The establishment of rotational polarity in the airway and ependymal cilia: analysis with a novel cilium motility mutant mouse

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Matsuo M, Shimada A, Koshida S, Saga Y, Takeda H. The establishment of rotational polarity in the airway and ependymal cilia: analysis with a novel cilium motility mutant mouse. Am J Physiol Lung Cell Mol Physiol 304: L736–L745, 2013. First published March 22, 2013; doi:10.1152/ajplung.00425.2012.—The airway is covered by multicilia that beat in a metachronous manner toward the mouth to eliminate debris and infectious particles. Coordinated one-directional beating is an essential feature of multicilia in the airway to guarantee proper mucociliary clearance. Defects in ciliary motility lead to primary ciliary dyskinesia (PCD), with major symptoms including bronchiectasis and other chronic respiratory diseases. Recent work suggested that ciliary motility and planar polarity are required in the process of ciliary alignment that produces coordinated beating. However, the extent to which cilia motility is involved in this process in mammals has not yet been fully clarified. Here, to address the role of ciliary motility in the process of coordinated ciliary alignment, we analyzed Kintoun mice mutants (Ktu−/−). Ktu−/− exhibited typical phenotypes of PCD with complete loss of ciliary motility in trachea and another ciliated tissue, the brain ependyma. Immunohistochemistry using antibodies against axonal dynein confirmed the loss of multiple axonal dynein components in mutant cilia. Observation of cilia orientation based on basal foot directions revealed that ciliary motility was not required in the alignment of airway cilia, whereas a strong requirement was observed in brain ependymal cells. Thus we conclude that the involvement of ciliary motility in the establishment of coordinated ciliary alignment varies among tissues.

primary ciliary dyskinesia; rotational polarity; ciliary motility; murine model

CILA PROJECT FROM THE SURFACE of most eukaryotic cells and consist of a microtubule-based axoneme surrounded by a specialized ciliary membrane. Cilia that contain dynein arms are motile and serve as a driving force of fluid transport in the vertebrate body (24). In the trachea, hundreds of motile cilia projecting from the apical surface of epithelial cells beat from lung to the oral direction to clear contaminants (mucociliary clearance). Mucociliary clearance plays a pivotal role in the prevention of respiratory disease. Fluid flow is also created by motile cilia in other organ systems such as oviduct and brain ventricles. Multiciliated cells lining the walls of brain ventricles generate directed cerebrospinal fluid (CSF) flow that helps CSF homeostasis by transporting secreted guidance factors and nutrients (25). Given its critical and diverse functions, motility defects in cilia cause a variety of human diseases such as primary ciliary dyskinesia (PCD). Recurrent respiratory infec-

To create a coherent directional fluid flow, multiciliated cells need at least two types of planar polarity, termed rotational polarity and tissue-level polarity. The former refers to the ciliary alignment within a cell and is manifested by the position of the basal foot, a structure on the basal body at the base of each cilium that points in the direction of effective stroke (3, 21). The latter is an intercellular polarity, coordination of the rotational polarity among the multiciliated cells within a tissue (16, 30). In addition to these two polarities, there is a third polarity in brain ependymal cells. Unlike most multiciliated cells where cilia (or basal bodies) cover the entire apical surface, the surface of ependymal cilia in the brain ventricle is only partially covered by clusters of cilia, and the position of these clusters is normally polarized to one end, which is referred to as translational polarity (12, 16). Because cilial disorientation was frequently reported in human PCD, understanding the relationship between ciliary motility and these polarities, particularly rotational polarity, has been a subject of recent studies (3, 21, 30), but how and to what extent ciliary motility contributes to their establishment remains largely unknown.

Previous reports showed complexity in the relationship between ciliary motility and the establishment of rotational polarity. First, in murine tracheal cilia, basal feet are initially oriented by the planar cell polarity (PCP) pathway before the onset of ciliary beating, although the involvement of cilia motility was not examined in this study (28). By contrast, elaborate work with Xenopus skin and mouse brain ependymal cells has revealed that basal bodies docked to the apical cell membrane reorient in one direction through coupling of ciliary-driven hydrodynamic forces and PCP-mediated planar polarity (5, 16, 17). Furthermore, the latter studies, the power balance between the two key determinants (i.e., ciliary motility vs. PCP pathway) is different in the establishment of rotational polarity: cilia-driven fluid flow was suggested to have a supportive role in Xenopus skin ciliary alignment, whereas, in mouse ependymal cells, it plays a dominant role in the decision of ciliary direction (5, 17). Although these studies were well performed, mouse ependymal cells used in these studies were taken from intraflagellar transport (IFT) mutant mice in which ciliogenesis was genetically blocked, that is, producing no protruding cilium. Because of this, the effect of ciliary loss and motility loss has not been clearly separated in these studies.

The mouse models with genetically disrupted ciliary motility reported so far still retained residual ciliary motility (15, 18). Therefore, to precisely determine to what degree ciliary alignment depends on ciliary motility, we need a model mouse that shows a complete loss of ciliary motility but with normal
ciliogenesis. For this purpose, we made and analyzed Ktu knockout mice (Ktu−/−). The ktu gene was first identified in a medaka mutant showing a defect in left-right patterning, and found to be mutated in PCD patients as well as in the pf13 mutant of Chlamydomonas. Subsequent genetic and biochemical analyses revealed that Ktu is required for preassembly of axonemal dyneins in the cytoplasm (19). In the absence of Ktu/PF13, outer and a part of inner dynein arms are missing, leading to a complete loss of motility. In the present study, we first characterized murine Ktu, followed by detailed phenotypic analysis of Ktu−/− mice. As expected, mutant mice exhibited a complete loss of ciliary motility in trachea and brain ventricles, providing an ideal model of PCD. With the analysis of Ktu−/− mice, we conclude that ciliary motility is required for the establishment of rotational polarity in brain ependymal cells, but not in tracheal ciliated cells, indicating that the involvement of ciliary motility in their orientation differs dramatically among organs.

MATERIALS AND METHODS

Generation of knockout mouse. We generated a targeting vector with a floxed neo cassette to replace the first exon of Ktu, which would thus remove a translational initiation codon to produce a null allele. The linearized vector (25 μg) was electropropated into TT2 ES cells (31). G418-resistant cell clones were further selected by PCR. Correct homologous recombination was confirmed by Southern blotting analysis, and targeted cell clones were aggregated with MCH (a closed homologous Ktu line was intercrossed to produce homzygous Ktu−/− mice (see Fig. 3A). Genotype of the mice was determined by PCR polymorphism. Amplification by primer set of one and two (1&2) generate DNA fragment of 600 bp, whereas amplification by primer set of one and three (1&3) generates a DNA fragment of 300 bp (primer 1: 5′-GGAGACATCCTGAGACATGTTGAG-3′, primer 2: 5′-TACCGCTACGCAACACACCCAG-3′, primer 3: 5′-GGCACGGAGCCCGGCGTCAG-3′); Ktu+/-: 1&2 (+) 1&3 (-), Ktu+/-: 1&2 (+) 1&3 (+), Ktu+/-: 1&2 (-) 1&3 (+). Ktu−/− were also identified visually by an enlarged head and abnormal location of the milk spot. All experiments were performed with the approval of the animal experiment ethics committee at the University of Tokyo according to the University of Tokyo guidelines for the care and use of laboratory animals.

Histology. Mice were killed, and tissues were dissected in ice-cold PBS. Brains were fixed in 4% paraformaldehyde (PFA) overnight at 4°C. After cryoprotection by 20% sucrose/PBS, brains were mounted in Tissue-Tek OCT compound (Sakura finetek) and sectioned into 20-μm slices. Sections were immersed in 70% ethanol for 4 h and dehydrated. They were then rinsed with water and stained with hematoxylin and eosin.

Western blotting. Tissues were dissected in ice-cold PBS and homogenized in HMEK buffer containing Nonidet P-40 (NP-40) [10 mM HEPES, 5 mM MgSO4, 5 mM EDTA, 25 mM KCI, complete protease inhibitor cocktail (Roche), 0.25% NP-40] as protein samples. Protein samples were boiled with 2× SDS buffer and separated by 5, 7, and 10% polyacrylamide gels. Gels were transferred onto polyvinylidine difluoride membranes (Millipore) and blocked with 5% skim milk for 1 h at room temperature. Primary antibodies were diluted in PBS or 1% skim milk and incubated for 2 h at room temperature or overnight at 4°C. After serial washing with PBST (PBS + 0.1% Tween 20), membranes were incubated with secondary antibodies.

Fig. 1. Kintoun (Ktu) is expressed in multiciliated tissues. A: immunoblotting of postnatal day (P) 14 mouse organs by anti-Ktu polyclonal antibody (pAb). Expression of Ktu was detected in multiciliated/flagellated tissues (oviduct, testsis, trachea, brain). B–F: anti-Ktu pAb was costained with anti-acetylated α-tubulin monoclonal antibody (mAb) (Ac-tub, a cilia marker, B), anti-Foxj1 mAb (multiciliated cell marker, C and D), S100β (epithelial cell marker, E), and TuJ1 (neuronal marker, F) in trachea epithelial cells (B and C) and brain ependymal cells in lateral ventricles (D–F). Ktu was detected in Foxj1-positive cells, indicating that it is expressed in multiciliated cells. Ktu also colocalized with S100β but did not colocalize with the neuronal marker TuJ1 in the lateral ventricles (yellow arrowheads). Dotted lines represent individual cells in B and C and the walls of the ventricle lumen in D and E. Scale bars: 5 μm in B and C; 10 μm in D–F.
Protein bands were visualized using the ECL prime kit (GE) or Chemilumine one kit (Funakoshi) and detected by ImageQuant (GE Healthcare).

Antibodies used in Western blotting are as follows: rabbit anti-Ktu polyclonal antibody (pAb) (1:1,500) (19), goat anti-actin pAb (1:3,000, sc-1616; Santa Cruz), anti-rabbit horseradish peroxidase (HRP) (1:3,000, A0545; Sigma), and anti-goat HRP (1:3,000, AP106P; Chemicon).

Production of antibody. Rabbit pAb against mouse DNAH5 were produced as follows. To produce antigen, mDNAH5 cDNA fragment (nuclear transcript 2752–3621 of NM_133365) was subcloned into Escherichia coli strain, BL21. The fusion protein was immunized to rabbits for pAb production. Antisera was purified and used for Western blotting and immunohistochemistry (IHC).

Section IHC. Mice were killed, and tissues were isolated in ice-cold PBS. Tissues were mounted in Tissue-Tek OCT compound (Sakura finetek) and frozen in liquid nitrogen. Frozen samples were sectioned by cryostat into 12- to 16-μm-thick sections (12 μm for trachea, 16 μm for brain). Sections were dried and fixed with 4% PFA/PBS for 10 min and permeabilized with 0.5% or 10% Triton X-100 for 10 min. When staining with anti-Tuj1 or anti-S100β, brain samples were dissected and fixed in 4% PFA/PBS overnight at 4°C. After cryoprotection by 20% sucrose/PBS, brains were mounted in OCT compound and sectioned as described above. After sections were washed with PBS or PBDT (PBS + 1% DMSO + 0.5% Triton X-100), they were blocked with 5% BSA or 5% skim milk diluted in PBS. Primary antibodies were diluted in PBS or 1% skim milk and incubated for 2 h at room temperature (or overnight at 4°C). After incubation, sections were washed in PBS or PBDT for 10 min, three times. Secondary antibodies were diluted in PBS or PBDT for 10 min, three times. For some samples, double or triple staining with DAPI or rhodamine phalloidin (R415; Invitrogen) was performed. Sections were mounted with 50% glycerol and analyzed by confocal microscopy (LSM 710; Zeiss). Primary antibodies used in IHC are as follows: mouse anti-Foxj1 monoclonal antibody (mAb; 1:250, 14–9965-80; ebioscience), rabbit anti-DNAI1 pAb (1:250, HPA021649; Sigma), mouse anti-acetylated α-tubulin mAb (1:500, T7451; Sigma), mouse anti-S100β mAb (1:300, S2532; Sigma), mouse anti-TUJ1 mAb (1:300, MMS-435P; Covance), and goat anti-IFT88 pAb (1:300, EB07088; Everest). Secondary antibodies are donkey anti-mouse Alex 488/555/647 (Invitrogen), donkey anti-rabbit Alexa 488/555 (Invitrogen), and donkey anti-goat Alexa 647. Secondary antibodies were used with a dilution of 1:250.

Whole mount IHC. Mice were killed, and brains were collected in ice-cold PBS. The lateral ventricle wall of the brain was dissected and immersed in 4% PFA/PBS at 4°C overnight. Isolated trachea were opened longitudinally and fixed in 4% PFA/PBS overnight at 4°C. When staining with Vangl1, Triton X-100 was added to a final concentration of 0.5% during fixation. After serial washing with PBS (20 min × 3), samples were blocked with normal goat serum. Primary antibodies were diluted in PBS and incubated for 4°C overnight. After serial washing with PBDT (30 min × 3), samples were incubated with secondary antibodies and rhodamine phalloidin for 4°C overnight. Samples were trimmed and mounted with 50% glycerol/PBS after serial washing with PBDT. Confocal images were obtained using a LSM710 (Zeiss) microscope. Primary antibodies were used....

Fig. 2. Ktu is expressed before ciliogenesis. A: a three-dimensional (3D) reconstruction of confocal microscopy images showing normal ciliogenesis in the mouse trachea epithelial cell (MTEC) culture system. B: Ktu is expressed in the Foxj1-positive cells. *Foxj1 (+) Ktu (-) cell. C: a 3D reconstruction of the MTEC immunostained with Ktu (green) and acetylated α-tubulin (Ac-tub, red). D: cells at different ciliogenesis stages are boxed in C. 1, Preciliogenesis period; 2, early ciliogenesis period; 3, cilia elongation period. Serial confocal images projected in the x-y plane are shown in the bottom. Ktu is expressed widely before and during ciliogenesis. E: DNAH5 (axonemal dynein protein) localized to cilia at an early ciliogenesis stage. Scale bars: 10 μm in B and 2 μm in E.
in the following concentrations: rabbit anti-Pericentrin pAb (1:300, PRB-432C; Covance) and rabbit anti-Vangl1 pAb (1:300, HPA025235; Sigma).

Electron microscopy. Tissues were isolated in ice-cold PBS and fixed in 2% glutaraldehyde and 2.5% PFA overnight at 4°C. After a series of washes in cacodylate buffer, samples were postfixed in 1% OsO4 for 2 h and dehydrated with a graded ethanol series. Samples were embedded in epixy resin (Nissin EM) and sectioned. Ultrathin sections were coated with iridium and contrasted with lead citrate (TAAB). After several washes in Milli Q water, sections were dried and observed by electron microscopy (JEOL).

Quantitative analysis of basal foot orientation. To quantify the alignment of cilia within each cell, the directionality of the basal foot was measured by standard protocols (5, 8) with modifications. A basal line was drawn for each picture. For each basal foot, a vector connecting the center of the basal body and the protrusion of the basal foot was drawn. The angle between this vector and the basal line was measured manually using ImageJ software. In brief, 7–14 basal feet were measured/cell, and 30–40 cells from 2–3 mice were used for each analysis. Mean angle was calculated for each cell using Oriana 4.0 software. Mean angle was defined as mean ciliary direction (shown as 0° in each circular plot graph). Deviation from the mean angle was calculated for all of the basal feet analyzed. Deviation angles of the basal feet were pooled and plotted on a circular graph using Oriana 4.0 software (on average, 300 basal feet, 20–30 cells, and 2–3 mice were used in each experiment).

Video microscopy of cilia motility. Animals were killed, and trachea and brain were isolated in PBS. Trachea were opened longitudinally, and lateral ventricles were dissected from brain ventricles. Tissues were sectioned into thin strips using a microsurgery knife (MANI) (~0.5 mm). Sections were placed on glass slides with holes and mounted with prewarmed Hanks’ balanced salt solution + 25 mM HEPES. Beating cilia were captured at 26 frames/s by an ARTCAM 130MI-BW (Artray) camera connected to a BX61 (Olympus) microscope.

In vitro mouse trachea epithelial cell culture. Mouse trachea epithelial cell (MTEC) culture was produced using detailed methods described previously (13, 29, 33). In brief, tracheal epithelial cells were collected from 3- to 5-wk-old mice by pronase digestion. After nonadherent cells were sorted, cells were seeded on polyester membrane dishes (Transwell) coated by rat tail collagen I. Cells were cultured in 5% CO2 at 37°C. Media filling upper and lower chambers were changed to a different composition. After several days of incubation, cultured cells were fixed with 0.5% PFA/PBS for 5 min followed by −20°C methanol for 7 min. Cells were permeabilized with 0.5% Triton-X 100 for 3 min. Samples were blocked with 5% BSA for 30 min and incubated in primary antibodies for 2 h at room temperature. After incubation, samples were washed with PBS three times and incubated with secondary antibodies. Dilution of primary and secondary antibodies was the same as the protocol for IHC.

RESULTS
Ktu is expressed in multiciliated tissues. Our previous study explored the function of Ktu in medaka, mouse, human, and Chlamydomonas (19), but the expression of Ktu in tissues and cells was not examined in detail except for in the medaka renal tubule. We thus started the analysis of murine Ktu with Western blots to various tissues from postnatal day (P) 14 mice using a specific antibody to murine Ktu. Ktu protein was detected as a band around 120 kDa as previously reported (19). Ktu is expressed in all tissues that harbor motile cilia or flagella, high in testis and moderate to low in oviduct, trachea, and brain. In contrast, Ktu expression was undetectable in other nonciliated organs such as spleen and heart (Fig. 1A). We then examined the subcellular localization of Ktu in multiciliated mouse tissues by IHC. Tracheal tissues taken from P14 were coimmunostained with anti-Ktu, acetylated α-tubulin (a marker for cilia), IFT88 (a component of the IFT machinery present in cilia), and Foxj1 antibodies. Foxj1 transcription factor is a master regulator of the motile ciliogenic program (34) and
exclusively expressed in cells that develop motile cilia (1, 6, 10). Thus Foxj1 demarcates ciliated cells in the tracheal epithelium that consists of multiciliated cells and nonciliated cells, including goblet cells. In the tracheal epithelium, Ktu is expressed only in Foxj1-positive cells and is present broadly in the cytoplasm but not in the ciliary compartment (Fig. 1, B and C).

The walls of the lateral ventricles of the brain at P7 are mostly lined by ependymal cells as shown by the localization of S100β (calcium-binding protein, a marker for ependymal cells) (10) (Fig. 1E), whereas neurons positive for TuJ1 (neuronal class III β-tubulin) are present underneath the ependymal layer (Fig. 1F). In lateral ventricles, Ktu is exclusively detected in ependymal cells, and, again, its subcellular localization is broad except for nuclei (Fig. 1, D-F). In the medaka renal epithelium, however, Ktu was reported to be localized in the apical portion of the cells (19). This apparent discrepancy in subcellular localization between mouse and fish could be because of the timing of observation; Ktu may change distribution depending on the step of ciliogenesis.

To monitor Ktu distribution at all steps of ciliogenesis, we adapted the MTEC culture system, which is known to faithfully model ciliogenesis in the tracheal epithelium in vivo (33) (Fig. 2A). The culture is started by seeding tracheal cells isolated from P28 mice onto a porous filter suspended in medium. Under submerged conditions, cells proliferate into a confluent state that resembles a polarized epithelium. Ciliogenesis is then induced by placing cells in an ALI. A certain proportion of cells begin ciliogenesis 2–3 days after induction and differentiate into a maximally ciliated epithelium at ~14 days. Similar to in vivo situations, Ktu is expressed exclusively in Foxj1-positive cells in MTECs (Fig. 2B). Ciliogenesis did not proceed synchronously among ciliated cells in vitro under our experimental conditions. Some cells (ca. 10%) began to express Ktu in the cytoplasm 2 days after induction. Ktu is broadly and evenly distributed throughout the cytoplasm, at time points at which cilia are not yet visible on the cell surface (Fig. 2, C and D). We noticed that a few cells express Foxj1 but not Ktu, suggesting that Ktu can be placed immediately downstream of Foxj1 (Fig. 2B). At subsequent stages, localization of Ktu did not change significantly (Fig. 2, C and D). Axonemal dynein heavy-chain DNAH5 was already localized to young cilia (Fig. 2E) in cells at the early ciliogenesis stage (as shown in cell 2 in Fig. 2C), suggesting that Ktu becomes functional in the cytoplasm to assemble axonemal dyneins before cells start morphological ciliogenesis.

Ktu⁻/⁻ mice exhibit PCD. Because ktu/pf13 mutation results in complete loss of ciliary motility in Chlamydomonas and medaka Kupffer’s vesicle, an organ equivalent to the mouse node, we expected the same in Ktu⁻/⁻ mice, which allowed us to examine the direct relationship between ciliary motility and orientation. We thus generated Ktu⁻/⁻ mice, in which exon 1 had been deleted through a Cre-recombinase-based technique (Fig. 3A). A Western blot analysis showed the absence of Ktu protein at 120 kDa in Ktu⁻/⁻ mice (Fig. 3B). Furthermore, cytoplasmic localization of Ktu was lost in tracheal epithelial cells (Fig. 3C). These results confirmed that no functional Ktu is produced from the mutated allele.

![Image](http://ajplung.physiology.org/)
Following normal Mendelian inheritance, ~25% of the pups born from Ktu\(^{-/-}\) intercrosses were homozygous Ktu\(^{-/-}\) mutants. Some of the Ktu\(^{-/-}\) mice died during the initial 1–2 days after birth. Most of the pups that survived the neonatal period showed a reduced rate of weight gain, resulting in small body size (Fig. 4A). More than 90% of the Ktu\(^{-/-}\) mice died before weaning. Translocation of circular (heart) and abdominal organs (stomach and spleen) was frequently reversed in Ktu\(^{-/-}\) pups (17 out of 43). This randomized positioning is a typical phenotype of PCD and referred to as situs inversus totalis (Fig. 4B). The ratio of situs inversus occurrences was 40% in Ktu\(^{-/-}\) (n = 43) (Fig. 4C). Ktu\(^{-/-}\) also exhibited another typical phenotype of PCD, hydrocephalus. Coronal sections of P14 brains of Ktu\(^{-/-}\) showed enlarged fluid-filled brain ventricles (Fig. 4D). Enlargement of the lateral ventricles was accompanied by thinning of the cerebral cortex. We further examined the development of hydrocephalus by making serial sections of mutant brains of different stages. At embryonic day (E) 18.5, brains from Ktu\(^{-/-}\) mice were indistinguishable from those of controls (heterozygous and wild-type littermates) and did not show any indication of hydrocephalus (data not shown). Coronal sections of P0.5 Ktu\(^{-/-}\) brains, however, exhibited enlarged lateral ventricles and thereafter gradually became enlarged during the course of development (Fig. 4E). As described above, the survival rate of homozygous mutants decreased as embryonic and postnatal development proceeded, and most mutants died before P21. This poor viability of Ktu\(^{-/-}\) mice prevented investigation of later-developing organs such as the oviduct, one of the well-studied ciliated tissues. We thus focused on the tracheal epithelium and ventricles of the brain in the following experiments.

PCD phenotypes were reported in other cilium-motility mutants such as Mdnah5 knockout mice (Mdnah5\(^{-/-}\)) that lack DNAH5, a major component of the outer dynein arm heavy chain (9). We thus investigated the expression and distribution of axonemal dyneins in Ktu\(^{-/-}\) mutant cells by IHC. In brain ependymal cells of P7 mice, DNAH5 and DNAI1 (a component of the intermediate chain) are both localized to cilia in Ktu\(^{+/+}\). However, positive signals were completely lost in mutant cilia (Fig. 5, A and B). The same was true for tracheal epithelial cells; no signal was detected for DNAH5 and DNAI1 (Fig. 5, C and D). Similar results were also obtained for DNAI2 and DNAH9, other components of the intermediate and heavy chains, respectively (data not shown). These results suggest that incorporation of axonemal dynein components to cilia is severely affected in Ktu\(^{-/-}\) mice.

To further confirm the above results, we performed transmission electron microscopy (TEM). TEM analysis revealed that both outer and inner dynein arms attached to the doublet of microtubules were lost in Ktu\(^{-/-}\) cilia of tracheal epithelial cells (Fig. 5E) and ependymal cells (data not shown). Finally, we examined the motility of cilia in Ktu\(^{-/-}\) mice at P7 by video microscopy. In wild-type mice, multicilia of both tracheal epithelial cells [Supplemental Movie 1 (Supplemental data for this article)] and tracheal epithelial cells showed normal motility of cilia (Fig. 5F). In Ktu\(^{-/-}\) mice, multicilia of both tracheal epithelial cells (Supplemental Movie 1) and tracheal epithelial cells (Supplemental Movie 2) were immobile.
ment (approximately 300 basal feet were analyzed for one experiment). Taken together, Ktu−/− mice completely lack cilia motility because of failure in dynein arm formation, despite normal ciliogenesis, thus providing an ideal PCD model. With Ktu−/− mice, we addressed the relationship between ciliary motility and rotational polarity in the trachea and ventricles of the brain.

Translational, but not rotational, polarity is normally established in mutant mice. Well-organized directional fluid flow created by multiciliated cells is guaranteed by two types of planar polarity, ciliary alignment within a cell (rotational polarity) and intercellular alignment (tissue-level polarity) (30). In brain ependyma, patches of cilia migrate toward one side of the cell, creating a third polarity called translational polarity. Previous studies showed that inhibition of PCP components (8) or loss of cilia (16) did not affect translational polarity. Thus translational polarity is established by a mechanism distinct from that for rotational and tissue-level polarities. However, the effect of ciliary loss and motility has not been clearly separated. We first examined whether translational polarity is established normally in ventricles of Ktu−/− and wild-type littermates. In mutant ventricles, basal bodies stained by anti-Pericentrin antibody migrated to the apical cell border by P10 as in the wild-type tissue (Fig. 6A). These results suggest that translational polarity is normally established in the absence of Ktu. However, the migration pattern of basal bodies in mutants was different from that in wild type in the following two aspects. First, probably because of general growth retardation, the migration is slower than that of wild type. Second, in wild-type ventricles, basal bodies tend to be tightly packed, forming a pattern commonly termed “crescent” during migration, but they migrate in a loosely packed form in mutant cells (data not shown). Nonetheless, consistent with polarized distribution of basal bodies, we observed the asymmetric localization of Vangl1, one of the core PCP proteins, in mutant ependymal cells. In both wild-type and Ktu−/− ependymal cells, Vangl1 localizes asymmetrically to the posterior cell cortex, forming a crescent shape (Fig. 6B), indicating the normal establishment of translational polarity in the absence of ciliary motility.

We then examined the rotational polarity in brain ependymal cells. For this, we quantified ciliary orientation at P10 by scoring orientations of basal feet in TEM sections. The mean angle of the basal foot projections was calculated for each cell. Deviation against the mean angle was then calculated for all

**Fig. 6.** Loss of cilia motility affects alignment of basal bodies in brain ependymal cells. A: double immunostaining of P10 lateral ventricles with anti-Pericentrin pAb (green, basal body marker) and rhodamine phalloidin (red, cell border marker). Basal bodies (arrows) migrated toward the anterior side of ependymal cells in Ktu+/+(top). Migration of basal bodies was unaffected in Ktu−/− (bottom). B: asymmetrical localization of Vangl1 (green) costained with actin (red) in the brain ependymal cells (P10). Asymmetrical localization of Vangl1 was retained in Ktu−/− (yellow arrowheads). C: schematic diagram of cilia and basal foot. The basal foot points toward the direction of ciliary beating. D: TEM sections of P10 brain ependymal cells. Unidirectional alignment of basal feet was observed in Ktu+/+(rotational polarity). Rotational polarity was disrupted in Ktu−/−. Basal feet directions are shown by yellow arrows. E: circular plots of deviation angles of the basal feet. Mean angles are pointed to 0°. Approximately 300 basal feet were analyzed for one experiment (Ktu+/+, 284 basal feet in 38 cells from 3 mice; Ktu−/−, 350 basal feet in 39 cells from 2 mice). Scale bars: 10 μm in A and B.
basal feet analyzed and pooled onto a circular plot (for details, see MATERIALS AND METHODS). As shown in Fig. 6D, the basal feet of Ktu-/- mice pointed in random directions compared with the basal feet of Ktu+/- mice that are aligned parallel to each other (Fig. 6D). Deviation angles of basal feet varied in mutant mice, suggesting a rotational orientation defect (Fig. 6E). These results indicate that cilia motility is required for normal cillum orientation in brain ependymal cells.

Cilium orientation is not determined by ciliary motility in trachea epithelial cells. Finally, we examined the rotational polarity in trachea epithelial cells of mutant mice. A recent paper reported that the establishment of ciliary orientation is largely determined by the PCP pathway in the trachea, but involvement of ciliary motility in this process has not yet been determined (28). In the mouse tracheal epithelium, ciliogenesis initiates at E16 in the trachea (27) and PCP-protein asymmetry emerges before the onset of ciliogenesis (28). We first examined the localization of Vangl1 at P10 (14, 28) and found that Vangl1 showed asymmetric distribution within the cell surface (cortex) in both wild-type and Ktu-/- mice (Fig. 7A), indicating that planar polarity is established normally in the absence of Ktu. We then examined the orientation of basal feet in tracheal epithelial cells by the method described above. Surprisingly, the orientation of basal feet in mutant cells at P10 was almost unidirectional, similar to those in control littermates (Fig. 7, B and C), indicating that the motility of cilia does not contribute to cillum orientation in the tracheal epithelium.

DISCUSSION

We have investigated the expression and subcellular localization of murine Ktu using a specific antibody. In adult mice, Ktu is highly expressed in organs that develop motile cilia/flagella, and in these organs only ciliated cells express Ktu (Fig. 1A). Broad and uniform distribution of Ktu was frequently observed in the cytoplasm of differentiated ciliated cells (Fig. 1B). The in vitro culture system (MTEC) further demonstrated that tracheal cells start to express Ktu just before the onset of ciliogenesis and maintain expression until cilia are fully developed (Fig. 2, C and D). Upon induction in MTECs, a certain proportion of tracheal cells develop multicilia de novo, and Foxj1 is expected to regulate the process of ciliogenesis as a master regulator (32). Ktu is likely to function immediately downstream of Foxj1 because, at an early step of differentiation, some cells express only Foxj1 but not Ktu, whereas Ktu is expressed exclusively in Foxj1-positive cells (Fig. 2B). Because Ktu/PF13 is known to facilitate preassembly of axonemal dyneins in the cytoplasm through interaction with dynein components and chaperones, Ktu could be one of the proteins essential for initiation of ciliogenesis downstream of Foxj1. MTEC also demonstrate that subcellular localization of Ktu does not significantly change during the entire process of ciliogenesis. This observation appears to conflict with our previous finding in the medaka renal epithelium in which Ktu mainly localizes to the apical cytoplasm (19). This may reflect a difference in cell type; medaka renal cells have a single motile cilium, whereas tracheal cells generate and maintain hundreds of cilia during differentiation and growth, requiring a large amount of ciliary components, including axonemal dyneins.

The Ktu-/- mice exhibited a complete loss of ciliary motility in tracheal and ventricular epithelia. This is consistent with the phenotype of respiratory cells taken from Ktu-defective human PCD patients caused by loss-of-function Ktu mutations (19). Among ciliary motility mutants reported thus far, the Mdnah5-/- mouse has been well characterized and served as a model of human PCD (9). Mdnah5 encodes a heavy chain of outer dynein arms, and a mutation of this gene was found in families of human PCD patients (18). In Mdnah5-/- mice, the beat frequency of ependymal cilia is severely reduced, irregular, and asynchronous, and hydrocephalus begins to emerge at P3–5 (9). In Ktu-/- mice, the symptoms of hydrocephalus were detectable at P0, much earlier than in Mdnah5 mutants. This difference in pathogenic timing could be explained by the severity of motility defects. While cilia are completely paralyzed in Ktu-/- mice, dynein mutants (18, 20) often retain residual ciliary motility although coherent CSF is not generated in brain ventricles. Because the Ktu mutation

Fig. 7. Alignment of basal feet in trachea epithelial cells is unaffected in a Ktu-/- mouse. A: trachea epithelial cells from P10 mice were immunostained with anti-Vangl1 pAb (green) and rhodamine phalloidin (red). Posterior view: left (Ktu+/+) and right (Ktu-/-). Scale bar: 10 µm. B: TEM sections of P10 trachea epithelial cells of Ktu+/+ (left) and Ktu-/- (right). Directionality of the basal feet is established in the Ktu-/- (yellow arrows). C: circular plots of deviation angles of the basal feet. Mean angles are pointed at 0°. (Ktu+/+, 319 basal feet in 31 cells from 2 mice; Ktu-/-, 341 basal feet in 29 cells from 3 mice.)

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assures the complete loss of ciliary motility without disruption of ciliogenesis at least in the trachea and brain ventricle, the *Ktu*−/− mouse provides an ideal model to study the pathogenesis of human PCD.

It was recently reported that the rotational orientation of tracheal cilia is largely determined by the PCP pathway (28). The tracheal epithelium exhibits a clear anterior-posterior polarity in which PCP components such as Vangl1 are asymmetrically localized in each cell. This tissue-level polarity is established by E14.5, and ciliogenesis and the formation of rotational polarity takes place in these molecularly polarized cells at E16.5 and onward. The timing in appearance of cellular and ciliary polarities supports the major contribution of the PCP pathway to initial cilium orientation. Once roughly oriented based on tissue-level polarity, cilium orientation is progressively refined at late embryonic and early neonatal (E17.5-P5) stages (28). Like in other multiciliated epithelia (discussed below), this refinement was also thought to require ciliary motility-driven fluid flow. However, in *Ktu*−/−, the refinement process appeared to normally take place, and at P10 the cillum orientation in mutant cells was indistinguishable from that in wild-type cells, suggesting that both initial orientation and the subsequent refinement process in tracheal cilia are independent of ciliary motility (Fig. 7, B and C). If so, what mechanism drives the refinement process of the immotile cilia in *Ktu*−/−? Because *Ktu*−/− cells showed proper alignment, we suspect that multicilia are able to sense extracellular cues despite the loss of motility. Indeed, there is emerging evidence that, like primary cilia, motile cilia in the respiratory and reproductive tracts of humans and mice can also function as sensors to external cues such as mechanical and chemical ones (5, 11, 26). Interestingly, it has been reported that bidirectional fluid flow is produced in the trachea through fetal breathing movements during late embryonic development, a period when the refinement takes place (4). Multicilia in *Ktu*−/− cells may sense this environmental flow. Further studies will be necessary to elucidate the mechanism responsible for the motility-independent refinement process in trachea.

In ependymal cells, the tissue-level and transatorial polarity was normally established in the absence of Ktu, as indicated by the asymmetric distribution of Vangl1 and clustered cilia (Fig. 6, A and B). However, rotational polarity was severely affected. Thus, in contrast to trachea, alignment of multicilia in ependymal cells depends on ciliary motility. These results are largely consistent with studies with IFT mutant mice (5, 16). However, the ciliary orientation in *Ktu*−/− cells is not totally random but exhibits some bias (Fig. 6E). It was previously shown that IFT mutant ependymal cells exhibit no bias in the orientation of basal body docking (5). This discrepancy, again, could be explained by the sensing ability of motile cilia; cilia in *Ktu*−/− cells, albeit lacking motility, would sense the initial CSF flow that is generated by CSF secretion in the choroid plexus and absorption in the subarachnoid cisterns (22). Taken together, our results from trachea and ependymal cells strengthen the idea that multicilia are not merely a fluid generator but a perception hub for environmental cues.

*Ktu*−/− mice provide concrete evidence that the dependency of motility for the establishment of ciliary orientation varies among tissues. What is the biological significance of this variety? The generation of flow requires a collection of ciliated cells working in unison, and feedback between flow and refinement would direct reorienting cilia in response to changes that occur during and after ciliogenesis. In the case of brain ventricles and the *Xenopus* epidermis, the surface of tissues continuously and dramatically changes in shape during development and growth. For these tissues, the continuous feedback loop through active beating needs to be functional to assure directional liquid flow on the surface. In the case of the trachea, however, one directional rostrocaudal axis is genetically determined for the future clearance of mucus, and this does not change for life. Thus, trachea cilia may not need the motility-driven feedback loop. Possibly because of this characteristic feature of organ development and function, the dependency of ciliary motility may have differentiated. Hence, the power balance between fluid flow and planar polarity in establishment of coordinated orientation of motile cilia may be determined by the stability of the tissue morphology during development. This notion would support the previous studies of quail oviduct and mouse node cilia in which planar polarity plays a major role in the establishment of ciliary orientation (2, 7). In conclusion, our findings provide new insights for the pathogenesis of PCD and better diagnosis of PCD.

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DISCLOSURES

No conflicts of interest, financial or otherwise are declared by the authors.

AUTHOR CONTRIBUTIONS

Author contributions: M.M., S.K., and H.T. conception and design of study; M.M., S.K., and H.T. performed experiments; M.M., S.K., and H.T. analyzed data; M.M., S.K., and H.T. interpreted results of experiments; M.M. drafted manuscript; M.M., S.K., and H.T. approved final version of manuscript; A.S., Y.S., and H.T. drafted manuscript.

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