regions of both sparse and more dense populations of ciliated cells (Fig. 3B, D). This radial splaying often exposes the microvilli-studded cell surface at the centers of Myo1d KO ciliated epithelial cells (Fig. 4A). Note that the domed appearance of non-ciliated cells in the KO example in Fig. 3D is not unique to the KO. The apical surface of both WT and Myo1d KO non-ciliated cells varies

from flat to prominently domed depending on the region of tracheal surface imaged. The loss of unidirectional cilia bending was also evident in transmission EM (TEM) of longitudinal sections of the apical domain of Myo1D KO tracheal epithelial cells (Fig. 5B).

To determine if the perturbation in ciliary organization was due to loss of coordinated basal body orientation, cross sections through lawns of cilia from WT and Myo1d KO were examined by TEM to determine if the orientation of the axonemal central pair of MTs was disordered in the Myo1d KO [Kunimoto et al. 2012]. This is determined 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 [Kunimoto et al. 2012]. The deviation from this angle is significantly greater in the Myo1d KO, indicating that the ciliary disorder observed in the KO is due to perturbations in rotational PCP (Fig. 6).

In WT tracheal epithelial cells, a number of PCP core proteins exhibit asymmetric localization to one side of the cell at the level of intercellular junctions. For example Vangl1 localizes to the distal, lung side of the apical junction [Kunimoto et al. 2012; Vladar et al. 2012]. Immunolocalization of Vangl1 in WT trachea showed the expected asymmetric localization, although because of the irregular surface of the tracheal mucosa, asymmetric localization pattern can only be observed in small patches of adjacent cells (Fig. 7A). In the Myo1d KO trachea the asymmetric distribution of Vangl1 is lost in the KO. Instead, Vangl1 is localized circumferentially, and there is staining of the cell periphery on both sides of cell-cell contact. This is particularly apparent at the junctional vertices of 3 cells (Fig. 7B).

Disruption of rotational PCP should also have effects on cilia-driven fluid flow (e.g. as shown in [Kunimoto et al. 2012]. To evaluate the effects of perturbed ciliary organization on fluid flow, time lapse movies of fluorescent beads placed over the surface of segments of WT and KO trachea were recorded. In WT, most beads moved in linear tracks, and adjacent beads moved in parallel. The average bead velocity over WT tracheal epithelium was $13.3 \pm 4.6 \mu\text{m/s}$ ($n=107$ beads). In the KO, track direction changed frequently and net rates of movement were reduced ($3.4 \pm 1.4 \mu\text{m/s}$, $n=116$ beads; Fig. 8). Beads imaged away from the tracheal epithelial surface exhibited Brownian movement, indicating that bead movement observed adjacent to the epithelium was driven by ciliary beating and not convective flow in the chamber (Supplementary Fig. 4).

It should be noted that despite the observed impaired ciliary flow, the Myo1d KO rats do not exhibit overt symptoms such as coughing or wheezing that have been documented for Odf-2 KO mice [Kunimoto et al. 2012].

There is increased apoptosis of Myo1d KO tracheal epithelial cells

In previous studies on other myosin KOs including Myo1a [Tyska et al. 2005] and mice lacking both Myo1a and Myo6 [Hegan et al. 2015] we noted a marked increase in apoptotic, terminal deoxynucleotidyl transferase dUTP nick end labeled (TUNEL) nuclei in the intestinal epithelium. However, unlike the intestinal epithelial mucosa, which turns over on a time scale of days, turnover of tracheal epithelial cells is quite slow, with a half-life of ~ 6 months in mice [Rawlins and Hogan 2008]. Nevertheless, TUNEL staining of nuclei in WT and Myo1D KO trachea revealed a marked increase in the frequency (~ 8 fold greater

corrected total cell fluorescence (CTCF); see Methods) of TUNEL positive tracheal epithelial cell nuclei (Fig. 9). The increased frequency of apoptosis could be a direct consequence of impaired fluid flow and resultant increased levels of cellular stress. Alternatively this could result from loss of Myo1d functions that are independent of its role in PCP; for example, impaired Myo1d-dependent endocytic membrane traffic [Huber et al. 2000].

Translational PCP is disrupted in ciliated ependymal epithelial cells

Although assessment of rotational PCP in the Myo1d KO was the primary focus of this study, we also have conducted ultrastructural studies to determine if PCP may be perturbed in the ciliated ependymal epithelial cells of the brain ventricle. These cells exhibit a second form of PCP in which all the basal bodies are aligned in a cluster at the anterior side of the cell. This has been termed translational PCP [Mirzadeh et al. 2010]. TEM of WT ependymal epithelium revealed the expected polarized clustering of basal bodies and cilia to one side of each cell (Fig. 10A). Although there is basal body/ciliary clustering in the Myo1d KO, many adjacent cells have the clusters on either side of the junctional complex between the two cells (Fig 10B–D) indicating that translational PCP is perturbed. This loss of anterior clustering was also apparent in ependymal epithelium stained with the basal body marker, ODF-2, and axonemal marker, acetylated α -tubulin (Fig. 11).

DISCUSSION

The results presented here demonstrate that Myo1d plays a role in establishing and/or maintaining rotational PCP in tracheal ciliated epithelial cells. Other key determinants of rotational PCP have been well characterized. These include homologs of PCP core proteins first identified in *Drosophila* including Frizzled, Vangl, Disheveled and Prickle as well as Inversin. Proteins thought to act down stream of these core PCP proteins include Fuzzy and Inturned (reviewed in [Kishimoto and Sawamoto 2012; Wallingford 2010]. We have shown that the asymmetric localization of the PCP core protein Vangl1 is lost in the Myo1d KO, suggesting that it plays a role analogous to its *Drosophila* homolog, Myo1A, which is required for asymmetric localization of DE-cadherin. Based on this initial observation, it is likely that Myo1d functions in tandem with at least Vangl1. It will be critical to determine if the asymmetric localization of any of the other PCP core proteins such as Vangl2, Frizzled 3, Disheveled 1 and 3 and Celsr1 [Vladar et al. 2012] is affected in the Myo1d KO tracheal epithelial cell.

A key player down stream of PCP core proteins in determining rotational PCP in tracheal epithelial cells is the cortical microtubule (MT) cytoskeleton. In these cells, MTs exhibit PCP, with MT minus ends associated with basal bodies and plus ends with lateral membrane domains at which the PCP core proteins Frizzled and Disheveled are localized. Treatment of cultured mouse tracheal epithelial cells with the MT inhibitor, nacadazole, disrupts rotational PCP [Vladar et al. 2012]. An important question to be addressed in future studies will be to determine if the PCP organization of the cortical MT cytoskeleton is perturbed. Such studies should preferably be done using primary cultures of tracheal epithelial cells which are far more suitable for MT localization than tracheal tissue [Vladar et al. 2012].

Another critical determinant of rotational PCP in the tracheal epithelial cell is the basal foot component Odf-2. In mice lacking Odf-2, basal feet are absent and rotational PCP is disrupted [Kunimoto et al. 2012]. Basal feet are present on the Myo1d KO tracheal basal bodies (Supplementary Fig. 5), so this myosin is not required for basal foot assembly.

The involvement of Myo1d in ependymal epithelial cell translational PCP was unexpected, since key determinants involved in rotational PCP are not involved in ependymal cell translational PCP [Kishimoto and Sawamoto 2012]. Two factors have been shown to contribute to translational PCP. Ablation of the primary cilium in the embryonic radial glial cells disrupts subsequent translational PCP in the ependymal cells [Mirzadeh et al. 2010]. Thus far the only protein shown to be involved in translational PCP in ependymal cells is nonmuscle Myo2. Pharmacological or RNAi knock down inhibition of Myo2 via ventricular injection in neonatal mice interferes with the anterior clustering of cilia [Hirota et al. 2010]. However, unlike Myo2 inhibition, which causes a loss of clustering and dispersal of basal bodies and attached cilia throughout the apical cell surface, the basal bodies remain clustered in the Myo1d KO. It is possible that Myo1d works in tandem with Myo2 to effect the anterior movement of basal bodies during ependymal cell differentiation. Alternatively it may play a role in maintenance of the anterior basal body cluster once translational PCP has been established. In this regard it would be of interest to examine whether basal body migration occurs normally during ependymal cell differentiation but then is subsequently lost. The fact that basal bodies are clustered at either anterior or posterior sides of the cell in the Myo1d KO suggests that it could play a role in polarity of migration, and not migration itself. Hopefully these initial findings will prompt other investigators in the PCP field to explore these and other questions taking advantage of this commercially available rat KO line.

It is important to note that not all cell types and tissues that exhibit PCP are affected in the Myo1d KO (reviewed in [Goodrich and Strutt 2011]). For example, these rats show no signs of hearing or vestibular dysfunction, an indication that inner ear neurosensory cell PCP mediated positioning of the kinocilium and stereo cilia are probably unaffected. Hair follicle directionality, a PCP-specified feature of the epidermis, is normal. As already noted, the rats do not exhibit *situs inversus* of the viscera indicating the PCP-dependent positioning of the motile cilia in the embryonic node is normal.

Another potentially exciting line of investigation utilizing the Myo1d KO will be to explore its utility as a novel rat model for ASD. The gene encoding Myo1d has been linked to ASD [Stone et al. 2007]. Assuming both rotational and translational PCP are disrupted in the Myo1d KO, it is likely that ventricular flow of cerebrospinal fluid (CSF) is impaired which in turn would result in increased ventricular fluid volume due to reduced rates of absorption by the ventricular subarachnoid spaces [Kishimoto and Sawamoto 2012]. Clinical studies have determined that there is increased CSF volume within the ventricles of ASD patients [Bradstreet et al. 2014; Hallahan et al. 2009; Shen et al. 2013]. A first step to explore the causal link between Myo1D and ASD would be to assess the KO rat for ASD-like behaviors using standard behavioral assays including assessment of social behavior, ultrasonic vocalization, anxiety levels and exploratory and repetitive behaviors [Zhang-James et al. 2014].

MATERIALS AND METHODS

Animals

The Myo1d KO rat line was provided to us for phenotypic screening by Transposagen, (Lexington, KY). It was generated in the Fischer rat background by transposon insertional mutagenesis [Lu et al. 2007] at the Medical College of Wisconsin under protocols approved by the Institutional Animal Care and Use Committee (IACUC) in accordance with the NIH Guide for Care and Use of Laboratory Animals. The Myo1d^{Tn(sb-T2/Bart3)2.334Mewi} strain (RGD ID: 2311691) harbors a gene trap *Sleeping Beauty* transposon insertion in intron 20 (Rn5; Chr10: 67,587,557) of *Myo1D* (NM_012983.2). The insertion was confirmed and genotyped using a three-primer PCR assay using primers Myo1D_F: CTGAAGTGTGGTTGGTGTGGG, Myo1D_R: CACACACAGACGGAGGATCAAG; and ITRL_L: CTGACCTAAGACAGGGAATT. Wild type male Fischer control rats were purchased from Charles River Laboratories (Wilmington, MA.) All data shown were generated using male rats. All procedures involving rats were performed in accordance with an approved Yale – IACUC animal protocol.

TEM and SEM

Tissues were processed for TEM as described in [Mooseker and Tilney 1975]. Tracheas were dissected from WT and Myo1d KO rats, gently flushed with 4° phosphate buffered saline (PBS). The tissue was cut into small pieces fixed in 2% glutaraldehyde, 0.2% tannic acid in 0.1M sodium phosphate buffer, pH 7.0 for 10 minutes at room temperature (RT), and then shifted to 4° for an additional 50 minutes. Following fixation the tissue was washed three times with 0.1M sodium phosphate buffer, pH 7.0, and then incubated on ice for 1 hour in 1% OsO₄ in 0.1M sodium phosphate buffer, pH 6.0. The tissue was then washed three times with cold water and incubated overnight at 4° in 1% uranyl acetate. The samples were dehydrated with a 25 – 100% ethanol series at room temperature, followed by two 10 minute incubations in propylene oxide. The tissue was embedded in EmBed 812 (Electron Microscopy Sciences (EMS), Hatfield, PA) following the manufacturer's recommended protocol. Imaging was done on a JEOL 1230 TEM (JEOL, Tokyo, Japan), and digital images were recorded with a Hamamatsu ORCA-HR digital camera (Hamamatsu, Hamamatsu City, Japan). For TEM analysis of brain ventricular ependymal epithelial cells, the brain of WT and Myo1d KO rats was dissected intact and bisected along the anterior/posterior axis to expose the ventricles. The tissue surrounding the ventricles was dissected as a pair of rectangles, and these were processed for TEM as described above.

Measurement of tracheal axonemal central pair angles—TEM micrographs of cross sections through tracheal epithelial cell cilia were used to determine if the coordinate asymmetric orientation of basal bodies was affected in the Myo1d KO as described in [Kunimoto et al. 2012]. A cross section was randomly selected on each micrograph and a line was drawn connecting the two central pair microtubules (MTs) and its angle arbitrarily assigned a value of 0–180°. The angle of lines drawn through central pairs of the other cilia in cross section in each micrograph was determined and recorded using ImageJ (<http://rsb.info.nih.gov/ij/>; NIH).

For SEM of tracheal epithelial cells, sheets of trachea were fixed as described above for TEM except tannic acid was omitted from the glutaraldehyde fixation step and the OsO₄ fixation was performed in 0.1M sodium phosphate buffer at pH 7.0. After OsO₄ fixation tissues were rinsed in several changes of 0.1M sodium phosphate buffer and then dehydrated in a graded (20–100%) series of ethanol solutions. Tissues were then critical point dried, glued to SEM specimen stubs and sputter coated with gold. Samples were viewed in an FEI XL-30 SEM at an accelerating voltage of 10 kV.

Tissue preparation for immunofluorescence microscopy

For immunolocalization studies on tracheal and ependymal epithelial cells, tissues were dissected as described above for TEM. Tissue pieces were fixed with 4 % paraformaldehyde (prepared from a freshly opened vial of 16% PFA from EMS) in PBS for 10 minutes at RT and then shifted to 4°C for an additional 50 minutes. The tissue was washed twice with PBS, followed by 30 minutes in each of 5%, 8%, 12%, and 16% sucrose in PBS at RT. The tissue was incubated overnight in 20% sucrose in PBS at 4°C, and the following day was incubated in 12% sucrose, 35% OCT (Sakura Finetek USA, Torrance, CA) in PBS for 1 hour at RT. The tissue was embedded in 12% sucrose + 35% OCT in PBS and stored at –80°C. Five micron sections were cut on a Leica CM 3050S cryostat (Leica, Wetzlar, Germany) and mounted on Superfrost + slides (Esco, Portsmouth, NH). The following steps were done with either PBS or TRIS buffered saline, with 0.05% Tween 20 as the buffer, depending on the primary antibody used. The sections were washed 3×5 minutes with room temperature PBS to remove OCT. The tissue was blocked for 1 hour at RT in 10% normal goat serum (NGS) in PBS. Primary antibodies were diluted in PBS + 5% NGS and incubated at RT for 1 hour, or overnight at 4°. The slides were then washed 3×10 minutes with PBS. Alexa fluor labeled secondary antibodies (Invitrogen, Carlsbad, CA) were diluted 1:200 in PBS containing 5% NGS, 0.33µM rhodamine phalloidin (Invitrogen) and 1µg/ml Hoechst dye (Sigma, St. Louis, MO) and incubated for 1 hour at RT in the dark. Slides were then washed 3×10 minutes with PBS and coverslips were mounted with Prolong Gold anti-fade media (Invitrogen).

For localization of Myo1d in tracheal epithelial cells, tissue was also embedded in paraffin and 6 µm sections were cut following standard methods (Research Histology, Yale School of Medicine). Slides were de-paraffinized with 4 × 5 minute washes in xylenes, 3 × 3 minute washes in 100% ethanol, and then washed/re-hydrated with 2 × 5 minute water washes. Slides were then boiled for 5 minutes in a pressure cooker in 10 mM sodium citrate buffer pH 6.0, and then incubated for 5 minutes in TBS. The tissue was blocked for 45 minutes at 37°C in 5% NGS, 5% bovine serum albumin (BSA), 1% Triton X-100 in PBS. Primary antibodies were diluted in 5% NGS, 5% BSA, 0.1% Triton X-100 in PBS and incubated with the tissue sections overnight at 4°C in a humid chamber. Following antibody incubation the slides were washed 2 × 10 minutes with PBS. Alexa fluor secondary antibodies (diluted 1:200) (Invitrogen) and 1µg/ml Hoechst dye (Sigma) were incubated with the tissue in the same buffer used for the primary antibodies. Following incubation with the secondary antibodies the slides were washed 2 × 10 minutes with PBS. Coverslips were mounted using Prolong Gold (Invitrogen).

Primary antibodies used in this study—Primary antibodies directed against the following proteins were used in this study for immunofluorescence microscopy: γ -tubulin, acetylated α -tubulin, actin, Vangl1 (Sigma, St. Louis, MO), Myosin 1d (H-6; Santa Cruz Biotechnology, Santa Cruz, CA), Odf-2 (Proteintech, Chicago IL). All primary antibodies were used at 10 μ g/ml. All immunofluorescence microscopy was done using a Zeiss (Thornwood, NY) LSM510 META laser scanning confocal microscope, and digital images were captured using ZEN 2009 software (Zeiss). When possible, WT and KO tissue sections were mounted on the same slide, allowing equal treatment during preparation and were imaged with identical microscope settings. Contrast enhancement, cropping, and all other image manipulation was done using ImageJ.

Preparation of tracheal and intestinal epithelial cell homogenates and isolation of intestinal brush borders for immunoblot analysis

Dissected WT and Myo1d KO trachea were rinsed with PBS and then cut open longitudinally and the epithelial mucosal surface was scraped with the edge of a glass slide. Scrapings were homogenized in 5% trichloroacetic acid and processed for SDS-PAGE and immunoblot analysis as described in [Hegan et al. 2012]. The same procedure was used to isolate intestinal mucosal epithelium from segments of WT and Myo1d KO duodenum, jejunum and ileum. BBs were isolated from segments of WT and KO duodenum, jejunum and ileum as described in [Hegan et al. 2012].

TUNEL labeling

Cryosections from WT and Myo1d KO trachea were washed 3 $\times$ 5 minutes with PBS. Tissue sections from both genotypes were mounted on the same slide in order to ensure equal treatment during the assay. The tissue sections were then blocked/permeabilized in 10% NGS, 0.1% Triton X-100 in PBS. The TUNEL assay (Roche Diagnostics, Indianapolis, IN) was done according to manufacturer's instructions; enzyme and label were prepared at a 1:10 ratio and the tissue was incubated at 37°C for 1 hour, and then washed 3 $\times$ 5 minutes with PBS. F-actin was stained with 0.33 μ M rhodamine phalloidin (Invitrogen) for 30 minutes at RT in the dark, followed by 3 $\times$ 5 minute washes with PBS. Coverslips were mounted with Prolong Gold anti-fade media (Invitrogen), and confocal images were taken and processed as described above. To quantify the results of the TUNEL assay, fluorescence intensity per μ m² of tracheal epithelium (sectioned longitudinally as shown in Fig. 9) was determined using Image J (function for determining corrected total cell fluorescence (CTCF)).

Tracheal bead movement assay

Trachea was rapidly dissected from either WT or Myo1d KO rat and cut into quarters to get relatively flat pieces of tissue. Tissue was then incubated in DMEM media (Life Technologies, Grand Island, NY) with 10% fetal bovine serum (Thermo Fisher Scientific, Pittsburgh, PA) at 37° C with 5% CO₂. Tissue pieces were then placed in 35mm glass bottom cell culture dishes (MatTek, Ashland, MA) containing 0.75 μ m Fluoresbrite microspheres (Polysciences Inc., Warrington, PA) diluted 1:50 in DMEM + 10% FBS media. Imaging was done using a Zeiss LSM510 META laser scanning confocal microscope

equipped with an incubator (5% CO₂) and stage warmer (30° C), and digital images were captured using ZEN 2009 software. Images were captured at a rate of 1 frame per second for 120 seconds and stacks were converted to AVI videos using ImageJ. Analysis and quantification of bead motility was performed using the Manual Tracking plugin (Fabrice P. Cordelieres) for ImageJ.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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Fig. 1. Myo1d is localized to the F-actin rich apical cortex of tracheal epithelial cells and is not associated with cilia

Localization of Myo1d with F-actin (A), acetylated α -tubulin (B) and γ -tubulin (C) in WT tracheal epithelial cells. (D) Immunostaining of Myo1d KO for Myo1d and acetylated α -tubulin. Bar: 15 μ m

Fig. 2. Tracheal ciliary organization is disordered in the Myo1d KO

Localization of acetylated α -tubulin (acTub) and F-actin in WT (A, B) and Myo1d KO (C, D) trachea. Nuclei are shown in blue. Cilia in the KO have a disheveled appearance compared to WT. Except for cilia, overall cellular morphology in the KO is comparable to WT. Bar, A, C: 35 μ m, B, D: 15 μ m.

Fig. 3. SEM reveals loss of unidirectional ciliary bending pattern in the Myo1d KO trachea
SEM of WT (A, C) and Myo1d KO (B, D) trachea in region sparsely (A, B) and more densely (C, D) populated with ciliated cells. Bar, A, B: 20μm; C,D: 20μm.

Fig. 4. The splayed ciliary bending pattern in Myo1d KO tracheal epithelial cells often exposes a bare region of cell surface at the center of ciliated cells

(A) SEM of a Myo1d KO tracheal ciliated cell showing the radial splaying of cilia and bare microvillus-studded cell surface at the center of the cell. (B) SEM of a WT ciliated tracheal cell. The distal ends of cilia overlap with cilia on a cell to the right. Bars: 2 μ m.

Fig. 5. TEM also reveals aberrant bending angles of cilia in the Myo1d KO tracheal epithelial cell

TEM of longitudinal sections through the apical domain of a WT (A) and Myo1d (B) ciliated tracheal epithelial cell. Note the pronounced splaying apart of the cilia in the KO. Bar, A, B: 500 nm.

Fig. 6. Rotational polarity of trachea cilia is disrupted by the loss of Myo1d

Representative TEM images of WT (A) and KO (B) trachea cilia cross- sections that were used to measure the angle of the central pair. Arrows are drawn through the central pair to indicate alignment and organization of adjacent cilia. Cilia from KO trachea have rotational polarity defects. Bar: 250 nm. (C, D) Quantification of cilia central pair angle from WT (C) and KO (D) trachea. 80% of WT central pairs are within $\pm 10^\circ$ from zero, while only 48% of KO central pairs fall into this range, and 30% of KO central pairs are $\pm 20^\circ$ from zero. $n = 227$ for each genotype. E. Statistical analysis of central pair rotation angle shows a significant increase in KO central pair angle as compared to WT. $n = 227$ for each genotype.

Mean angle of central pair relative to zero, WT = $\pm 6.4^\circ$, KO = $\pm 13.9^\circ$. Standard deviation for WT = 5.2 and KO = 13.5. $p = 2.8 \times 10^{-13}$.

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Fig. 7. The asymmetric localization of Vangl1 is lost in the Myo1d KO tracheal epithelium
Localization of Vangl1 in WT (A) and Myo1d KO tracheal epithelium (B). In WT tracheal epithelial cells Vangl1 is asymmetrically concentrated on the distal side of the cells. Cells in which asymmetric Vangl1 localization is apparent are marked with arrowheads. In the KO, Vangl1 is uniformly localized around each cell. Bar: 10 μ m.

Fig. 8. Unidirectional fluid flow driven by Myo1d KO ciliated tracheal epithelial cells is disrupted

Tracings of individual beads moving over the surface of WT (A) and Myo1d KO (B) trachea. Each dot represents the bead position at 1 second intervals. WT and KO bead velocities in the two movie traces shown were $12.7 \pm 4.6 \mu\text{m/s}$ ($n=14$) and $3.1 \pm 0.93 \mu\text{m/s}$ ($n=16$) respectively. The bead at the start of each trace is marked (*). Arrows indicate general direction of flow at the start of the movie. Bar: $50 \mu\text{m}$

Fig. 9. There is increased apoptosis of tracheal epithelial cells in the Myo1d KO

F-actin staining and TUNEL staining of nuclei in WT (A–C) and Myo1d KO (D–F) trachea. WT CTCF = 43803 $\pm$ 19862; Myo1d KO CTCF = 348610 $\pm$ 125418. $P = 0.00015$. Values were determined from 8 WT and KO images using Image J function for determining CTCF. Bar: 50 μ m

Fig. 10. Translational PCP is disrupted in the Myo1d KO ependymal epithelial cell
 TEM of thin sections through the ciliated ependymal epithelium of WT (A) and Myo1d KO (B–D). Note that the polarized clustering of basal bodies and cilia in the WT is lost in the Myo1d KO. Cluster boundaries are marked with double headed arrows. Bars: (A): 2μm; (B): 2μm; (C): 1μm; (D): 1μm.

Fig. 11. Basal body cluster position is not fixed to the anterior position in the Myo1d KO ependymal epithelial cell

Localization of the basal body marker, Odf-2 (red) and acetylated α -tubulin (green) in WT (A) and Myo1d KO (B) ependymal epithelial cells. Hoechst stained nuclei are displayed in blue. Basal body/ciliary clusters are highlighted with arrowheads. Bar: 10 μ m.