The ciliary GTPase Arl13b regulates cell migration and cell cycle progression

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ABSTRACT

Primary cilia are ‘cellular antennae’, receiving and amplifying a wide range of signals due to their large surface area to volume ratio and acting as a hub for cross talk between different signaling pathways. Originating from the mother centriole, they are closely linked to the Golgi apparatus, which may act like a compass by relocating toward the leading edge to direct cell migration. They are absent during cell division and when the cell is migrating because the centrosome dissociates from the cilium to facilitate those processes. Nevertheless, during pauses in cell migration, cilia re-emerge until movement is resumed, suggesting that they might play a role in sensing directional cues. An inability to sense extracellular cues of both chemical and physical nature is thought to be the main cause of ciliopathies; diseases associated with the primary cilium. Ciliopathies include diverse developmental phenotypes ranging from kidney disorders to nervous system disorders (e.g. encephalocele, mental retardation and cerebellar hypoplasia). In terms of cellular mechanism it is unclear how specific ciliopathies arise since the effects on morphology of the cilium itself are often limited to size alteration.

The requirement for cilia in neuronal migration in vivo has been controversial as some data indicate that cilia do not emerge until after cellular migration within the neocortex is complete. Other reports show cilia in the developing brain, so the negative observations might be linked to episodes of cell proliferation. When the cilium is present on the cell it is responsible for sensing a wide range of developmental cues including Sonic hedgehog (SHH), WNT and platelet derived growth factor (PDGF), with conflicting evidence regarding epidermal growth factor (EGF). Although it is clear that a shorter cilium would be inhibited from sensing these cues, it is still uncertain how much of the developmental deficits can be contributed to cilium independent effects of the ciliopathic genes.

The hennin immortalised mouse embryonic fibroblast (HNN) cell line carries a null mutation of Arl13b which manifests itself in alteration of the ciliary axoneme structure and neural tube defects. Arl13b is a gene involved in Joubert...
Syndrome, which results in cerebellar hypoplasia, retinopathy, kidney malfunction and mental retardation, and has been most thoroughly studied in the context of the developing nervous system, especially neuronal migration. ARL13B is a small GTPase, which in WT cells is localized to primary cilia and plays a part during the initiation of the organelle during centrosome docking. Outside of the cilium it plays a role in actin cytoskeleton polarization at the leading edge of the cell and its downstream effects might contribute to microtubule organization. Phenotypically, cells lacking ARL13B have a cilium that is about half the WT cilium length. This is thought to be a result of disrupted Intraflagellar Transport (IFT) complex association.

Studies on HNN cells have demonstrated effects on key developmental patterning and signaling molecules, including defects in SHH signaling, elevated levels of Smoothened and defects in bone morphogenic protein signaling, which can result in exencephaly and spina bifida. Cilia are thought to sense directional cues because in a scratch assay cilia face the direction of migration, with reorientation of the centrosome, Golgi apparatus and nucleus. Even though cells, including neurons, exist in a natural electric field in situ, the role of cilia in sensing extracellular direct current electric fields (DCEF) has never been explored. Hence, it remains unknown whether cilia play a role in sensing extracellular voltage gradients.

The role of WNT signaling in DCEF directed migration has been well established and cilia are known to facilitate this pathway. Although EGF signaling has been found to be vital for DCEF, the evidence behind the cilium’s role facilitating this signaling remains controversial. The short HNN cilium also possesses defects in receptor trafficking, making it likely not to sense the signals necessary for DCEF migration and cell cycle progression.

Here we evaluated the role of Arl13b in cell cycle progression and in directed migration. We demonstrated that some of the migration deficits associated with cilia may relate to their inability to detect the presence of extracellular voltage gradients, while cell cycle changes are likely to be associated with a diminished ability to respond to chemical signals.

**Results**

**HNN cells have significantly shorter cilia than WT cells**

The HNN fibroblasts used here possessed stunted cilia compared to WT cells (Fig. 1). Cilia measured 3.2 ± 0.2 μm in HNN cells versus 5.3 ± 0.4 μm in WT cells, (P < 0.01, n = 4, Fig. S1). Our results are consistent with previous findings.

**HNN and WT cells migrate cathodally, but HNN cells migrate more slowly**

To test directly the role of cilia in detecting extracellular voltage gradients cell migration of HNN and WT cells...
was monitored during DCEF exposure. Both cell types migrated cathodally (toward the negative pole; Figure 2B, D, E), showing that HNN cells can sense the direction of the electrical cue despite having smaller cilia. Nevertheless, HNN cells migrated more than 50% more slowly than WT cells (Fig. 2E–F), reminiscent of neurons with faulty ARL13B, in which migration speed was reduced in chemical gradients. Although the DCEF increased the velocity in WT cells compared to controls (without a DCEF), HNN cells showed no increase in velocity with the DCEF. To determine if the change in velocity was linked with a change in cell adhesion we performed a cell adhesion assay and found that HNN cells displayed significantly less adhesion (Supplementary Fig. 2). Therefore, HNN cells showed migration defects upon DCEF stimulation, indicating that cilia contribute to increased speed of cell migration upon electrical stimulation.

**Cilia do not orient with respect to DCEF**

DCEFs are present in the developing brain and other systems, so many cells, including neurons develop normally within DCEFs. Primary signaling pathways in cilia are also associated causally with responses to DCEFs so we explored the role of cilia in sensing an extracellular

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**Figure 2.** HNN cells show a decreased migratory response to an electric field. (A–D) The paths of migration of WT and HNN cells in the presence and absence of an electric field. The x-axis shows the movement along the electric field axis, with the negative values showing movement toward the cathode and the positive values toward the anode. The y-axis shows displacement in the plane perpendicular to the electric field. The intersection of the 2 axes denotes the starting position of each cell, the lines show the path taken by it and the dot indicates the cells position at the end of the experiment. Black denotes cells showing net migration to the cathode, while red those to the anode. (E–F) Show graphs of the various parameters of cell migration. Panel A shows the x-Forward Direction Index, which measures the direction of cell migration with respect to the DCEF. There is a general effect of the electric field on both cell types \((p < 0.01)\), as well as an effect for each of the specific lines (WT, \(p < 0.05\); HNN, \(p < 0.05\)). Panel (F) shows the Velocity for each cell line, defined as displacement from the point of origin in time. The DCEF had a significant effect on the WT cells \((p < 0.05)\); there was also a significant difference between WT and HNN cells at 400 mV/mm \((p < 0.01)\) and a general difference between both cell lines \((p < 0.05)\).
voltage gradient by measuring the angle at which cilia protruded from cells and comparing this to the EF vector. Cilia on WT cells did not reorient with respect to the EF (Fig. 3). Therefore, the role of cilia in detecting the extracellular electric field does not require the cilium to reposition itself with respect to the DCEF vector. However, the reduced migration speed of HNN cells in DCEFs relative to WT speed, suggests that primary cilia still play a role in detecting or interpreting the presence of the field.

**HNN cells have a slower proliferation rate than WT cells**

Mutations of ARL13B result in Joubert Syndrome, which is characterized by the underdevelopment of specific brain regions; therefore the cell cycle of HNN cells was assessed to determine whether defective proliferation contributes to this phenotype.

Growth-curves revealed that by day 4 HNN cells showed significantly less proliferation, with the population size about one third of the WT (Fig. 4). Cell death is unlikely to contribute to the difference because no decrease in cell survival (99.9% for both cell types) was observed using live/dead assays.

**Fewer HNN cells are in S phase and more are in the G2/M phase compared to WT**

Prompted by the diminished HNN proliferation rate, we compared the cell cycle distribution of WT and HNN cells during mitosis using flow cytometry. Propidium iodide staining revealed that fewer HNN cells were in S phase compared to WT cells and HNN cells were more frequently in the G2/M phase than WT cells (Fig. 5). Flow cytometry

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**Figure 3.** WT cilia do not orientate with respect to the electric field. Cilia point in all 4 directions (cathodally, anodally, up, and down) with equal frequency irrespective of the presence of an electrical field. Cilia therefore do not respond directionally to the electric field.

**Figure 4.** HNN cells show reduction in growth rate. Cells were plated at 2000 cells per cm² and cultured for 4 d and cell density was assessed every 24 h. By day 4 HNN cells show a significantly lower cell concentration than WT cells (ANOVA, ***, p < 0.001; error bars show standard error of mean).

**Figure 5.** HNN cells show differences in cell cycle compared to WT cells. The cell cycle was assessed using flow cytometry. No significant difference was found in the G1/G0 phase and cells showing more than 4 chromatids (˃4 N) (ANOVA, p>0.999, p = 0.678 respectively). HNN show a decrease in the fraction of cells in the S phase (ANOVA, p < 0.001) and an increase in the fraction of cells in the G2/M phase (ANOVA, p = 0.002). Error bars show standard error.

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**Defective Arl13b changes expression of extracellular signal-regulated kinase (ERK1/2) and suppressor of fused**

To shed some light on the possible causes of these alterations we probed for several markers using Western Blot analysis. ERK1/2 activity has been associated with ciliary mechanosensation and growth factor responses. We detected a significantly decreased level of ERK1
(p44 MAPK), but not in ERK2 (p42 MAPK) phosphorylation in HNN cells (Fig. 6A, B, D), indicating reduced activity in this pathway. This points toward an impairment of responses to growth factors in HNN cells, possibly slowing the cell cycle and inhibiting migration.7,9,20,20

Suppressor of Fused (SUFU) is associated with the distal tips of cilia and its activity depends on the IFT proteins.15,40,41 It is associated with hedgehog signaling, which can affect its levels through degradation.42,43 We found an increase in SUFU protein in HNN cells compared to WT cells (Fig. 6C, D), which is consistent with effects on the HNN cell cycle and suggests further that HNN cells might not respond effectively to growth factors in the medium, thus slowing the cell cycle.

**Higher microtubule stabilization is associated with faulty Arl13b**

Microtubule function is essential for proper cell division and migration. Tyrosination abnormalities are associated with changes in neuronal network organization and the cell cycle.44,45 Prompted by the observed changes in the cell cycle and the reduced migration of HNN cells we investigated their tubulin tyrosination.

HNN cells had an increased ratio of detyrosinated α-tubulin (Glu-tubulin) to tyrosinated α-tubulin (Tyr-tubulin) (Fig. 7 and Fig. 8). Glu-tubulin is enriched in stable microtubules, but its prevalence is considered a consequence of their stabilization and not its cause.46,47 Therefore HNN cells can be viewed as less dynamic, which corresponds with the reductions seen in cell proliferation and in migration rate.

**Discussion**

Prompted by the established role of cilia in sensing extracellular chemical signals and the developmental abnormalities present in Joubert Syndrome, we assessed the influence of the hennin genotype of Arl13b on cell proliferation, and migration in DCEFs. The role of cilia in sensing extracellular voltage gradients has not been tested previously. Consistent with previous reports of ARL13B defective neuronal migration in chemotactic gradients we found that HNN cells migrated more slowly in DCEFs than WT cells, but with no directional impairment in response to an external electrical gradient.6 HNN cells showed a slower proliferation rate however, with an altered cell cycle compared to WT cells. These findings highlight the important role of cilia in cell proliferation and show that the organelle affects cell migration beyond just sensing directional chemotactic cues.

**Ciliopathic phenotypes are not limited to the direct sensory function of the cilia**

Our data show that although HNN cells exhibit a significantly reduced cilia phenotype (Fig. 1) this did not affect

![Figure 6. HNN cells show reduced expression of phosphorylated Erk but increased SUFU. (A) HNN cells show a decrease of phosphorylated ERK1 (T-test, \( p = 0.018 \); error bars show standard error) (B) but no significant difference of Erk2 (t-test, \( p = 0.224 \); error bars show standard error). (C) HNN presents significantly increased expression of SUFU (t-test, \( p = 0.022 \); error bars show standard error). (D) Typical Images of blotted protein extracts.](image-url)
the cell’s ability to read the directional electrical cue (Fig. 2). Using DCEFs as a tool to induce directional migration we have shown that the migratory deficits of cells with defective cilia cannot merely be a problem in sensing the direction of this particular cue (Figs. 2E and 3). This is largely in concordance with previous findings where cilia were proven to be important in migration, but no explanation was given regarding how they might affect directionality.2

One hypothesis is that a faulty interaction between cilia and the centrosome results in its improper function in both cell division and migration, for which this organelle is crucial.48-50 Greater attention has been given recently to this interaction suggesting that the cilium should not be treated in isolation from its environment.51-54 Importantly, some proteins associated with Joubert Syndrome, including ARL13B, have been linked with cilium initiation, which involves the docking of the centriole.24,55

The affected cilium could alter the release of the centrosome during migration and cell division. Since the centrosome is the microtubule organizing center, the truncated cilium might exert a deleterious effect on it, resulting in increased levels of glu-tubulin (Figs. 7 and 8) decreasing the dynamic instability of the cell.47,56

Hence, the primary cilium could be making a binary decision about whether to let the cell migrate (or not) rather than sensing the direction of the electrical cue. This would be consistent with previous reports that electrotaxis is mediated by a rearrangement of several proteins on the cell membrane and an intracellular gradient of GTPases, as well as with the periodical re-emergence of cilia during migration.6,37

The possible effects of the truncated cilium and faulty ARL13B function are summarized in Figure 9.

Some ciliary developmental effects rely on extracellular signal sensing

Cilia sense extracellular chemical cues and ARL13B interacts with inositol polyphosphate-5-phosphatase E which plays a role in mediating cell proliferation, so the slower proliferation rate of HNN cells (Fig. 4) might be due to attenuated responses to growth factors.25,28,57,58 In fibroblasts, ERK1/2 is phosphorylated upon exposure to growth factors, so reduced ERK1 phosphorylation (Fig. 6A, B, D) in HNN cells indicates their inability to respond to these factors.9,59,60 ERK is also crucial for polarization of the Golgi apparatus and repositioning the centrosome; events important for migration.2,5,36 Another marker associated with growth factor response and cilia is SUFU, a negative regulator in the hedgehog pathway.15 Its deletion
increases SHH activity and causes premature cell cycle exit. Together, the increase in SUFU levels (Fig. 6C, D) and the decrease in ERK phosphorylation (Fig. 6A, B, D) suggest that, at least in part, slower HNN cell proliferation lies in a diminished response to growth factors.

Insights into cell cycle

DNA replication and (usually) centriole replication occur during S phase of the cell cycle, whereas final preparations for mitosis and its execution occur in the G2/M phase. Several cell cycle checkpoints exist here.

Figure 8. Immunocytochemistry staining showing the α-Tubulin composition. Compared with WT cells (A, C, E, G), HNN cells (B, D, F, H) show a stronger signal of Glu-Tubulin in the distal parts of HNN cells. White arrows point to the areas where tubulin expression between both cells differ. Scale bars = 10 μm.
controlling for a range of defects from DNA damage, chromosome attachment to spindles and Golgi apparatus division, so perturbation of the cell cycle may contribute to developmental defects.\(^{65}\)

It is difficult to pinpoint the nature of the changes in cell cycle progression (Fig. 5). As the proportion of cells located in the G1/G0 phase is the same as in WT cells, these changes are unlikely to be caused by a diminished response to serum.\(^{9}\) Therefore, these changes in cell cycle might be mediated via downstream action of ARL13B. One proposal is via Aurora kinase A activity, which is important for the G2-M phase transition, and which co-localizes with the centrosome and plays a role in establishing cell polarity.\(^{25,27,28,58}\) Faulty Aurora kinase A activity might cause stagnation at the G2/M phase as a consequence of poor interaction with ARL13B. Hence, it is possible that deficits seen in HNN cells are not exclusively due to aberrant development of cilia, but might be linked also with cilia independent effects of ARL13B.

**Conclusion**

The small GTPase ARL13B exerts its effects on both cell migration and cell proliferation. Migration deficits have been reported in cilia defective cells, which are proposed to be related to the organelle’s role in sensing chemical cues, so we tested whether the cilium also contributed to directional migration in DCEFs and to cell cycle progression. Our data show that the primary cilium contributes to directional migration in DCEFs and to cell cycle regulation. We conclude that synergistic deficits a shorter primary cilium and faulty ARL13B function contribute to these developmental deficits.

**Materials and methods**

**Cell culture and growth curve analysis**

HNN cells and control WT cells obtained from C3H/HeJ background mice were kindly provided by Prof. Tamara Caspary (Emory University, USA).

Cells were maintained in DMEM with pyruvate, GlutaMAX and 4.5 g of glucose /L (Gibco) supplemented with 10% FBS (Invitrogen) at 37°C and 5% CO2. The cells were passaged before reaching 100% confluence.

For the growth curve analysis, cells were plated at 2000 cells/cm² (4 dishes for each cell lines, 3 repeats) and medium was changed every 2 d. Cells were trypsinized and collected and counted using a haemocytometer after 24 h, 48 h, 72 h and 96 h respectively. The final value was obtained by calculating the mean from all 3 repeats.

**Live/dead assay**

Similar to the growth curve analysis, with the exception that the medium in which the cells were grown, as well as the PBS which was used to rinse the plate before trypsinization were spun down together with the cells collected after trypsinization. Trypan blue was used during the cell count to differentiate dead cells from live cells.

**Immunocytochemistry**

For immunocytochemical analysis of the cytoskeleton, cells were plated at a density of 40000 cells/cm² on glass coverslip (VWR) placed in a 4 well plate (Nunc, Nunclon). For cilia staining, cells were plated at 50000 cells/cm² and after 22 hours medium was changed to serum free. Cells were fixed using 4% paraformaldehyde on ice and subject to primary antibody incubations overnight at 4°C. Secondary antibody and Hoechst incubations were done in 2% donkey serum in PBS at room temperature for 1.5 h. The antibodies were used at the following concentrations: AlexaFluor 488 (1:500, Molecular Probe), AlexaFluor 594 and 647 (1:1000, Molecular Probe), Hoechst (1:2000, Molecular Probe), Alexa Fluor 488 Phallodin (1:50, Molecular Probe), anti-Glu-tubulin
Cell size, cillum length measurement and cell adhesion assay

Cell size was assessed by measuring the area of a cell using Image J software. Alexa Fluor 488 phallodin was used to visualize the cell’s actin skeleton from images taken using a 100x objective. The “Find Edges” and “Threshold” processes were used to obtain the cell perimeter in 8-bit format. The perimeter was then selected and the area was measured using the “Analyze Particles” function with the “Include Holes” option selected. Cell size was confirmed using flow cytometry (see below). Cillum length was measured from anti-acetyl-α-tubulin immunofluorescence images (63x) using Image J software equipped with a NeuroJ plugin.

The cell adhesion assay was performed using an established protocol. Briefly, both WT and HNN cells were maintained in 96-well-plates at a seeding density of 5 × 10^5/ml for 20 minutes. Then they were fixed with 5% glutaraldehyde followed by crystal violet staining (0.1%) for 60 minutes with staining absorbance at 570 nm read using a microplate reader (Bio-Tek).

Immunoblotting

Cells were harvested at 80% confluence and proteins were extracted with RIPA buffer (Cell Signaling) containing complete protease and phosphatase inhibitor (Roche). Equal amounts of protein (15 μg) were fractionated by SDS-PAGE and transferred to nitrocellulose membranes. The membranes were blocked and incubated with primary antibodies overnight at 4°C, incubated with HRP-conjugated secondary antibody (Invitrogen) and developed using an enhanced chemiluminescence kit (Millipore).

The following primary antibodies were used: Glut-tubulin (Chemicon); Tyr-tubulin (Chemicon); phosphorylated ERK (Cell Signaling), total ERK (Cell Signaling) and SUFU (Cell Signaling); HRP-conjugated anti-GAPDH (Proteintech). All secondary antibodies were purchased from Sigma.

Electrotaxis assay

For migration in direct current electric fields the cells were plated at 20000 cells/cm² in a 1 cm × 4 cm chamber 40 minutes before the start of the experiment. The chamber was made in a 96 mm tissue culture dish using no.1 coverslips secured using adhesive silicone (Dow Corning RTV 3140). The silicone was left to cure at least over night before plating the cells. Barriers to prevent medium from spilling to other parts of the dish were made out of a non-curing silicone compound (Dow Corning, DC4). A third coverslip was secured to the top/roof of the chamber using the non-curing silicone compound (Dow Corning, DC4) before plating the cells. The cells were kept in 6–8 ml DMEM supplemented with 10% FBS and 2% 1 M HEPES buffer and migration took place in a microscope incubation chamber heated to 37°C. The electric field was applied to the chamber via salt bridges (1% w/v agar in Steinberg’s solution: 58 mM NaCl, 0.67 mM KCl, 4.6 mM Trizma Base, 0.44 mM Ca(NO₃)₂, 1.3 MgSO₄, pH 7.9). One end of each bridge was immersed at one end of the cell chamber and the other was kept in a beaker with Steinberg’s salt solution with an Ag/AgCl electrode at each side connected to DC power supply in series with a variable resistor. The electric field was 400 mV/mm over a period of 3 hours. Control cultures were treated the same way, but were not connected to a power supply. The voltage was checked and re-adjusted every hour if needed and 1 ml of medium was changed for fresh medium from the anodal side. Images were collected at 10 minute intervals with a 10x objective on Nikon Eclipse TE2000-U microscope, using SimplePCI software Version 5.3.1 (Compix Inc.)

The images were analyzed using Image J software (National Institute of Health, USA). The Manual Tracking plugin (Institut Curie, France) was used to track cell movement and the Chemotaxis plugin (Ibidi GmbH, Germany) was used to analyze the output. x-Forward Migration Index is defined as the displacement along the x-axis divided by the total distance traveled, speed was defined as Euclidean distance (total displacement) divided by time (180 minutes).

Cilia orientation assessment

Cells were plated at 50000 cells/cm² in a 10 cm dish with 3 4 cm microscope slides in it (1.5 thickness, with the letters ‘L’, ‘R’, ‘T’, ‘B’ scratched onto it for orientation on the side not intended for the cells to attach), using the culture medium as above. After 22 h, medium was renewed, except no serum was added; this was preceded by a PBS wash. After 21 h, the cells were subjected to the same DCEF protocol as described above, with the difference that instead of plating the cells on the dish, the microscopy slides with the cells already present were attached to the migration chamber and that the medium in which the cells migrated did not contain serum. Cells were exposed to the electric field for 3 hours, the medium taken off, the cells washed once with PBS and fixed with PFA. Cells were stained with anti-α-acetylated Tubulin (1:5000, Sigma) to image primary cilia and with antipericentrin (1:1000, Abcam) to image the centrosome.
Images were taken on a Zeiss AxioImager M2 using a 40x objective, and the slide orientation assessed. Images were analyzed in AxioVision LE64 V 4.9.1.0 (Zeiss); using the ‘Angle 3’ option a vertical line was drawn from the source of Pericentrin staining near the base of the primary cilium, and another originating from the same point and going through the tip of the cilium. Using the value of that angle, each cilium was assigned a direction: cathodal, anodal, top or bottom.

Flow cytometry

Cells were trypsinized when they reached a confluence of ~50% and centrifuged at 800 g for 5 minutes. The cell pellets were washed with PBS and centrifuged for an additional 5 minutes. The supernatant was then removed and the pellets were resuspended in 1 ml of ice cold 70% ethanol diluted with H2O (v/v) and stored at −20°C for further processing. Before the procedure, the cells were washed twice with PBS + 1% (w/v) BSA, spun at 1000 g for 5 minutes and re-suspended in 1 ml of staining buffer (50 μg/ml of propidium iodide, 50 μg/ml of ribonuclease A, 0.1% (v/v) Triton-X 100 in PBS). Samples were incubated in the dark at room temperature and analyzed on the BD Calibur followed by using FlowJo software.

Statistical analysis

Data was analyzed using ‘R’ version 3.1.0 (The R Foundation for Statistical Analysis). Data was analyzed using the t-tests and ANOVA. p < 0.05 was taken to be significant.

Abbreviations

ARL13B ADP-ribosylation factor-like 13B
DCEF Direct current electric fields
ERK Extracellular Signal-Regulated Kinase
Glu-tubulin detyrosinated α-tubulin
HNN hennin immortalised mouse embryonic fibroblast
IFT Intraflagellar Transport
SHH Sonic hedgehog
SUFU suppressor of fused
Tyr-tubulin tyrosinated α-tubulin
WT wild type

Disclosure of potential conflicts of interest

No potential conflicts of interest were disclosed.

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Author contributions

BL and CM conceived the study. BL, AMR, YD and CM designed and supervised experiments. MP carried out most of the described work and designed the cilia orientation experiment. ZY and HC performed some immunoassays and cell adhesion assay. All authors assisted with data analysis and interpretation. MP, BL and AMR drafted the initial version of the manuscript. All authors read and edited subsequent drafts and all approved the final manuscript.

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