Mechanical Forces Program the Orientation of Cell Division during Airway Tube Morphogenesis

Highlights

- Two different spindle dynamic behaviors present in the developing airway epithelium
- Different spindle behaviors lead to different dividing angle distributions
- The differences in spindle dynamic behaviors are directly dictated by cell shape
- Mechanical forces control both cell shape and the orientation of cell division

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In Brief
Tang, Hu et al. demonstrate that a combination of two spindle dynamic behaviors explains the global cell division orientation in the developing airway epithelium. The authors provide insights into how mechanical forces, cell geometry, and oriented cell division function in a highly coordinated manner to ensure normal airway tube morphogenesis.
Mechanical Forces Program the Orientation of Cell Division during Airway Tube Morphogenesis

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SUMMARY
Oriented cell division plays a key role in controlling organogenesis. The mechanisms for regulating division orientation at the whole-organ level are only starting to become understood. By combining 3D time-lapse imaging, mouse genetics, and mathematical modeling, we find that global orientation of cell division is the result of a combination of two types of spindles with distinct spindle dynamic behaviors in the developing airway epithelium. Fixed spindles follow the classic long-axis rule and establish their division orientation before metaphase. In contrast, rotating spindles do not strictly follow the long-axis rule and determine their division orientation during metaphase. By using both a cell-based mechanical model and stretching-lung-explant experiments, we showed that mechanical force can function as a regulatory signal in maintaining the stable ratio between fixed spindles and rotating spindles. Our findings demonstrate that mechanical forces, cell geometry, and oriented cell division function together in a highly coordinated manner to ensure normal airway tube morphogenesis.

INTRODUCTION
Oriented cell division is essential for tissue and organ morphogenesis (e.g., body axis elongation, gastrulation, etc.) (Baena-Lopez et al., 2005; Gong et al., 2004; Saburi et al., 2008). During morphogenesis, the orientation of cell divisions has to be constantly monitored and dynamically regulated in response to changes in both tissue shape and mechanical tension within the tissue. However, there have been few studies focused on the dynamic regulatory mechanisms that control the orientation of cell divisions on the whole-tissue/organ level (Legoff et al., 2013; Mao et al., 2013; Xiong et al., 2014). It was noted 150 years ago that the cell shape controls the orientation of cell division (Hertwig, 1884; Hofmeister, 1863). These and other classic observations led to what is now known as the “long-axis rule,” which states that a mitotic cell tends to divide along its long axis (Minc et al., 2011). However, given that some cells have been shown to divide without following the long-axis rule, this rule simply cannot be the sole determinant of the orientation of cell division (Gong et al., 2004).

During early mouse lung development (E10.5 to E11.5), airway tubes elongate more than they widen. The orientation of cell division with respect to the longitudinal axis of the airway tube is thought to have a major effect on the airway shape of the tube (Tang et al., 2011): there is an enrichment of cell divisions orienting parallel to the airway longitudinal axis, while the rest of the cell divisions are randomly oriented relative to the airway longitudinal axis. Despite the importance of oriented cell divisions in controlling airway tube shape, we still know very little about how oriented cell division is controlled dynamically during tube morphogenesis.

Previous studies of oriented cell division during airway morphogenesis have been conducted mainly with fixed tissues. This poses a limiting restriction on studies of morphogenesis, as fixation can provide only static information about the orientation of cell division. For example, it is not possible to use fixation-based methods to monitor a single cell over time to determine the influence of cell shape on cell division orientation. It is thus clear that the ability to monitor cellular processes in real time (e.g., cell shape, mitotic spindle orientation, etc.) could facilitate important discoveries about the mechanisms that control the orientation of cell divisions. In addition, the spatial and temporal information of a dividing cell of the airway epithelium could provide insight into how an airway epithelial cell determines the orientation of its division for its position in the developing airway epithelium at a given development stage. Knowledge about these processes will significantly improve our understanding of the function and the regulation of the oriented cell division in tube morphogenesis. However, it is technically challenging to measure the spatial and temporal status of dividing cells in the developing airway epithelium. One would have to obtain the three-dimensional (3D) orientation of all dividing cells along the whole airway tube epithelium and would have to track the spindle dynamics during airway development.
Using mouse genetics, ex vivo live imaging, quantitative cell biology, and mathematical modeling, we sought to understand how the orientation of cell divisions is controlled during airway tube morphogenesis. We found that there are two types of mitotic spindle dynamic behaviors that selectively follow the long-axis rule in developing airway epithelium. Interestingly, the two types of mitotic spindle behaviors are correlated with different distributions of cell dividing angles. The ratio between the two types of spindles remains stable over time. By both a cell-based mechanical model and lung-explant-stretching experiments, we show that the mechanical forces in the developing airway epithelium function to maintain the stable ratio of spindle types. Our study provides new insights into how epithelial cells orchestrate complex cellular changes such as cell shape and oriented cell division during airway morphogenesis.

RESULTS

Two Different Spindle Dynamic Behaviors in the Developing Airway Epithelium

To investigate the control of oriented cell divisions in the developing airway epithelium, we conducted an ex vivo time-lapse study to monitor cell divisions in developing airway tubes. 

H2B-GFP fusion proteins are widely used to monitor the dynamics of chromosomal changes during the cell mitosis process (Liang et al., 2015). We were easily able to distinguish the highly condensed chromosomes at metaphase from the less condensed interphase and prophase chromosomes (Figures S1A–S1C). During metaphase, the chromosomes form a plane at the equator of the cell. Depending on the orientation of the mitotic plane, the H2B-GFP-labeled chromosomes show either a rod-like shape (Figure S1D, view 1) or a rosette-like shape structure (Figure S1D, view 2) in the images. Using lungs dissected from Shh-Rosa26-Cre; Rosa26-TTA; teto-H2BGF mice (Harfe et al., 2004; Tumbar et al., 2004; Wang et al., 2008), we were able to monitor the orientation of mitotic spindles in airway epithelial cells. The whole left epithelial tube was imaged every 3 min for 4 hr (Movie S1, STAR Methods). All of the lungs maintained their normal shape changes during the imaging process (Figure S1E).

The inner surface, outer surface, and central axis of an airway tube are required to determine the orientation of mitotic spindles in three dimensions. We developed a suite of computational tools (implemented in MATLAB) to reconstruct the 3D geometry of the entire airway tube (Figure S1F), allowing us to obtain precise geometric information on the imaged airway tube. The position and the direction of the chromosome plane of all mitotic spindles were recorded at each imaging time point. We then mapped these spindles to the computationally reconstructed tube geometry and determined the orientation of spindles with respect to the longitudinal axis of the airway tube within a plane tangent to the tube surface (Figure 1A). Our imaging analysis tools allow us to quantitatively analyze the spatial and temporal changes of mitotic spindles in developing epithelial tubes.

Monitoring chromosome dynamics using our computational tools reveals that all of the mitotic spindles had aligned within the epithelial plane; the epithelial plane is perpendicular to the apical-basal axis. Given this, we only determined the planar angles of spindles (II) with respect to the longitudinal axis of airway epithelial tubes (Figures 1A and S1G–S1I) (Tang et al., 2011).

We identified at least two distinct spindle behaviors: some spindles stopped rotating soon after the cells entered metaphase and remained stationary until the cell had finished dividing (Figures 1B, 1D, and S1J; Movie S2); the other spindles did not stop rotating within the epithelial plane (Figures 1C, 1E, and S1K) until just before anaphase.

The Two Spindle Dynamic Behaviors Correspond to Different Distributions of Dividing Angles

We analyzed the planar spindle angles (II) of all dividing cells throughout the entire duration of metaphase in five E11.5 mouse lungs (Figures S2A and S2B). By tracking the chromosome changes, we found that a population of spindles became fixed rapidly within 6 min after the cells entered metaphase (Figure S2A). In the remaining spindles (Figure S2B), we observed two types of dynamic trajectories: some spindles kept rotating until they divided (Figure 1C); the other spindles stopped rotating temporarily before beginning to rotate again (Figures S2C and S2D). An important theoretical question raised by our observations is whether these apparently different spindle behaviors can be accounted for with a one-behavior model in which all spindles follow the same probabilistic rule but exhibit different outcomes owing to random noise, or if a more complicated model with multiple rules is required to explain the different spindle behaviors. The distribution of time points at which all spindles stop rotating can be predicted by these two models. We then compared the predicted values with the experimental results that we obtained in our live-imaging analysis. We found there is a significant discrepancy between the results predicted from the one-behavior model and the experimental results (Figure 2A, red dashed line, p < 10^{-6}, Kolmogorov-Smirnov test), suggesting it is unlikely that the different spindle dynamic behaviors that we observed in lung tubes are controlled by a single rule. However, the spindle trajectories predicted by a two-behavior model fit the experimental results very well (Figure 2A, green line, p = 0.65, Kolmogorov-Smirnov test). There are two distinct types of spindle behaviors in the airway epithelium in the two-behavior model: one type of spindle stops rotating in the first 6 min of metaphase; the other type of spindle apparently switches randomly between a rotating phase and a resting phase throughout metaphase. We henceforth refer to the spindles that stop rotating within 6 min as “fixed spindles” and refer to the spindles that do not stop rotating within 6 min as “rotating spindles.” The changes in spindle angle observed for each imaging time point throughout metaphase differed significantly between the fixed spindles and the rotating spindles (Figure 2B). We found that all examined E11.5 lungs contain 37% ± 3% fixed spindles and 63% ± 3% rotating spindles (Figure 2C). Furthermore, E10.5 lungs contain 38% ± 2% fixed spindles and 62% ± 2% rotating spindles (Figure S2E), suggesting that some regulatory mechanism maintains a stable ratio of these two types of spindle dynamic behaviors during airway development.

To investigate whether spindle dynamic behavior is related to the orientation of cell division, we analyzed the cell dividing angles of fixed spindles and rotating spindles. Interestingly, most fixed spindles divided at an angle less than 30° (Figure 2D), while the dividing angles of rotating spindles were uniformly distributed between 0° and 90° (p = 0.07; Kolmogorov-Smirnov test; Figure 2E). Note that the mean metaphase duration did not differ

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between cells with fixed spindles and cells with rotating spindles (Figure 2F). These results demonstrate a connection between spindle dynamic behaviors and cell dividing angles in the developing airway epithelium.

The Spatial and Temporal Relationships between Fixed Spindles and Rotating Spindles

Our results showed that spindles exhibiting two different behaviors in airway epithelial cells have differential effects on tube morphogenesis. To explore potential regulatory mechanisms underlying these two spindle behaviors, it will be important to investigate whether these two spindle behaviors are correlated in space and/or time within the developing airway epithelium. We thus developed computational tools that can reconstruct the 3D airway tube geometry directly from our imaging data (Movies S3 and S4; Figures S3A and S3B); these reconstructions allow us to extract the spatial and temporal information for individual spindles.

To explore the spatial relationship between fixed spindles and rotating spindles, we mapped individual spindles onto a...
two-dimensional epithelial plane that was obtained by unfolding an airway tube into an epithelial sheet (Figure S3C). As the two types of spindles can be clearly distinguished in the time-lapse images, the positions of the two spindles on the plane of the airway epithelium can be monitored throughout the observation period (Figure 3A). In our analysis, we computed Moran’s $I$, which can be used to determine the correlation in space between positions of the two types of spindles (Gittleman and Kot, 1990). A positive $I$ indicates that neighbor spindles tend to be of the same spindle type, whereas negative $I$ indicates that neighbor spindles are of opposite type. We found that the Moran $I$ values in all of the analyzed lungs were neither significantly positive nor significantly negative, indicating that there is no significant local correlation between the position of cells containing fixed spindles and the position of cells containing rotating spindles in the airway epithelium (Figure 3B, $p = 0.25$).

To explore the temporal relationship between the fixed spindles and the rotating spindles, we recorded the time at which each of the spindles entered metaphase and tracked individual spindles throughout metaphase for individual sample lungs (Figure 3C). In theory, if the occurrence of a fixed spindle and the occurrence of a rotating spindle are independent of each other, the sequential emergence should behave like an asymmetric one-dimensional random walk (Figure 3D) (Van Kampen, 2007). In this random walk model, we define the weighted deviation $X_i$ as:

$$X_i = 0.63 \times \text{number of fixed spindles} - 0.37 \times \text{number of rotating spindles}.$$  

$X_i$ measures the deviation of the ratio of rotating spindles after the $i$th division from the average ratio of rotating spindles (recall that the measured ratio from our live-imaging analysis was 63%; Figure 2C). At any given step, if the ratio of rotating spindles equals 63%, $X_i = 0$. The larger the difference between the ratio of rotating spindles and the average ratio, the farther away $X_i$ is from 0. Using the observed emergence sequence of fixed spindles and rotating spindles in each lung, we calculated $X_i$ after each cell division (Figure 3E). Strikingly, in all five of the lungs we analyzed, $X_i$ always remained close
The maximum deviation observed in any of the five lungs was much smaller than that of random samples generated based on an asymmetric one-dimensional random walk simulation (p < 0.001). The \( \text{Xi} \) distribution from our imaging data suggests that the emergence pattern of spindles is tightly controlled; that is, it seems clear that the occurrence of a fixed spindle and the occurrence of a rotating spindle over time are not independent of each other. This implies that some regulatory mechanism is responsible for maintaining a stable ratio of the two spindle types over time.

**Figure 3. Spatial and Temporal Relationships between Fixed Spindles and Rotating Spindles**

(A) The spatial positions of all imaged fixed-spindle cells (orange circles) and rotating-spindle cells (blue circles) on the airway epithelial planes of five lungs.

(B) Spatial correlation between fixed-spindle cells and rotating-spindle cells in five lungs based on Moran’s I test. The histograms of the I values of the random control samples are shown as black solid curves. The red line corresponds to the observed I in the experimental samples, and the blue dashed line marks the 95% confidence region in the random control samples.

(C) Time-series information of individual spindles in five lungs. Orange and blue indicate fixed-spindle cells and rotating-spindle cells, respectively. The metaphase starting points and endpoints are labeled in solid squares.

(D and E) The weighted deviation \( \text{Xi} \) after the \( i \)th divided cell computed from the experimental data (E) was much less variable than \( \text{Xi} \) as predicted by a random walk model (D) (p < 0.0001, statistical analysis is described in STAR Methods). Dashed red lines mark the 95% confidence region. See also Figure S3 and Movies S3 and S4.
A Stable Ratio of the Two Spindle Types Is Required for Normal Airway Shape

Given our findings about the spatial and temporal relationships between fixed spindles and rotating spindles, we hypothesized that a regulatory mechanism may function to maintain a stable ratio of fixed-spindle cells and rotating-spindle cells (37:63). To evaluate this hypothesis, we needed to establish an in vivo experimental method to disturb the normal ratio of rotating-spindle cells. Our previous work revealed that increasing ERK1/2 signaling by expressing constitutively activated BRAF in airway epithelial cells leads to a uniform distribution of cell dividing angles (Tang et al., 2011). Given our findings that rotating spindles led to uniformly distributed division angles (Figure 2E), we hypothesized that activated BRAF signaling should increase the population of rotating spindles in the airway epithelium. We investigated this by analyzing the mitotic spindle dynamics in E11.5 Shh-Cre; BrafCA, Rosa26-tTA; teto-H2B-GFP (BRAFCA) lungs (n = 3) (Dankort et al., 2007). The epithelial cell proliferation rate is not different between wild-type and BRAFCA mutant lungs at E11.5 (data not shown). Strikingly, 94% of spindles in airway epithelial cells of BRAFCA lungs are rotating spindles (indistinguishable from the rotating spindles of wild-type lungs) (Figures 4 A, 4B, and S4A–S4C). Thus, expressing BRAFCA in some epithelial cells allows us to increase the ratio of rotating spindles in vivo. These results demonstrate that this experimental system based on mosaic expression can be used to selectively alter the spindle angle distribution in a sub-population of cells.

We generated Shh-CreER; Rosa26-ZsGreen1; BrafCA (inducible BRAFCA) mice (Dankort et al., 2007; Madisen et al., 2010). After a tamoxifen injection, constitutively activated BRAF was expressed in a mosaic manner in the airway epithelial cells of inducible BRAFCA mice (Figure 4C). Note that the cells expressing activated BRAF could be identified because they were also positive for GFP (Figure 4C). After tamoxifen injection, constitutively activated BRAF was expressed in a mosaic manner in the airway epithelial cells of inducible BRAFCA mice (Figure 4C). Note that the cells expressing activated BRAF could be identified because they were also positive for GFP (Figure 4C). In addition, the levels of p-ERK1/2 were higher in the mitotic GFP(+) cells, indicating that activated BRAF expression was occurring in these cells (Figure S4 D). We then measured the airway shape of lungs with various proportions of BRAFCA epithelial cells (ranging from 20% to 60%) (Figures 4D, S4E, and S4F). The airway shapes of inducible BRAFCA lungs were not significantly changed even though there were 40% BRAFCA epithelial cells (Figures 4D and S4E).
Further, when we measured the spindle angle distribution of all of the dividing cells in the lungs with 40% BRAF\textsuperscript{CA}-expressing cells, it was indistinguishable from the spindle angle distribution in wild-type lungs (\(p = 0.27\); Kolmogorov-Smirnov test; Figure 4E versus Figure 4F). One explanation for the normal spindle angle distribution in the inducible BRAF\textsuperscript{CA} lungs is that the constitutively activated BRAF may not function in a cell-autonomous manner. An alternative explanation could be that a compensatory change in the cell division angles occurs in the cells lacking BRAF\textsuperscript{CA} expression; such a change would restore a normal global distribution of division angles throughout the airway tube. Considering these two possibilities, we used immunostaining to measure the cell division angles separately in GFP(+) and GFP(-) cells of mosaic lungs. In GFP(+) cells, the cell dividing angles were indeed uniformly distributed between 0° and 90° (Figure 4G), just as in BRAF\textsuperscript{CA} lungs, indicating that the influence of BRAF\textsuperscript{CA} on the spindle dynamics occurs in a cell-autonomous manner. Strikingly, in GFP(-) cells, the percentage of cells with division angles <30° was more than 65%, significantly higher than the 50% seen in wild-type lungs (Figure 4H, \(p < 0.001\), Student’s t test), indicating that the spindle angle distribution in wild-type cells is affected by the presence of BRAF\textsuperscript{CA} cells in the mosaic tissue, apparently in a compensatory manner. Thus, the experimental results obtained by expressing BRAF\textsuperscript{CA} in a mosaic manner support the idea that some regulatory mechanism functions to maintain a stable ratio of fixed-spindle cells and rotating-spindle cells during airway tube development.

### All Fixed Spindles Follow the Cell Long-Axis Rule

It was noted 150 years ago that mitotic cells tend to divide along their long axis; this has come to be known as the long-axis rule (Gibson and Gibson, 2009; Gibson et al., 2011; Hertwig, 1884; Minc et al., 2011; Nestor-Bergmann et al., 2014; Saburi et al., 2008; Thery and Bornens, 2008; Xiong et al., 2014). However, many studies have also provided evidence that certain cells do not obey this long-axis rule during some developmental processes (Gong et al., 2004; Minc et al., 2011). Almost nothing is known about the relationship between cell division orientation and the cell long axis during airway tube morphogenesis. By imaging lungs of Shh-Cre; Rosa26-1TA; Rosa26-mTmG; tet-OH2B-GFP mice, in which both a membrane-localized GFP reporter and an H2B-GFP fusion protein are specifically expressed in airway epithelial cells (Figure 5A), we were able to monitor changes of both cell shape and chromosomes in dividing airway epithelial cells. We first analyzed whether the shapes of fixed-spindle cells and rotating-spindle cells are different prior to entering prophase. Interestingly, the aspect ratio of all fixed-spindle cells is less than 1.53, whereas the aspect ratio of all rotating-spindle cells is larger than 1.53 (Figure 5B). We thus chose 1.53 as the aspect ratio threshold. If the aspect ratio of the cell is above 1.53, the corresponding cell will become a fixed-spindle cell, whose cell division follows the long axis of the cell. If the aspect ratio of a cell is less than 1.53, this cell will become a rotating-spindle cell, whose cell division is randomly oriented. We first tested whether the system should approach an equilibrium state in which the ratio between cells whose aspect ratio is >1.53 and cells whose aspect ratio is <1.53 remains approximately constant over time. We started with two different initial states: in one case most cells have an aspect ratio <1.53, and in another case most cells have an aspect ratio >1.53. As the system evolves, the proportion of cells whose aspect ratio is >1.53 does indeed approach a fixed value that matches with the proportion of fixed spindles observed in the experiment (37%) (Figures S6A and S6B).

Our live-imaging results showed that two newly generated daughter cells always position themselves within the airway epithelium along the orientation of cell division (Figures S6D and S6E), which could generate a change in mechanical tension within the airway epithelium. Given that cell division will cause anisotropic mechanical force around the dividing cell depending on the division angle, and considering that fixed spindles and rotating spindles tend to have different dividing angle distributions, the fixed spindles and rotating spindles could contribute differentially to the mechanical forces of the
tissue. These forces change the shape of surrounding cells and thus determine their spindle dynamic behaviors when they divide. We hypothesize that this kind of mechanical tension generated by cell divisions functions as a signal for cells to communicate with each other to keep a stable ratio between fixed spindles and rotating spindles. To test if our cell-based mechanical model could recapitulate the spatial and temporal relationship between fixed spindles and rotating spindles, we ran a large number (n = 1,000) of simulations. In each simulation, we tracked 85 cell divisions and analyzed the spatial and temporal distributions of the fixed spindles and rotating spindles. The fixed-spindle to rotating-spindle ratio was well maintained in the cell-based mechanical model (Figures 6 B and 6C). The variance of the weighted deviation $X_i$ predicted by the cell-based mechanical model was significantly smaller than the predicted weighted deviation $X_i$ from random cases (model predicted: variance of $X_i$ is 10.82, n = 1,000; random predicted: variance of $X_i$ is 19.77, n = 100,000, p < 10^{-6}, Student’s t test). Moreover, the spatial distributions of fixed spindles and rotating spindles are not significantly correlated (Figures 6D and 6E), similar to what we observed in the live-imaging analysis (Figures 3A and 3B).

Our cell-based mechanical model is thus able to recapitulate all of our experimental observations, suggesting that mechanical forces acting at the level of cell shape, when combined with the long-axis rule, are sufficient to account for the maintenance of stable ratios of fixed-spindle cells and rotating-spindle cells in the developing airway epithelium.

**Stretching of Lung Explants Alters the Distribution of Mitotic Spindle Angles in the Developing Airway Epithelium**

The cell-based mechanical model predicted that the application of different levels of external mechanical force along the longitudinal axis of the airway epithelium would significantly increase the percentage of cells whose aspect ratio is larger than 1.53, and as a result the percentage of cells with division angles <30° increases (Figures S7A and S7B).
To verify this supposition empirically in a biological system, we performed dose-dependent lung-stretching experiments with lung explants cultured on a silicon membrane. Our imaging analysis revealed that the long axis of airway epithelial cells tends to align with the long axis of the airway tube (Figure S5B). Based on this observation, we applied external mechanical forces along the longitudinal axis of the airway epithelium. The two separate levels of force that we applied in the stretching treatments resulted in an immediate 5% or 10% increase in the length of lung explants (Figures 7A, 7B, and S7C). We found that the average cell aspect ratio is positively correlated with the level of applied stretching force (Figures S7D–S7G). Stretching treatment did not affect the proliferation rate of epithelial or mesenchymal cells (Figure S7H). Importantly, similar to the predicted dividing angle distributions from the cell-based mechanical model, we found that the percentage of cells with division angles <30° increased significantly within 3 hr in stretched lungs, not only in stretched wild-type lungs but also in stretched BRAFCA mutant lungs (Figures 7C and S7I). The increased percentage of cells with division angles <30° is positively correlated with the levels of external forces along the longitudinal axis of the airway epithelium, indicating that mechanical forces are sufficient to control the mitotic spindle angle distribution by regulating the cell shape in the developing airway epithelium. This empirical result is consistent with our conclusions from the cell-based mechanical model. We also analyzed the airway...
shape of both stretched and non-stretched lungs after lungs were stretched for 8 hr. The airway shape factor ($r$) of stretched wild-type and BRAF$^{CA}$ mutant lungs decreased significantly compared with non-stretched control lungs (Figures 7 D–7G).

**DISCUSSION**

Using several approaches, including mouse genetics, quantitative cell biology with 3D time-lapse imaging, and mathematical...
modeling, we have identified two types of spindles that have different dynamic behaviors in the developing airway epithelium: one type fixes their orientation within the first 6 min of metaphase (fixed spindles); the other type does not come to a complete stop during metaphase (rotating spindles). Cells with fixed spindles divide along with the cell long axis, and this orientation is established before they enter metaphase. Our finding that both fixed-spindle cells and rotating-spindle cells tend to align with the long axis of the airway tube (Figure S5B) raises a question about whether fixed spindles follow the long axis of cells or follow the long axis of the airway tube. We conclude that fixed spindles follow the classic cell long-axis rule, which is supported by our observation that a few cells with fixed spindles did not align their cell long axis with the airway long axis (Figure S2A, data not shown). In contrast, cells with rotating spindles do not strictly follow the classic long-axis rule and determine their division orientation during metaphase.

In addition, we found that rotating-spindle cells are less elongated than are fixed-spindle cells, suggesting that the differences in spindle rotation behavior are directly dictated by cell shape. The spindle angle distribution in wild-type cells is affected by the presence of BRAFCA cells in the mosaic tissue in a compensatory manner (Figures 4E–4H), raising a question whether there is a similar “compensation” in terms of cell aspect ratio. However, we cannot label wild-type cells and activate BRAFCA in other cells of a single lung in our experimental system. We choose to address this question in our cell-based mechanical model. In our models, when a given percentage of BRAFCA cells (modeled as cells with reduced aspect ratio) is presented, the portion of wild-type cells whose aspect ratio is >1.53 increases (Figures S6C). This result supports the idea that some compensatory mechanism helps to maintain the ratio of fixed-spindle cells and rotating-spindle cells by functioning on the cell shape. One intriguing possibility is that some cells are not able to elongate in the rapidly proliferating airway epithelium. In this scenario, these cells would continue to adjust the orientation of their mitotic spindles to achieve the appropriate distribution of cell division orientation that is required for tubule morphogenesis (Chenet and Martin, 2014; Fink et al., 2011; Nestor-Bergmann et al., 2014). We show that ERK1/2 signaling regulates the orientation of cell divisions by controlling the interphase cell shape. The cell shape can be influenced by many factors, including cortical tension and cell-cell interactions. ERK1/2 signaling has been shown to modulate actin cortex mechanics (Logue et al., 2015), to promote cell contractility (Klemke et al., 1997), and to regulate cell adhesion (Roskoski, 2012). Further investigation will be required to understand whether these ERK1/2-regulated cellular mechanisms are involved in controlling the interphase cell shape in the developing airway epithelium. It remains an interesting question how the interphase cell shape is “memorized” to control the orientation of mitotic spindles in the airway epithelium. In Drosophila, Mud/NuMA is localized to the tricellular junctions of epithelial cells at interphase and functions as a sensor of the interphase cell shape to help control the orientation of cell division (Bosveld et al., 2016). As with the Drosophila epithelial cells, we found that mitotic cell rounding also occurs in lung airway epithelial cells. However, NuMA is mainly localized in the nucleus of many mammalian cells at interphase, including the airway epithelial cells (data not shown) (Radulescu and Cleveland, 2010). Furthermore, not all mitotic cells become round during mitosis (Campinho et al., 2013; Minc et al., 2011), which suggests that there are other mechanisms involved in regulating relationships between interphase cell shape and mitotic spindle orientation.

Mechanical forces can affect tissue development and growth by directly modulating cell behavior during many steps of embryonic development, from gastrulation to organogenesis (Savin et al., 2011). At the tissue scale, mechanical forces have been shown to act as a key component in regulating cell behaviors such as changes in cell shape and mitotic spindle movements (Fink et al., 2011; LeGoff and Lecuit, 2015; Strieder et al., 2015). During tissue morphogenesis, the mechanical environment of developing tissues undergoes rapid changes owing in part to high rates of cell proliferation (Chenet and Martin, 2014; Mammo and Ingber, 2010; Patwari and Lee, 2008; Thery and Bormens, 2008). One important regulatory mechanism is the coordination of the orientation of cell divisions and interphase cell shape in response to mechanical tension in the tissue (Campinho et al., 2013; Gibson and Gibson, 2009; Legoff et al., 2013; Mao et al., 2013; Minc and Piel, 2012; Nestor-Bergmann et al., 2014). The influence of the local tension on cell shape can be assumed to occur transiently if the relaxation time of the local mechanical tension caused by individual cell divisions is short. This may explain why there is no significant local correlation between the position of cells containing fixed spindles and the position of cells containing rotating spindles in the airway epithelium with an imaging period lasting about 4 hr. Our cell-based mechanical modeling result also shows that although cells whose aspect ratio is >1.53 and cells whose aspect ratio is <1.53 may transiently form small patches in the tissue, the fixed spindles and rotating spindles among the divided cells tend to be randomly distributed over a longer time period. Taken together, both the cell-based mechanical model and the dose-dependent lung-stretching experiments support the notion that mechanical forces function to coordinate cell geometry and spindle orientation during airway morphogenesis (Figure 7H).

Spindle rotating behavior has been observed in epithelial tissues in other model experimental systems such as corneal epithelia and neural tubes (Geldmacher-Voss et al., 2003; Haydar et al., 2003; Tibber et al., 2004). Our findings reveal the emergent cellular mechanism, involving complex relationships among cell geometry, oriented cell division, and mechanical tension during epithelial tube morphogenesis. Future investigations that pair 3D live imaging and quantitative modeling at the tissue level will continue to shed light on the sophisticated cellular mechanisms that control tissue morphogenesis.

STAR METHODS

Detailed methods are provided in the online version of this paper and include the following:

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- Statistical Analysis

SUPPLEMENTAL INFORMATION
Supplemental Information includes seven figures, four movies, and one data file and can be found with this article online at https://doi.org/10.1016/j.devcel.2017.12.013.

ACKNOWLEDGMENTS
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AUTHOR CONTRIBUTIONS
Z.T., N.T., and W.M. conceived the experiments. Z.T., Z.W., and K.W.J. performed experiments and collected data. C.Z. set up two-photo microscopy. Z.T. set up the live-imaging system. Y.H. developed the mathematical model and image analysis tools. Z.T., Y.H., N.T., and W.M. analyzed the data and wrote the manuscript. All authors discussed results and edited the manuscript.

DECLARATION OF INTERESTS
The authors declare no competing interests.

REFERENCES
STAR METHODS

KEY RESOURCES TABLE

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Chemicals, Peptides, and Recombinant Proteins

- Dulbecco’s Modified Eagle Medium Nutrient Mixture F-12 (DMEM/F12) | Thermo Fisher Scientific | Cat# 11039021 |
- Insulin-Transferrin-Selenium Supplement (100X) | Thermo Fisher Scientific | Cat# 41400-045 |
- Vitamin C | Sigma-Aldrich | Cat# A4544-25G |
- Penicillin-Streptomycin | Thermo Fisher Scientific | Cat# 15140122 |
- SeaPlaque Agarose | Lonza | Cat# 50100 |

Experimental Models: Organisms/Strains

- Mouse: Shh-Cre | (Harfe et al., 2004) | N/A |
- Mouse: Shh-CreER | (Harfe et al., 2004) | N/A |
- Mouse: Rosa26-LNL-tTA (Rosa26-tTA) | (Wang et al., 2008) | N/A |
- Mouse: teto-H2BGFP | (Tumbar et al., 2004) | N/A |
- Mouse: BrafCA/CA | (Dankort et al., 2007) | N/A |
- Mouse: Rosa26-CAG-mTmG (Rosa26-mTmG) | (Muzumdar et al., 2007) | N/A |
- Mouse: Rosa26-CAG-ZsGreen (Rosa26-ZsGreen) | (Madisen et al., 2010) | N/A |

Software and Algorithms

- Imaris x64 | Bitplane | Version 7.7.0 |
- ImageJ | (Schneider et al., 2012) | https://imagej.net/NIH_Image |
- MATLAB R2017b | The Mathworks, Inc. | N/A |
- R 3.4 | Open source | N/A |
- Airway tube 3D reconstruction tool | https://github.com/hydrays/AirwayTubeReconstruction.git | N/A |

CONTACT FOR REAGENT AND RESOURCE SHARING

Further information and requests for reagents and resources should be directed to and will be fulfilled by the Lead Contact, Dr. Nan Tang (tangnan@nibs.ac.cn).

EXPERIMENTAL MODEL AND SUBJECT DETAILS

Mice

Shh-Cre and Shh-CreER (Harfe et al., 2004), Rosa26-LNL-tTA (Rosa26-tTA) (Wang et al., 2008), teto-H2BGFP (Tumbar et al., 2004), BrafCA/CA (Dankort et al., 2007), Rosa26-CAG-mTmG (Rosa26-mTmG) (Muzumdar et al., 2007), and Rosa26-CAG-ZsGreen (Rosa26-ZsGreen) (Madisen et al., 2010) mice have been described previously. Mice had mixed genetic backgrounds. Noon of the day on which a vaginal plug was detected was considered E0.5. Embryos at E10.5 and E11.5 were staged more precisely by counting.
the number of somites posterior to the hindlimb bud, and scoring the first one counted as somite 26. The sex of embryos was not characterized at these stages. All experiments were performed in accordance with the recommendations in the Guide for Care and Use of Laboratory Animals of the National Institute of Biological Sciences, Beijing.

METHODS DETAILS

Time-Lapse Imaging of Embryonic Lung Explants
Lungs at E11.5 were dissected and immediately embedded in 0.4% low melting-point agarose (Lonza) dissolved in a medium including DMEM-F12 (Gibco), 1% insulin-transferrin-selenium (Gibco), 1% vitamin C and 1% v/v penicillin/streptomycin (Gibco). The lung explants were first cultured in a tissue culture incubator (5% CO₂, 37 °C) for 1h. The culture dish with the embedded lung was then placed on a 37 °C heated platform. The culture medium was perfused with oxygen and preheated in a 37 °C water bath. Time-lapse images were taken with a two-photon microscope (FV1000, Olympus) using a 25x water immersion objective. Imaging stacks of 512 × 512 pixels × 30 optical sections (xyzt sampling: 0.994 × 0.994 × 5 μm × 3 min) were acquired for 4h. It normally takes about 1.5 minutes to acquire a single 3D stack for a particular time point. Cells in the stalk region of the left airway tubes were analyzed.

Live Imaging the Cell Shape and Mitotic Spindle Behaviors
The green fluorescent intensity of H2B-GFP is much higher than membrane GFP. The pregnant females that carry Shh-Cre; Rosa26-tTA; Rosa26-mTmG; teto-H2B-GFP embryos were given doxycycline water (0.5 mg/ml) at E10.5 to reduce fluorescence intensity of H2B-GFP, allowing simultaneous imaging of both H2B-GFP and membrane GFP. The Shh-Cre; Rosa26-tTA; Rosa26-mTmG; teto-H2B-GFP mouse lungs were imaged as described above, except the z stack was 2.5μm. We fit the cell with an ellipse before their chromosomes condensed. The major axis of the ellipse defines the long-axis of the cell.

Lung Explant-Stretching Experiment
The stretching device was described previously (Liu et al., 2016). Briefly, first assemble a customer-made cylinder holder, an indenter ring, and an O-ring with a piece of silicone membrane so that the silicone membrane can be attached to the bottom of the indenter ring. Then put this assembled device between a screw-top and a screw-bottom so that the screw-top can be turned down to the indenter ring that stretches the silicone membrane. The lungs were then seeded on a silicone membrane and embedded in 0.4% low melting-point agarose until the agarose was solidified. Note that the width of the silicone membrane is smaller than the diameter of indenter ring for performing a uniaxial stretch. The lung explants were then stretched along its longitudinal axis until the length of the lung explant increased by 5% or 10%. For mitotic spindle angle analysis, lung explants were collected after being stretched for 3 hours. For wholemount staining, lung explants were collected after being stretched for 8 hours.

Immunostaining
For frozen sections, mouse lungs were fixed in 4% paraformaldehyde for 1 hour at 4 °C, immersed in sucrose, and then embedded in OCT. Immunostaining was performed using the following primary antibodies: Rat anti-E-Cadherin (Invitrogen clone ECCD-2, 1:500); Rabbit anti-p-ERK1/2 (Cell signaling 4370s, 1:50); Chicken anti-GFP antibody (Abcam, ab13970, 1:1000); Rabbit anti-pH3 (Millipore 06-570, 1:100); Mouse anti-γ-tubulin antibody (Sigma T5326, 1:500). The following secondary antibodies were used: Donkey anti-chicken, Alexa Fluor 488 (Jackson Immuno Research, 703-545-155); Goat anti-mouse, Alexa Fluor 568 (Molecular Probes, A11031); Goat anti-rat, Alexa Fluor 633 (Molecular Probes, A21094); and Goat anti-rabbit, Alexa Fluor 568 (Invitrogen, A-11036). All of the Alexa Fluor coupled secondary antibodies were used at 1:500 dilutions.

Modeling the Dynamic Behavior of Spindles
The planar angle \( \theta(t) \) describes the planar rotational dynamics of a spindle. Here, \( t \) is the metaphase time of the spindle (t=0 corresponds to entry to metaphase). \( t \) takes the values 0 min, 3 min, 6 min, etc. Within each time step, the spindle orientation may rotate (diffusive phase) or hold still (resting phase). After a diffusive step, the planar angle \( \theta \) of the spindle may change by a small amount \( \Delta \theta \). The angle change \( \Delta \theta \) between two successive diffusive steps appears to be uncorrelated (with a correlation value equal to -0.03, which is not significantly correlated according to a bootstrapping test using random permutation of samples), suggesting that the spindle rotation is erratic rather than persistent. During the resting phase, the planar angle \( \theta \) of the spindle does not change (\( \Delta \theta = 0 \)). After a resting phase, a spindle may resume rotating or remain still throughout the remainder of metaphase. If a spindle does not change its orientation until anaphase, we consider it to have stopped. We also consider spindles that have entered anaphase as stopped (by this definition, all spindles are stopped at the end of metaphase, and technically we cannot distinguish if a spindle is stopped or is in the resting phase).

One-Behavior Model
First we try to explain the spindle dynamics using a “one behavior model”, in which the spindle dynamical behavior is described as a Markov chain model. According to the model, before coming to a complete stop, a spindle may randomly switch between the diffusive phase and the resting phase. A spindle in the diffusive phase would go to the resting phase with probability \( p_{12} \), and stay in the diffusive phase with probability \( p_{11} \) in the next step. Similarly, a spindle in the resting phase would go to the diffusive phase with probability \( p_{21} \), or stay at the diffusive phase with probability \( p_{22} \). Moreover, a spindle may come to a full stop according to certain stopping
rules (specified below; see Stopping rule I and II). Once a spindle has fully stopped, this spindle will not change its orientation throughout the rest of the metaphase.

The transition probability matrix $P = (p_{11}, p_{12}, p_{21}, p_{22})$ can be estimated from a sub-population of spindles that have not yet stopped (note that by definition we have $p_{12} + p_{11} = 1$ and $p_{21} + p_{22} = 1$). At each time step (excepting the first step), we counted the number of spindles that remained in the same phase (diffusive or resting) or switched their phase. We found that $p_{11}$ and $p_{21}$ are almost identical throughout the metaphase (as are $p_{12}$ and $p_{22}$), and can be fitted as a linear function of the metaphase time $t$,

$$p_{11} = p_{21} = 0.9 - 0.01 \times t.$$

The fact that $p_{11} = p_{21}$ means that, before coming to a full stop, a spindle will switch to the diffusive phase with probability $p_{11}$ to the resting phase with probability $1 - p_{11}$, irrespective of its current state. Additionally, $p_{11}$ and $p_{21}$ decrease with $t$, meaning that it is more likely that a spindle will be found in the resting phase nearer the end of metaphase. We call this the “rest-and-diffuse model”, and use it to describe the spindle dynamics before a spindle stops.

➢ Stopping rule I: At each time step, if a spindle falls in an attraction zone $0 < \theta < \theta_i$, the spindle will stop with probability $p_{\text{capture}}$, and the boundary of the attraction zone, $\theta_i$, are parameters that can be tuned to fit the data.

➢ Stopping rule II: There is a $\theta$-dependent probability density function $p_{\text{capture}}(\theta)$ so that, at each time step, a spindle with a planar angle $\theta$ may fully stop rotating with a probability $p_{\text{capture}}(\theta)$. $p_{\text{capture}}(\theta)$ can be tuned to fit the data.

Combining the rest-and-diffuse model of rotational dynamics and the stopping rule, the planar angle $\theta(t)$ can be described by the process summarized below:

➢ Set planar angle $\theta(0)$ to a random variable uniformly distributed in $(0, 90^\circ)$. Sample the duration of metaphase $L$ from the empirical distribution of metaphase duration.

➢ For $t=3, \ldots, L$, determine if the spindle is stopped or not, according to the given stopping rule.

- If stopped, set $\Delta \theta = 0$, and record the stopping time.
- If not stopped, randomly assign the spindle in the diffusive phase with probability $p_{11}(t)$, or the resting phase with probability $1 - p_{11}(t)$.
  - If in the diffusive phase, sample $\Delta \theta$ as a Gaussian distribution with mean 0 and standard deviation $38^\circ$.
  - If in the resting phase, $\Delta \theta = 0$

➢ Update $\theta(t) = \theta(t-3) + \Delta \theta$

**Two-Behavior Model**

As the one population model fails to explain the experimental data, we consider a two-behavior model hypothesizes in which there are two distinct types of spindle behaviors. One type of spindles (fixed-spindles) tends to stop soon after the beginning of metaphase and fix their orientation in alignment with the tube axis. The second type of spindles (rotating-spindles) retains their rest-and-diffusion motion until the end of metaphase. This model can easily explain the data (Figure 2A in the main text, green curve).

**Spatial and Temporal Correlation Analysis**

For spatial correlation analysis, each dividing cell was mapped to a two-dimensional epithelial plane that was formed by unfolding the airway tube (Figures 3A and 3B in the main text). Then, for each lung, we computed Moran’s $I$ (Gittleman and Kot, 1990), defined as

$$I = \frac{N \sum_{i,j} \sum_{1}^{N} \omega_{ij} (y_i - \bar{y}) (y_j - \bar{y})}{\sum_{i=1}^{N} (y_i - \bar{y})^2}.$$

Here, $y_i$ specifies the type of the $i$-th cell; $y_i = 1$ for fixed-spindle and $y_i = 0$ for rotating-spindle. $\bar{y}$ is the average of $y_i$ among all $N$ observed cells in one airway tube. $\omega_{ij}$ is the inverse of the distance between cell $i$ and cell $j$. The distance is defined as the length of the shortest path on the cylinder between two points, which is independent of the cutting position (red dash line in Figure S4C in the main text). If cells $i$ and $j$ are the same type, then $(y_i - \bar{y})(y_j - \bar{y}) > 0$. Otherwise, $(y_i - \bar{y})(y_j - \bar{y}) < 0$. In general, it could be expected that if nearby cells tend to have similar spindle dynamics (a positive correlation), then $I$ would tend to be greater than 0, and if nearby cells tend to have different spindle dynamic behaviors (a negative correlation), then $I$ would tend to be less than 0. For each case, we computed $I$ for 100,000 random samples to estimate the confidence region (Figure 3B in the main text).

To study the temporal relationship of the occurrence of fixed-spindles and rotating-spindles, we followed the number of cell divisions in each lung and compare them with a one-dimensional random walk model (Van Kampen, 2007). More precisely, we consider a random walk model in which the walker is initially located at $X_0 = 0$. Each time a cell divides, with a probability of 0.63, the walker moves to the left with a step size of 0.37 (corresponding to a rotating-spindle); alternatively, with a probability...
of 0.37, the walker moves to the right with a step size of 0.63 (corresponding to a fixed-spindle). The selection of these particular step sizes ensured that position 0 always marks the “equilibrium state” with 63% rotating-spindles. Thus, after the i-th cell division, the walker’s position $X_i$ measures the deviation from the equilibrium state in the ratio between the rotating-spindle cells and fixed-spindle cells. $X_i$ is defined as the weighted deviation in the main text. The mean squared displacement of $X_i$ in the random walk model should grow linearly with respect to the number of cell divisions $i$. To compare the experimental data with the random walk model, for each lung, we compute the weighted deviation according to the observed spindle sequence over time. Figures 3D and 3E in the main text correspond, respectively, to simulated trajectories of $X_i$ obtained using the random walk model and trajectories computed from the experimental data. We computed the maximum deviation among all five observed lungs, $\max_{i} \max_{l} |X_{il}|$, where $l = 1, 2, ..., 5$ indicate the five lungs.

**Cell-Based Mechanical Model**

The model is a simplified version of the one proposed by Jennings (Jennings, 2014). A similar model has been used by Harris (Harris et al., 2012) to study the effects of mechanical tension on cell division. Details of the model are described in Data S1.

**QUANTIFICATION AND STATISTICAL ANALYSIS**

**Quantification of Airway Shape ($\rho$)**

The airway circumference and length were measured as described previously (Tang et al., 2011). Briefly, following staining in whole mount with anti-E-CAD monoclonal antibody, lungs were sectioned in the transverse plane at 50 μm along their entire proximal to distal lengths. Each transverse section was imaged using a Leica confocal microscope, with a 6 μm step size between optical sections. Outer circumference (basal surface) of the airway epithelium was measured in each optical section using Image J software, and average circumference for the entire airway was determined. Length of the airway epithelium was determined by summing the number of sections and multiplying by section thickness. The airway shape is defined as the ratio of average tube circumference to length.

**Reconstructing the 3D Airway Tube from the Raw Imaging Data**

We developed a suite of computational tools (implemented in MATLAB and available online at https://github.com/hydrays/AirwayTubeReconstruction.git) to reconstruct the three-dimensional (3D) geometry of the entire airway tube. The input is the raw imaging data, and the output is the airway tube geometry and the central axis of the tube. Details of the 3D tube reconstruction workflow are described in Data S1.

**Determining Mitotic Spindle Angle $\theta$ and Angle Change $\Delta \theta$**

After reconstructing the 3D airway tube, we can obtain the central axis of the tube $R(s) = (x(s), y(s), z(s))$. The position and orientation of the spindle in each dividing cell are manually extracted from the time-series images (analyzed using Bitplane). The position and orientation of a spindle can be described by three points $A = (x_1, y_1, z_1)$, $B = (x_2, y_2, z_2)$, $C = (x_3, y_3, z_3)$ on the mitotic plane. These points are manually picked as spots with relatively high fluorescence intensity on the condensed chromosome structure (Figure S1J). The center of the spindle $O = (x_0, y_0, z_0)$ is the center of the circumscribed circle of the triangle $ABC$, which can be computed using the MATLAB function “circumcenter”. The orientation of the spindle is the normal direction of the plane determined by points $A$, $B$, $C$, which can be computed by

$$n = \frac{\mathbf{AB} \times \mathbf{AC}}{||\mathbf{AB} \times \mathbf{AC}||}. $$

Let $P = R(s_0)$ be the point on the central axis $R(s)$ that is closest to the point $O$. At $R(s_0)$, the tangent direction along the central axis is $\frac{\partial R}{\partial s}|_{s = s_0}$, which is approximated by

$$m = \frac{R(s_0 + \Delta s) - R(s_0) - \Delta s}{||R(s_0 + \Delta s) - R(s_0) - \Delta s||}. $$

The spindle angle $\theta$ is defined as the angle between $n$ and $m$ projected on the plane passing point $O$, parallel with $m$ and perpendicular with $OP$ (such a plane is uniquely determined). To compute $\theta$, we build a new Cartesian coordinates $x'y'z'$ whose center is at point $O$, and with $m$ to be its x-axis direction and $\mathbf{OP}$ to be its y-axis. After coordinate transformation, in the new coordinates $n = (x', y', z')$, and $\theta = \arctan(\frac{y'}{\sqrt{x'^2 + z'^2}})$.

To study the spindle dynamics we also need to compute $\Delta \theta$, which is defined as the amount of change of $\theta$ between two successive imaging time points. Suppose initially the spindle orientation is set to $n_1$, and three minutes later (the interval between two imaging time points) the orientation becomes $n_2$. The actual change in orientation can be either $n_1 \rightarrow n_2$ or $n_1 \rightarrow -n_2$. In our analysis, we always choose $\Delta \theta$ as the smaller angle change between the two paths.
Statistical Analysis
All data are presented as mean ± s.e.m. (as indicated in figure legends). Experimental analyses were not blinded. The data presented in the figures were collected from multiple independent experiments performed on different days using different mice. Unless otherwise mentioned, most of the data presented in figure panels are based on at least three independent experiments. We used two-tailed Student’s t-tests to assess differences between means. We used a two-sample Kolmogorov-Smirnov (KS) test to assess the difference between the spindle angle distributions from two data samples, or a one-sample KS test to assess the difference between the observed spindle angle distribution against a uniform distribution. In both cases, n refers to the number of spindles measured, and a larger P-value (which varies from 0 to 1) suggests that it is more likely that the two samples are drawn from the same distribution (in the two-sample case) or the sample is randomly drawn from a uniform distribution (in the one-sample case).
Supplemental Information

Mechanical Forces Program the Orientation of Cell Division during Airway Tube Morphogenesis

Figure S1

A, B, C: Images showing the progression of interphase, prophase, and metaphase with DAPI, GFP, PH3, γ-tubulin, and Merge channels.

D: Diagram showing the orientation of views 1 and 2.

E: Table showing control lungs and lungs after imaging with values for C (μm), L (μm), and ρ.

F: Diagram illustrating the orientation of the airway longitudinal axis with respect to the X, Y, and Z axes.

G, H: Diagrams showing the basal and apical views with airway longitudinal axis.

I: Sequence of images showing steps 1 to 4.

J, K: Images showing the progression from 0 min to 21 min with arrows indicating changes.
Figure S1. Related to Figure 1. Analyze the orientation of mitotic spindles in a three-dimensional airway tube.

(A-C) The H2B-GFP labeled chromosomes at interphase, prophase, and metaphase can be easily distinguished. Lung sections dissected from Shh-Cre; Rosa26-tTA; teto-H2BGFP mice were stained with antibodies against GFP (green), PH3 (white), and γ-tubulin (red). (D) Depending on the orientation of the mitotic plane, the H2B-GFP labeled chromosomes show different shapes (view 1 or view 2). (E) The shape factor of lungs after the imaging experiment was measured and compared with that of control lungs (mean ± s.e.m., n=5 lungs). Control lungs were immediately fixed after the dissection. (F) The three-dimensional (3D) geometry of the whole airway tube can be reconstructed computationally from time-lapse Z-stack images. (G, H) Diagrams illustrating when angle θ equals 0° (G) or 90° (H). (I) Diagram illustrating how to determine the angle θ. The chromosome forms a rosette-like torus shape (Step 1). We then manually picked three points in the rosette that had the strongest fluorescence intensity (Step 2). The coordinates of the three points determined the rosette plane (green circle, Step 3). The angle θ is defined as the angle between the spindle axis projected on the epithelial plane and the tube longitudinal direction. (J, K) A fixed-spindle (J) or a rotating-spindle (K) on the glancing section of the airway epithelium. Scale bar: A-C: 5 μm; J, K: 5 μm.
Figure S2

(A, B) The mitotic spindle angle $\theta$ of each dividing cell was measured at each time point. Mitotic spindle angles $\theta$ of all of fixed-spindles (orange lines, A) or mitotic spindle angles $\theta$ of all of rotating-spindles (blue lines, B) are shown from five wild type mouse lungs at the 47 somite (som) stage. (C, D) A rotating-spindle that temporarily stopped rotating from 3 to 6 minutes (black arrow in D), and then began to rotate again. (E) The average ratio of cells with fixed-spindles and cells with rotating-spindles were calculated in imaged E10.5 lungs (n=5 lungs, total 199 cells). Scale bar: C: 5 μm.
**Figure S3**

**Figure S3.** Related to Figure 3. Obtaining spatial and temporal information of fixed-spindles and rotating-spindles.  
(A) A maximum intensity projection image of confocal Z-stacks of an airway epithelium expressing H2B-GFP. (B) We developed a computer program that can reconstruct the airway geometry from three-dimensional two-photon images. Dark grey color lines represent the apical surfaces and light grey color lines represent the basal surfaces of airway tubes in individual Z sections. Orange color lines and blue color lines represent fixed-spindles and rotating-spindles, respectively. (C) The diagram shows how we obtained the airway epithelial plane from an epithelial tube. The fixed-spindles (orange circles) and rotating-spindles (blue circles) can be positioned on the epithelial plane. Scale bar: A: 50 μm.
Figure S4

A. fixed-spindle cell distribution over metaphase time (min).

B. rotating-spindle cell distribution over metaphase time (min).

C. Distribution of dividing angle θ(°) for fixed-spindle and rotating-spindle cells in BRAFCA and WT lungs.

D. Percentage of mitotic cells (%).

E. Graph showing p-ERK1/2 level for GFP(-) and GFP(+) cells.

F. Images showing control, 20% BRAFCA cells, and 60% BRAFCA cells.

G. Aspect ratio of BRAFCA cells in lungs.
Figure S4. Related to Figure 4. Analysis of mitotic spindle dynamic behaviors in lungs expressing constitutively activated BRAF (BRAF<sub>CA</sub>).

(A, B) Mitotic spindle angles θ of each dividing cell at each time point were determined in three Shh-Cre; Braf<sup>CA</sup>; Rosa26-tTA; teto-H2B-GFP (BRAF<sub>CA</sub>) lungs at 47 som. Mitotic spindle angles θ of all fixed-spindles are shown in orange lines (A); mitotic spindle angles θ of all rotating-spindles are shown in blue lines (B). (C) The spindle angle distribution of all dividing cells in BRAF<sub>CA</sub> lungs (mean ± s.e.m., n=3 lungs) in comparison with the spindle angle distribution in wild type lungs (mean ± s.e.m., n=5 lungs). (D) Quantification of p-ERK1/2 levels (normalized to background; mean ± s.e.m., n=52 cells) in individual mitotic cells in inducible BRAF<sub>CA</sub> lungs. (E) The circumference (C), length (L), and shape factor (ρ) of inducible BRAF<sub>CA</sub> lungs that have different ratios of epithelial cells expressing BRAF<sub>CA</sub> (mean ± s.e.m., n=3 lungs). (F) Frontal sections of lungs were assayed for E-cadherin (red color) and GFP (green color) by immunofluorescent antibody staining. Inducible BRAF<sub>CA</sub> lungs contain 20% cells expressing BRAF<sub>CA</sub> or contain 60% cells expressing BRAF<sub>CA</sub> (green cells). (G) The aspect ratio of interphase epithelial cells of BRAF<sub>CA</sub> lungs (mean ± s.e.m., n= 69 cells) Scale bar: F: 50μm. * P<0.05; **, P<0.01, Student's t-test.
Figure S5. Related to Figure 5. The relationship between the cell shape and cell division orientation.

(A) Scatter plot of the deviation angle versus the cell aspect ratio for individual cells. Linear regression analysis (black solid lines) shows that there is no dose-dependent effect between the deviation angle and the aspect ratio of the cell among cells with aspect ratios < 1.53. Nor is there a dose-dependent effect between the deviation angle and the aspect ratio of the cell among cells with aspect ratios > 1.53. (B) The angle between the long-axis of cells at interphase and the long-axis of the airway tube. The long-axis of epithelial cells tends to be aligned with the long-axis of the airway tube.
Figure S6. Related to Figure 6. A cell-based mechanical model explains that mechanical forces control cell shape.

(A, B) The ratio between cells whose aspect ratio >1.53 and cells whose aspect ratio <1.53 remains approximately constant over time. In the model, initially, almost all cells are cells whose aspect ratio <1.53 (red curves) or cells whose aspect ratio >1.53 (blue curves). Different curves correspond to different simulation samples. The black-dashed line marks the position of 0.37 (A). Distribution of cell aspect ratio at time 2000 (B). The grey-dashed line marks the position of 1.53 (B). (C) Cumulative distribution functions obtained from the cell-based mechanical model. The blue curve corresponds to the distribution of cell aspect ratio in a simulation. The red curve corresponds to cell aspect ratio of the GFP (-) cells in a simulation in which GFP (+) cells (40% of total cell number) were introduced. The grey dashed line marks the position of 1.53. (D) Epithelial cells of Shh-CreER; Rosa26-mTmG lungs were labeled in a mosaic manner after tamoxifen injection. (E) Images were collected every 10 minutes. After the cell division, two daughter cells always reinserted themselves into the airway epithelium along the orientation of cell division. Scale bar: E: 10 µm.
Figure S7. Related to Figure 7. Mechanical forces alter the distribution of mitotic spindle angles in the developing airway epithelium.

(A) Cumulative distribution functions of cell aspect ratio in the cell-based mechanical model. The grey dashed line marks the position of 1.53. Applying stretching forces would increase the percentage of cells whose aspect ratio larger than 1.53 as compared with non-stretched control. (B) Dividing angle distribution predicted by the cell-based mechanical model using the
same settings of A. (C) The shape of cells in the BRAF$^{\text{CA}}$ lungs before and after the stretching treatment were imaged and quantified by a membrane GFP reporter. (D, E) The epithelial cell aspect ratio of airway epithelial cells in 5%-stretched (D) or 10%-stretched (E) wild type lungs was quantified before and after being stretched. (F, G) The epithelial cell aspect ratio of airway epithelial cells in 5%-stretched (F) or 10%-stretched (G) BRAF$^{\text{CA}}$ mutant lungs was quantified before and after being stretched. (H) The cell proliferation rate of epithelial or mesenchymal cells in stretched and non-stretched wild type lungs were analyzed by phospho-histone 3 (PH3) antibody staining. The bar graph shows the mean percentage of mitotic cells (mean ± s.e.m., n= 3 lungs each group). (I) The distributions of mitotic spindles in non-stretched, 5%-stretched, and 10%-stretched BRAF$^{\text{CA}}$ lungs. Scale bar: C: 5 μm. N.S., not significant; **, P<0.01; ***, P<0.001, Student’s t-test.