Tubulin polyglutamylation is essential for airway ciliary function through the regulation of beating asymmetry

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Airway epithelial cilia protect the mammalian respiratory system from harmful inhaled materials by providing the force necessary for effective mucociliary clearance. Ciliary beating is asymmetric, composed of clearly distinguished effective and recovery strokes. Neither the importance of nor the essential components responsible for the beating asymmetry has been directly elucidated. We report here that the beating asymmetry is crucial for ciliary function and requires tubulin glutamylation, a unique posttranslational modification that is highly abundant in cilia. WT murine tracheal cilia have an axoneme-intrinsic structural curvature that points in the direction of effective strokes. The axonemal curvature was lost in tracheal cilia from mice with knockout of a tubulin glutamylation-performing enzyme, tubulin tyrosine ligase-like protein 1. Along with the loss of axonemal curvature, the axonemes and tracheal epithelial cilia from these knockout (KO) mice lost beating asymmetry. The loss of beating asymmetry resulted in a reduction of cilia-generated fluid flow in trachea from the KO mice. WT mice displayed a significant accumulation of mucus in the nasal cavity, and also emitted frequent coughing- or sneezing-like noises. Thus, the beating asymmetry is important for airway ciliary function. Our findings provide evidence that tubulin glutamylation is essential for ciliary function through the regulation of beating asymmetry, and provides insight into the molecular basis underlying the beating asymmetry.

ciliary dyskinesia | ciliopathy | glutamylase | flagella | microtubule

Cilia are evolutionarily conserved organelles that protrude from the cell surface and perform many different functions. Protists (e.g., paramecium) use these organelles for locomotion. Mammals use motile cilia to generate fluid flow on the cell surface. Mammalian airway epithelial cilia are part of the innate defense system that protects the respiratory system from inhaled harmful materials, including bacteria, viruses, and pollutants, by propelling mucus. The mucus flow is generated by the vigorous asymmetric beating of the cilia, which can be divided into effective strokes and recovery strokes (1). The importance of ciliary activity and mucociliary clearance in airway health has been well documented; for example, primary ciliary dyskinesia (PCD) is an inherited disease that can be caused by mutations resulting in a partial or complete loss of ciliary activity (2–4). In many cases of PCD, components of the dynein arms (the molecular motors responsible for cilia beating) are missing or defective (2–4). In contrast, fewer studies have investigated the mechanisms regulating the asymmetry of ciliary beating, and neither the importance of nor the essential components controlling the beating asymmetry has been elucidated.

The ciliary axoneme has a highly organized structure consisting of nine outer doublets and a central pair of tubulin fibers (i.e., a 9 + 2 structure) (5, 6). The major protein component of the ciliary axoneme, tubulin, undergoes glutamylation, a unique posttranslational modification (7, 8). Our group and others have recently identified glutamylation-performing enzymes known as glutamylases in tubulin tyrosine ligase (TTL)-like family members (9–12). The exact role of tubulin glutamylation in ciliary or flagellar function is unclear (13); however, the regulation of tubulin glutamylation is known to be critical for normal ciliary function (14, 15). The involvement of tubulin glutamylation in the regulation of ciliary or flagellar motilities has been suggested by studies in which a modification-specific antibody was injected into living cells (16–18); however, a recent study using a glutamylase-deficient protist revealed that glutamylases are not essential for ciliary motility in this system (19). To clearly investigate the role of tubulin glutamylation in the structure and motility of mammalian airway motile cilia, we generated knockout (KO) mice deficient in a tubulin glutamylase and analyzed both the in vivo phenotype and the effects on ciliary activity in vitro.

Results

Generation of Ttll1-KO Mice. Tubulin glutamylases belong to a family of proteins that contain a TTL domain. These TTL-like proteins are divided into classes based on their ability to initiate or elongate the glutamate chain and their preference for tubulin subunits (12). In this study, we disrupted the Ttll1 gene (TTL-like family member 1 gene) that codes for the catalytic subunit of a tubulin glutamylase (9, 11). Ttll1 has an initiating activity with a preference for α-tubulin, but also shows partial activity on β-tubulin (19). We replaced the exon containing the start codon of the Ttll1 gene with a TKneo cassette via homologous recombination (Fig. 1 A and B). Complete disruption of the Ttll1 gene was verified by detection of the loss of Ttll1 protein in lysates of respiratory organs, including the trachea and lung, from Ttll1-deficient homozygotes (Fig. 1C). Heterozygous matings produced littersmates according to Mendelian law (Fig. 1D), indicating that the Ttll1-KO mice show neither embryonic nor postnatal lethality.

To examine the effect of the Ttll1 deletion on airway cilia, we first examined whether the Ttll1 deletion resulted in a loss of tubulin glutamylation using a monoclonal antibody, GT335; that recognizes both monoglutamylated and polyglutamylated tubulin (20). Immunolabeling of tracheal tissue sections revealed less staining with GT335 in the tracheal cilia of Ttll1-KO mice (Fig. 1E). Importantly, Western blot analyses revealed that the tracheal tissue lysates lost the majority of both glutamylated α- and β-tubulins (Fig. 1F). Other types of modifications, such as acetylation, detyrosination/tirosination, and removal of penultimate glutamate (shown as Δ2), were not significantly affected by the deletion of Ttll1 (Fig. 1F and Fig. S1A). To directly examine whether the loss of glutamylated tubulin occurs in ciliary axonemes, we isolated axonemes from tracheae of WT and Ttll1-KO mice. Isolated ciliary axonemes...
Loss of Axonemal Curvature in Ttll1-KO Mouse Tracheal Epithelial Cilia. Although the foregoing investigations demonstrated no obvious structural changes in cilia from the Ttll1-KO mice, we did observe an apparent alteration in the shape of the Ttll1-KO ciliary axonemes. Of special interest, we noticed that ciliary axonemes isolated from Ttll1-KO mice were often straightened (Fig. 1G; arrowheads), whereas those from WT mice were typically curved (Fig. 1F). To eliminate possible artifacts introduced by the immunostaining processes, we directly observed the shape of axonemes after isolation and quantified the bend angle by assuming the axoneme to be a simple arc (Fig. S4). WT axonemes showed a clear curvature (Fig. 2A) with an average bend angle of about 50 degrees (Fig. 2B and C). In contrast, a major portion of Ttll1-KO axonemes lost the curvature (Fig. 2A, arrows). The bend angles of Ttll1-KO axonemes were concentrated at <40 degrees (Fig. 2B and C).

A possible explanation for this difference is that the axonemal curvature observed in the WT axonemes results from fixation of the axoneme in an active position of the ciliary beating cycle, which can result from strong rigor states of dynein motors (22, 23). To address this issue, we produced axonemes in a relaxed state by exposing them to low concentrations of ATP (<10 μM), as reported previously (22, 23). In our protocol, 2 μM ATP caused the smallest average bend angle (Fig. S5). Even under this condition, WT axonemes retained a similar degree of curvature, whereas Ttll1-KO axonemes remained in a more straightened form (Fig. 2D and E). This suggests that the axonemal curvature is a structurally intrinsic property of the axoneme and is independent of dynein rigor state. The loss of curvature was not accompanied by altered axonemal length in the Ttll1-KO tracheal cilia (Fig. 2F). Based on these two parameters, representative axonemes from WT and Ttll1-KO mice clearly showed a loss of structural curvature in the Ttll1-KO axoneme (Fig. 2G). Taken together, our findings demonstrate that tubulin glutamylation is required for the structural curvature of ciliary axonemes, which is independent of dynein-rigor bridges and inherent in axoneme.
axonemes from the...

...this condition, all cilia curved in one direction (Fig. 3A) and that the curvature was lost in axonemes of Ttll1-KO tracheal cilia (Fig. 2D, E, and G). To test this hypothesis, we prepared isolated ciliary axonemes from WT and Ttll1-KO tracheas, reactivated the axonemes by adding ATP, and compared their motilities. The use of isolated axonemes for analysis avoided some of the difficulties of investigating ciliary beating asymmetry in tracheal preparations. For example, the fluid flow generated by the large number of cilia on tracheal epithelia could positively affect (i.e., enhance or amplify) the ciliary beating asymmetry (24), or rows of neighboring cilia could interfere with the beating pattern of adjacent cilia (25, 26).

Thus, we first examined whether ATP-reactivated Ttll1-KO axoneme lost beating asymmetry. We recorded the motility of 1 mM ATP-reactivated axonemes by means of a microscope equipped with a high-speed digital camera (Movie S2 and Movie S3). Frame-by-frame photographs are shown in Fig. 3C; kymographs of the movies are shown in Fig. 3D. To quantify the axoneme motility, we wrote a simple software program that allowed us to map the position of the ciliary tips in each frame. Trajectories, in which the beating plane was fitted on the y-axis, are shown in Fig. 3E (Top). We expanded the y-axis trajectory on a time axis (Fig. 3E, Middle). From the trajectory, we calculated y-axis velocity (Fig. 3E, Bottom). The ratio of maximum velocities between effective and recovery strokes was significantly decreased and almost reached 1 in Ttll1-KO axonemes (Fig. 3F), demonstrating that the Ttll1-KO axonemes lost beating asymmetry (i.e., the effective and recovery strokes were similar). Ciliary axonemes isolated from Ttll1-KO mice also showed a 1.5-fold increase in ciliary beating frequency compared with ciliary axonemes from WT mice (Fig. 3G). Our findings indicate that tubulin glutamylation is required for generating the asymmetry between the effective and recovery strokes in the beating pattern of ciliary axonemes.

**Loss of Ciliary Beating Asymmetry in Ttll1-KO Trachea.** We next examined whether the beating asymmetry of intact cilia is affected in tracheas from Ttll1-KO mice. For this experiment, isolated tracheas were placed in organ culture and the tips of the cilia were labeled with a diluted solution of Indian ink. Ciliary motility was recorded and analyzed as above (Movie S4 and Movie S5). Frame-by-frame photographs of the movies are shown in Fig. 4A. The direction of the effective (E) and recovery (R) strokes were determined by the orientation of the trachea. Kymographs of the movies are shown in Fig 4B. Analyses of ciliary beating pattern were performed with the same procedure used to analyze axoneme beating pattern (Fig. 4C). The ratio between the velocities of the effective stroke and recovery stroke also was significantly decreased in Ttll1-KO cilia (Fig. 4D). The ciliary beating frequency was also 1.5-fold higher in Ttll1-KO mice than in WT mice (Fig. 4E). Thus, the results obtained using intact tracheas are identical to the results obtained with isolated axonemes, also supporting the conclusion that tubulin glutamylation is essential for the establishment of ciliary beating asymmetry.

**Primacy Ciliary Dyskinesia–Like Respiratory Phenotypes in Ttll1-KO Mice.** It is widely known that defects in ciliary motility, usually described as partial or complete lack of activity, impair the ability of cilia to generate proper fluid flow and result in the phenotypic features of PCD (27–30). In contrast, there are no reports exam-

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**Fig. 3.** Loss of beating asymmetry in Ttll1-KO ciliary axonemes. (A and B) Relationship between bend direction and beating direction. (A) Three representative cilia are highlighted. In the relaxed state, all cilia retained bends to the left (yellow). After reactivation, the cilia beat to the left (blue to red). (Scale bar: 5 μm.) (B) Note that the bend direction is the same as the direction of the effective stroke. (C) Time-lapse photography of ATP-reactivated ciliary axoneme. (Scale bar: 3 μm.) (D) Kymograph of an ATP-reactivated ciliary axoneme. (E) Analyses of axonemal motility. The beating plane of the original trajectory was fitted on the y axis (Top). The y-axis trajectory was plotted as a time function (Middle). The velocity in the y-direction was plotted against time (Bottom). (F) Distinction between effective and recovery strokes. The ratio between the maximum and minimum velocity was decreased significantly in axonemes from the Ttll1-KO mice and approached a value of 1, indicating a symmetric beating pattern (P = 0.001, one-way ANOVA; n = 56 (***), 65 (**)). (G) Frequency of axonemal beating. Axonemes isolated from Ttll1-KO mice showed a significantly increased ciliary beating frequency compared with axonemes isolated from WT animals (P < 0.001, one-way ANOVA).

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Fig. 4. Loss of beating asymmetry of tracheal cilia in Ttll1-KO mice. (A) Time-lapse photography of labeled cilia. The white line shows a trace of ciliary motility during the effective (E) and recovery (R) strokes. (Scale bar: 10 μm.) (B) Kymograph of ciliary strokes. (C) Analyses of ciliary motility. The ciliary trajectory was plotted as a time function after being fitted onto the y axis (Upper). The velocity in the y-direction was plotted against time (Lower). (D) Distinction between effective and recovery strokes. The ratio of velocities between effective and recovery strokes was significantly decreased in Ttll1-KO cilia (P < 0.001, one-way ANOVA; n = 40). (E) Frequency of ciliary beating. Ttll1-KO cilia showed a significantly higher beating frequency than WT cilia (P < 0.001, one-way ANOVA).

Fig. 5. Ciliary dysfunction, rhinosinusitis, and respiratory defects in Ttll1-KO mice. (A and B) Cilia-generated flow. In A, 500-ms trajectories of polystyrene beads deposited on the tracheal surface are depicted in 66-ms intervals. (Scale bar: 3 μm.) Quantified data are shown in B. The velocity of each bead was estimated by dividing the width of the field of view (50 μm) by the time each individual bead takes to pass through the field. Data are presented as mean ± SEM [n = 15 (**), 10 (***), ***P < 0.001, one-way ANOVA]. (C) Orientation of ciliary beating. The mean deviation showed a slight, but statistically significant increase in Ttll1-KO mice. P values were analyzed with the Mann-Whitney U test [n = 279 (**), 304 (***), ***P < 0.001]. (D and E) Orientation of ciliary basal feet. (D) The basal feet of both WT and Ttll1-KO mice point in the same direction (white arrows) in the transmission electron microscopy (TEM) micrographs. (E) Rose diagrams of basal foot orientation. There was no statistically significant difference using the Mann-Whitney U test [n = 121 (**), 146 (***), MD, mean deviation. (F) Alcian blue (Top) and H&E (Middle) staining of nasal cavity. In Ttll1-KO mice, accumulated mucus was clearly visible (asterisks), whereas WT mice showed no mucus accumulation. (Bottom) A magnified photograph of the boxed region of the H&E-stained KO sample. (Inset) Neutrophils in the nasal cavity of a Ttll1-KO mouse. [Scale bars: 0.4 mm (Top and Middle), 50 μm (Bottom).] (G and H) Coughing/sneezing-like phenotypes in Ttll1-KO mice. (G) Strong spikes (arrows) were observed only in spectograms recorded from KO animals. (H) Quantification of the frequency of coughing/sneezing-like noises observed in Ttll1-KO mice. ***P < 0.001, one-way ANOVA (n = 3). No coughing/sneezing-like noises were recorded from WT mice.

In humans with PCD and mouse models alike, defects in mucociliary clearance result in a high incidence of rhinosinusitis. Thus, we examined whether the Ttll1-KO mice exhibited any evidence of mucus accumulation in the nasal cavity. Paraffin sections of the nasal cavity from WT and Ttll1-KO mice were stained with Alcian blue to clearly detect any accumulated mucus. Alcian blue–positive mucus was clearly detected in the nasal cavity and sinuses of Ttll1-KO mice.
whereas no evidence of mucus accumulation was observed in WT mice (Fig. 5F, Upper). Staining with H&E revealed many infiltrating neutrophils are present in the accumulating mucus (Fig. 5F, Lower, Inset), providing additional evidence of rhinosinusitis.

In addition to rhinosinusitis, patients with PCD typically present with chronic cough (31). Thus, we examined the respiratory phenotypes of Ttll1-KO mice. We observed that they frequently made coughing- or sneezing-like noises (Movie S8 and Movie S9), whereas WT animals were mostly silent (Movie S10). To evaluate the severity of the phenotype, we counted the frequency of coughing- or sneezing-like noises. We recorded 1-min audio files from WT and Ttll1-KO mice and displayed the data as a sound spectrogram. The coughing-/sneezing-like noises are clearly shown in the spectrogram as strong spikes (Fig. 5G, arrows). By counting the spikes, we found that Ttll1-KO mice made the coughing-/sneezing-like noises about 30 times per minute (Fig. 5H). The incidence of the phenotype was 100%, because we were able to correctly identify Ttll1-KO homozygotes by the presence of coughing- or sneezing-like noises before performing PCR-based genotyping. The onset of this coughing phenotype was at least 2 weeks after birth.

To confirm this observation, we examined another mouse model of PCD in which a deletion was introduced into the gene coding for axonemal dynein intermediate chain Dnaic1 (32). Two groups of animals were examined by similar techniques; eight of nine PCD animals produced frequent coughing-/sneezing-like sounds (Audio S1), whereas the control animals did not (Audio S2). An investigator blinded to the genotype identified 18/19 animals correctly after listening to a 1-min audio recording. Thus, similar to PCD patients, mice with defects in mucociliary clearance apparently use coughing/sneezing as a backup mechanism to clear their airways.

Discussion

Our findings demonstrate that generation of an asymmetric ciliary beating pattern requires axonemal tubulin glutamylation. An important question that remains is how tubulin glutamylation results in ciliary beating asymmetry. At least two hypotheses can be raised. First, tubulin glutamylation per se might be asymmetrically distributed. An asymmetric distribution of glutamylated tubulin has been reported in murine sperm flagellar axonemes. Glutamylated tubulin is distributed predominantly in axonemal outer doublets 1, 5, and 6 (33). In a ciliary axoneme, these three doublets are located closest to the plane of beating, with doublets 5 and 6 positioned on the side of the effective stroke direction and doublet 1 on the opposite side—that is, the side of the recovery stroke direction (1). Thus, the centroid of tubulin glutamylation is not in the center of the axoneme, but rather is shifted in the direction of the effective stroke and doublet 1 on the opposite side of the recovery stroke direction (1). Thus, the centroid of tubulin glutamylation would eliminate the asymmetry has not been reported for mammalian cilia, however.

We also have found that ciliary axonemes have an intrinsic structural curvature dependent on tubulin glutamylation (Fig. 2). A microphotograph of a preparation of ciliary axonemes isolated from porcine trachea showing a bending tendency of the axonemes was published in 1986 (36). The mechanism for this inherent ciliary curvature had not been studied until now, however, because it was interpreted as simply an “instantly-fixed” shape of the normal ciliary beating cycle (37), similar to that observed in rigor waves of sperm flagella (22, 23). Our data demonstrate that the curvature is independent of the rigor state of the dynein motors (Fig. S5). A number of previous reports support our findings. For example, ATP-relaxed sperm flagella retain a curved shape with an average bend angle of 50 degrees (23), which is equal to the median bend angle of relaxed ciliary axonemes in the present study. Immotile human airway epithelial cilia that lack the outer axonemal dynein heavy chain DNAH5 retain their structural curvature (38). Moreover, KIu-KO mice that completely lack outer dynein arms and have a partial deficit of inner dynein arms have curled cilia, even though the cilia are completely immotile (39). Thus, the structural curvature of ciliary axoneme is neither a “still image” of the normal ciliary beating cycle nor a result of dynein rigor bridges. The inherent structural curvature is consistent with the concept that the resting position of beating cilia is at the end of the effective stroke, that is, the most bent position (40). The axoneme-intrinsic bending force generating the axonemal curvature could bias beating toward the direction of curvature, as suggested by the finding that external forces (i.e., flow) generate beating asymmetry (24).

In this study, we provide direct evidence of the importance of ciliary beating asymmetry in ciliary function. The Ttll1-KO mice exhibited many of the phenotypic traits of PCD, including a reduced rate of cilia-generated flow (Fig. 5A and B), defective formation of sperm flagella, and decreased motility of rhinosinusitis (Fig. 5F–H). Interestingly, however, they did not present other phenotypes commonly seen in mouse models of PCD. In particular, we found no animal with situs inversus or severe hydrocephalus among the more than 50 animals observed. This perhaps can be explained by the fact that Ttll1-KO cilia remained motile. Specialized nodal cilia, which determine the left-right axis of the body (41), might not require TTLL1 to be functional. Because ciliary beating asymmetry and cilia-generated flow were partially maintained in the trachea (Figs. 4D and 5C), sufficient fluid flow could remain in the brain ventricles to prevent the development of hydrocephalus. The residual flow also could explain the mildness of the beating orientation impairment. Alternatively, the presence of residual tubulin glutamylation might explain the mildness of the phenotypes observed, given that the Ttll1-KO did not result in complete loss of tubulin glutamylation (Fig. 1E–H).

Two additional findings warrant mention. First, Ttll1-KO mice did not have any statistically significant difference in either the rate or the waveform. Second, Ttll1-KO cilia had an increased beating frequency (Fig. 4E). A similar increase in beating frequency has been reported in cases of human PCD caused by mutations in the outer axonemal dynein heavy chain DNAH11 (42, 43). In contrast, ciliary or flagellar beating frequency is decreased in Tetrahymena (9, 10), although Ttll1 was originally isolated from murine brain as a catalytic subunit of a glutamylase complex with a preference for modifying α-tubulin (9, 11). This is perhaps not surprising, however, because Tetrahymena Ttll1 has detectable levels of β-tubulin glutamylation (19) and accounts for both α- and β-tubulin glutamylation in the cytoskeletal fraction of Tetrahymena (19). Ttll1 might have different specifications in different species or cell types, or in different subcellular locations. Second, Ttll1-KO cilia had an increased beating frequency (Fig. 4E). A similar increase in beating frequency has been reported in cases of human PCD caused by mutations in the outer axonemal dynein heavy chain DNAH11 (42, 43). In contrast, ciliary or flagellar beating frequency is decreased in Tetrahymena with a reduced level of glutamylated β-tubulin (44) and also in Chlamydomonas that grossly loses glutamylated α-tubulin (45). However, the loss of glutamylated tubulin resulted in an increase in microtubule-sliding activity of inner arm dyneins in mutants lacking outer dynein arms. These results indicate that many complex interactions between glutamylated α- and β-tubulins and inner and outer dynein arms are likely involved in the regulation of ciliary beating frequency.

The ciliary (and flagellar) axoneme has a highly complex, organized structure (5, 6). Furthermore, the axoneme is composed of hundreds of proteins present in widely varying amounts (46, 47). Some previous studies have suggested that the structural asymmetry might be responsible for the asymmetry of the beating cycle (34, 35).
In addition, some mathematical simulations have been performed (24–26, 48). These hypotheses have not yet been tested by loss-of-function models, however. Our work demonstrates, by means of a loss-of-function animal model, that ciliary beating asymmetry depends on tubulin glutamylation. These findings provide insight into the molecular basis responsible for generating the effective and recovery strokes in the ciliary beating cycle.

Materials and Methods

Animals. The Tll1-KO mice were newly developed (Fig. 1 A). The conditional Dna11c-KO mice were established previously (32). All animal use experiments followed protocols approved by the Animal Care and Use Committees of the respective institutions.

Preparation and Reactivation of Ciliated Cortices and Cilia. Ciliated cortices were prepared from murine trachea as described previously (49) with some modifications. Cilia isolation and reactivation were carried out as described previously (36) with an extensive scale-down of procedures. Immunoblotting and immunostaining were performed as described previously (50, 51). Bend angles were calculated as shown in Fig. 54.

Supporting Information

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SI Materials and Methods

Animals. To disrupt the TTLL1 gene, we replaced exons 2–4 with a TKNeo cassette (derived from pMC1-Neo polyA vector; Stratagene) by homologous recombination in ES cells (129/Sv/Ev background). Genotyping of mice was performed by PCR with the following primers: 5’-TACGTCCTCCTGGCTTGTTGAAGG-3’, 5’-GGTGATACACTGTCACCGTC-3’, and 5’-GGTGGAAGATGCGCGGCG-3’. All analyses used litters derived from mating heterozygous mice on a hybrid 129Sv/C57BL6 background, the backcross generations of which range from N3 to N7. The conditional Draic1-KO animal was described previously (1). All animals were treated according to guidelines approved by the Animal Care and Use Committees of the respective institutions.

Antibodies. Anti-TTLL1 polyclonal antibody was raised in guinea pigs immunized with purified recombinant TTLL1 fused to GST. The recombinant protein was expressed in an Escherichia coli strain BL21 (DE3) (Stratagene) and purified with glutathione Sepharose beads (GE Healthcare). Anti-polyglutamylated tubulin monoclonal antibody, GT335 (2) was a gift from Dr. Carsten Janke (Institut Curie). Monoclonal antibodies against α-tubulin (DM1A), acetylated tubulin (6-11B-1), and tyrosinated tubulin (1A2) and polyclonal antibodies against actin and γ-tubulin were purchased from Sigma-Aldrich. Polyclonal antibody for β-tubulin was obtained from Lab Vision. Monoclonal antibody for GAPDH (6C5) and polyclonal antibodies for detyrosinated tubulin and Δ2-tubulin were purchased from Chemicon International. Anti-Tektin2 mouse polyclonal antibody was obtained from Abnova. Alexa fluorophore-conjugated secondary antibodies were purchased from Invitrogen. HRP-conjugated secondary antibodies for Western blot analysis were obtained from Jackson ImmunoResearch Laboratories.

Analysis of Ciliary Axoneme Bend Angle. For analyzing the bend angle directly after isolation, resuspended ciliary axonemes were seeded on MAS-coated glass slide and fixed with 4% paraformaldehyde (PFA). When axonemes were relaxed, isolated axonemal pellets were suspended in cilia-reactivating solution as described previously (3). The relaxation of axonemes was carried out by incubating the axonemes with 2 μM ATP at room temperature (22–25 °C) for 15 min. The relaxed axonemes were fixed with 0.1% glutaraldehyde (GA) as described previously (4). This lower GA concentration was adopted to avoid axonemal distortion from a higher GA concentration (5). The effectiveness of this lower GA concentration was verified by observing its effect on stopping ciliary motility. The majority of axonemes examined demonstrated a clear curve; a few axonemes appeared damaged or had a small curvature in only a small part of the entire axoneme (Fig S4). Whereas obviously damaged axonemes were excluded from this assay, axonemes with small areas of curvature were analyzed. Axonemal lengths and areas between the arc and the chord were measured by tracing the whole length of individual axonemes using AquaCosmos image-analyzing software (Hamamatsu Photonics). Given the two measured values, bend angles were calculated as shown in Fig S4. The representative axonemal shape in Fig. 2G was depicted as a simple arc with the median axoneme length and the median bend angle using commercial software (Adobe Illustrator; Adobe Systems).

Manipulation of Ciliary Beating. Ciliated cortices were prepared from murine trachea as described previously (6) with some modifications. CHAPS was used as the membrane-removing detergent instead of Triton X-100. Relaxation and reactivation of ciliary axonemes were carried out according to published procedures (6). A microperfusion system using coverslips and cotton paper was used to replace solutions as described previously (6). Ciliary motility was recorded with a CCD camera (C4742-95; Hamamatsu Photonics) at 54 fps. Recording was done at 24–26 °C.

Analysis of Ciliary Motility. For analysis of isolated axonemes, an aliquot of axonemes suspended in reactivation buffer was placed in a chamber formed between two pieces of cover glass. To analyze intact cilia, tracheal tissue was transiently cultured in DMEM with 10% FBS. Cilia tips were labeled with Indian ink diluted with culture medium at 1:100. Ciliary motility was recorded with a high-speed CCD camera (C9100-02 or C9300-201; Hamamatsu Photonics) at 127 fps. Recording was done at 24–26 °C. Four independent animals for each genotype were examined. Fast Fourier transformation was done with Origin (Origin Lab). Data are given as mean ± SEM (n = 40 for ink-labeled cilia of both genotypes; n = 56 for isolated axonemes of WT; n = 65 for those of KO). Ciliary beating frequencies were determined by subjecting original traces to fast Fourier transformation using Origin. Statistical analyses were performed by one-way ANOVA.

Electron Microscopy. For observation of central doublets and central sheath, tracheas were fixed with 2% GA/2% PFA. For observation of dynein arms, isolated cilia axonemes were fixed with 2% GA and 0.1% tannic acid. Scanning electron microscopy was performed after dehydration and coating of the fixed tissues with Pt and Pd. For TEM, the fixed tissues were embedded in epon resin after dehydration. Uranium-lead–stained ultra-thin sections (~50 nm) were observed under a JEM-1230 electron microscope (JEOL). The central sheath was observed after averaging 32 independent photomicrographs using Photoshop (Adobe Systems). The number of dynein arms was counted in >10 axonemes from three independent samples.

Analysis of Cilia-Driven Flow. Ciliary transport on the surface of intact tracheas was analyzed in transient organ culture. To visualize cilia-generated fluid flow, 10-μm-diameter polystyrene beads (Invitrogen) were loaded onto the tracheal surface. The movement of the beads was observed with a Nomarski microscope (Zeiss) equipped with a water-immersible 20× objective lens (Zeiss) and a CCD camera (Hamamatsu Photonics). The data were recorded with household digital video camera (Hitachi) for 5 min. The velocity of each bead was estimated by dividing the width of the field of view (50 μm) by the time each individual bead took to travel across the field.

Analysis of Ciliary Beating Orientation. Three independent samples and at least 10 fields of view in each sample were analyzed. The orientation of beating was calculated from the slope of the beating plane using Microsoft Excel. Analyses were performed as described previously (7–9).

Histological Analysis. Mice were perfused with 4% PFA/1% GA under anesthesia with diethyl ether. Then the heads were removed, skinned, and further fixed in Carnoy’s fixative at 4 °C for 2 h. After acid decalcification (~24 h), the heads were embedded in paraffin and sectioned at ~4 μm thickness. Sections were stained with H&E or Alcian blue.

Analysis of Coughing- or Sneezing-Like Phenotypes. To quantify the frequency of coughing/sneezing-like noises, animals were recor-
ded using a household digital video camera (Hitachi) for 1 min in a silent room. The recorded audio file was extracted, and a spectrogram of the recording was drawn by free software working in Windows Media Player (Microsoft). Spikes shown in the spectrogram were counted. Three animals for each genotype were analyzed. Data are given as mean ± SD.


![Fig. S1.](image1.png) Quantification of results of Western blot analyses. The intensity of each band was quantified by ScionImage. Data are shown as mean ± SEM (A, n = 4; B, n = 5). P values were analyzed by the Welch test.

![Fig. S2.](image2.png) Morphology of sperm. Sperm was obtained by dissection of the epididymis. Sperm from Ttll1-KO mice (−/−) had obviously shorter flagellum (arrowheads) compared with sperm from WT mice (+/+). (Scale bar: 50 μm.)
**Fig. S3.** Electron microscopy of tracheal epithelial cilia. (A) Representative photograph of scanning electron microscopy of tracheal epithelial cilia. Cilia were normally formed in Ttll1-KO (-/-) mice. (Scale bar: 4 μm.) (B) Transverse views of cilia. Ttll1-KO (-/-) cilium had the normal 9 + 2 structure. (Scale bar: 50 nm.) (C) TEM of isolated cilia axonemes. Axonemes of WT (+/+ ) and Ttll1-KO (-/-) tracheal cilia are shown. (Scale bar: 50 nm.) (D) Quantification of dynein arms. Three independent animals were examined. More than 10 axonemes were analyzed in each sample. Data are shown as mean ± SEM. Neither outer (ODA) nor inner (IDA) dynein arms were decreased in Ttll1-KO (-/-) mice. (E) Averaged TEM of cilia axonemal central apparatus; magnified images of central doublets and central sheaths. Shown are averaged images of 32 independent photographs. There is no obvious difference between WT (+/+) and Ttll1-KO (-/-) samples. (Scale bar: 50 nm.)

**Fig. S4.** Method of bend angle quantification. To simplify the quantification protocol, the ciliary axoneme was assumed to be a simple arc. This assumption allows easy calculation of the bend angle (θ) because the bend angle is equal to the arc angle (θ). Thus, we were able to mathematically calculate the angle (θ) by measuring two parameters, arc length (l) and the area between the arc and chord (A). The original equation was expanded with Maclaurin’s expansion up to n = 5, and the polynomial equation was solved by the “Goal Seek” tool mounted in Microsoft Excel. The table shows the accuracy of the angle calculations. The majority of axonemes appeared as a smooth curve (black examples in the left-top box). In rare cases, small numbers of axonemes appeared to be damaged (red example) or had only a small curvature (green example). Among the rare cases, obviously damaged axonemes (red) were excluded from this assay. Axonemes that had a small curvature in a part of the axoneme (green) were analyzed by the same method, resulting in an average bend angle over the entire length of the axoneme.
**Fig. S5.** Relaxation of ciliary axonemes by 2 μM ATP. Bend angles were decreased when ciliary axonemes were incubated with 2 μM ATP for 15 min, compared with untreated axonemes. Axonemes reactivated by 0.1 mM ATP showed larger bend angles, resulting from fixations of actively beating cilia by glutaraldehyde. *n* = 263 (no ATP), 244 (2 μM ATP), and 219 (0.1 mM ATP).

**Fig. S6.** A model of ciliary asymmetry. The structural centroid is placed in the center of the axoneme (blue circle). If tubulin polyglutamylation is distributed unevenly in cilia, as described for sperm flagella (10), then the centroid of modification moves to the side of doublets 5 and 6 (red circle), which is located in the direction of the effective stroke (11).

**Movie S1.** Ciliated cortices purified from WT mouse tracheal epithelia. The first half of the movie shows relaxed axonemes exposed to 5 μM ATP. The last half shows reactivated axonemes exposed to 0.1 mM ATP. Replacement of solution was done with a perfusion system.

**Movie S1**
Movie S2. Motility of 1 mM ATP-reactivated axonemes isolated from a WT mouse. The movie is recorded at 127 fps and run at 12.7 fps (i.e., it is presented in 10-fold slow motion).

Movie S3. Motility of 1 mM ATP-reactivated axoneme isolated from a Ttll1-KO mouse. The movie is recorded at 127 fps and run at 12.7 fps (i.e., it is presented in 10-fold slow motion).

Movie S4. Cilia motility in transient organ culture of trachea prepared from a WT mouse. Cilia tips are labeled with Indian ink to facilitate visualization of the cilia motility. The movie is recorded at 127 fps and run at 12.7 fps (i.e., it is presented in 10-fold slow motion).

Movie S5. Cilia motility in transient organ culture of trachea prepared from a Ttll1-KO mouse. Cilia tips are labeled with Indian ink to facilitate visualization of the cilia motility. The movie is recorded at 127 fps and run at 12.7 fps (i.e., it is presented in 10-fold slow motion).
**Movie S6.** Transport of 10-μm beads over the surface of a WT trachea by normal mucociliary function. Left, the side of lung; right, the side of larynx. The movie is run at real speed. (Scale bar: 10 μm.)

**Movie S7.** Transport of 10-μm beads over the surface of a Ttll1-KO trachea. Left, the side of lung; right, the side of larynx. The movie is run at real speed. (Scale bar: 10 μm.)

**Movie S8.** A Ttll1-KO mouse exhibiting coughing-like noises. (Please watch the movie with the speakers or headphones of your PC turned on.)
Movie S9. A Ttf1-KO mouse exhibiting both sneezing-like and coughing-like noises. (Please watch the movie with the speakers or headphones of your PC turned on.)

Movie S9

Movie S10. A WT mouse with no coughing- or sneezing-like noises. (Please watch the movie with the speakers or headphones of your PC turned on.)

Movie S10

Audio S1. Sounds recorded from a Dnaic1-KO mouse, with frequent coughing-sneezing-like noises.

Audio S1
Audio S2. Sounds recorded from a WT mouse, with no coughing/sneezing-like noises.