Rotational motion during three-dimensional morphogenesis of mammary epithelial acini relates to laminin matrix assembly

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Our understanding of the mechanisms by which ducts and lobules develop is derived from model organisms and three-dimensional (3D) cell culture models wherein mammalian epithelial cells undergo morphogenesis to form multicellular spheres with a hollow central lumen. However, the mechanophysical properties associated with epithelial morphogenesis are poorly understood. We performed multidimensional live-cell imaging analysis to track the morphogenetic process starting from a single cell to the development of a multicellular, spherical structure composed of polarized epithelial cells surrounding a hollow lumen. We report that in addition to actively maintaining apicobasal polarity, the structures underwent rotational motions at rates of 15–20 μm/h and the structures rotated 360° every 4 h during the early phase of morphogenesis. Rotational motion was independent of the cell cycle, but was blocked by loss of the epithelial polarity proteins Scribble or Pard3, or by inhibition of dynein-based microtubule motors. Interestingly, none of the structures derived from human cancer underwent rotational motion. We found a direct relationship between rotational motion and assembly of endogenous basement membrane matrix around the 3D structures, and that structures that failed to rotate were defective in weaging exogenous laminin matrix. Dissolution of basement membrane around mature, nonrotating acini restored rotational movement and the ability to assemble exogenous laminin. Thus, coordinated rotational movement is a unique mechanophysical process observed during normal 3D morphogenesis that regulates laminin matrix assembly and is lost in cancer-derived epithelial cells.

extracellular matrix | tubulogenesis | plasticity

Tissues are composed of a community of cells that act in a coordinated manner to achieve and maintain normal architecture and perform their function. The mechanisms by which cells coordinate their behavior during tissue morphogenesis is poorly understood in part due to technical bottlenecks associated with studying the process in vivo. Three-dimensional (3D) culture systems provide an environment in which normal tissue morphogenesis can be recapitulated and thus is a powerful tool for investigating the molecular signals that specify epithelial tissue architecture (1–3). Unlike monolayer cultures, epithelial cells grown in 3D recapitulate numerous features seen in vivo, including the formation of acini-like spheroids with a hollow lumen, apicobasal polarization of cells comprising these acini, and the deposition of basement membrane components collagen IV and laminin-332 (2).

The initial stages of 3D morphogenesis are characterized by the ability of epithelial cells to detect surfaces with which they come in contact. Cells use integrins and dystroglycans to contact the extracellular matrix (ECM), and cadherins and desmosomes to contact their neighbors (3). These initial contacts trigger a series of cell remodeling events, which result in polarization of cells to create a basolateral surface and an apical surface with asymmetric distribution of membrane proteins. Whereas development of membrane protein asymmetry can occur in monolayer cultures, formation of multicellular structures of finite size and with a hollow central lumen is unique to 3D cultures. Epithelial cells immediately surrounding the lumen point their apical pole toward the lumen. This ability of cells to polarize is needed for morphogenesis and inhibiting polarization affects lumen formation and laminin assembly around 3D cysts (4). Exogenous laminin-1 rescues polarity and lumen formation, suggesting laminin assembly is an important step that initiates and promotes morphogenesis (4). The mechanisms by which cells establish polarization and assemble matrix around them are poorly understood.

To better understand 3D morphogenesis, we engineered Michigan Cancer Foundation (MCF)-10A human mammary epithelial cells to express fluorescent protein reporters and followed them by high-resolution four-dimensional imaging. We report a surprising observation that adds a unique insight into the morphogenetic process. We find that the 3D structures undergo a coordinated rotational movement during the early stages of morphogenesis and that this process is required for assembly of laminins and collagen around the 3D structures. Both 3D structures derived from MCF-10A cells lacking expression of polarity proteins and cancer-derived cell lines fail to undergo rotational motion and are defective in their ability to assemble laminin matrix.

Results

MCF-10A Cells Maintain Cell Polarity and Display Rotational Motion During 3D Morphogenesis. To investigate the events that occur during in vitro 3D morphogenesis of human mammary epithelial cells, we created an MCF-10A reporter cell line stably coexpressing fluorescent histone 2B (H2B)–Venus and Golgin A2-mCherry (5) fusion proteins to mark the nucleus and the Golgi apparatus, respectively. Using these cells grown fully embedded in a Matrigel matrix, we performed live-cell imaging of acinar morphogenesis over a period of 10 d with a time resolution of 2 h, starting with single cells and culminating in multicellular acini. Live-cell imaging data were processed using Imaris software (Bitplane). Three-dimensional intensity thresholding and tracking analysis functions allowed us to identify and track nuclei and Golgi movements over time. Golgi orientation was used as a surrogate marker of the apical pole of a cell undergoing morphogenesis. We discovered that cell polarity, as monitored by the presence of the apical pole, is actively maintained during multiple rounds of cell division, despite marked cell movement and reorganization (Fig. L4 and Movie S1). Because MCF-10A cells do not develop tight junctions, a hallmark of apicobasal membrane polarity, it is likely that development and maintenance of the apical pole is independent of the cell’s ability to establish apicobasal membrane polarity.


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To better understand the morphogenetic process from a single cell to multicellular acini, we increased the time resolution, scanning 3D structures every 10 min for 2 h every day from day 1 to day 9. Surprisingly, we found that cells moved following rotational movements along the periphery and diagonal movements across the lumen during morphogenesis (Fig. 1B). The structure rotated 360° every 4 h (Fig. 1C). These observations highlight the dynamic nature of the morphogenetic program.

**Rotational Motion Occurs During Early Stages of 3D Morphogenesis and Depends on Microtubule Networks.** Detailed analysis of coordinated rotational movement from day 0 to day 8 showed that, whereas on day 0 single MCF-10A cells did not rotate (Fig. S1A), beginning from day 1 the cells displayed rotational motion for the first 4 d of morphogenesis (Fig. 2A), which stopped by day 5, despite the fact that the structures continued to grow. The cells, however, continued to show random movement until day 7 and all types of movement stopped in mature (day 8 or older) acini. Quantifications showed that 90–100% of days 2–3 acini showed rotational motion and more than 70% of structures underwent rotational movement on day 4 of 3D morphogenesis (Table S1) with speeds ranging from 13 μm·h⁻¹ to 17 μm·h⁻¹ (Fig. 2B). No preference for left- or right-hand orientation was detected, and few structures changed the direction of rotation during the course of morphogenesis. The random movement we observed after day 5 is similar to the noninvasive movement previously reported in MCF-10A acini (6), and the meandering movement of epithelial cells in salivary bud (7); however, in both cases, no movement was observed in mature acini, consistent with our observations. These observations demonstrate that rotational and random movements are observed during early stages of morphogenesis and that random movements can occur in the absence of rotational movements, suggesting that rotational and random movements may be regulated by distinct mechanisms.

The actin cytoskeleton plays a critical role during morphogenesis and actomyosin contractility is required for noninvasive movement (9). We first investigated the role of actin fibers during rotational motion. To monitor changes in actin dynamics during morphogenesis, we generated MCF-10A cells expressing red fluorescent protein-tagged Lifeact (Lifeact-TagRFP), a 17-amino-acid peptide that labels filamentous actin (F-actin) (Fig. S1B). Interference of F-actin with cytochalasin D or inhibition of myosin II with blebbistatin is known to block stress fiber formation. Treatment of day 3 acini with 5.0 μM cytochalasin D or 50.0 μM blebbistatin disrupted cortical actin (Fig. S1B) and strongly inhibited both rotational and random movements (Fig. 2C). To test whether increased actomyosin contractility can induce rotational motion, the acini were treated with lysophosphatidic acid (LPA), an extracellular signaling phospholipid known to activate myosin II by stimulating phosphorylation of myosin light chain (MLC) (10, 11). However, we did not observe accelerated rotational motion in 3D acini treated with LPA (Fig. S1E). Thus, inhibiting actomyosin contractility reduces both rotational and random cell movements in 3D acini.

To test the relationship between cell cycle progression and rotational motion, we treated day 3 MCF-10A acini with mitomycin C, a DNA cross-linking agent, for 7 h before imaging (Fig. S1C). Rotational motion was still detectable in acini treated with mitomycin C (Fig. 2C) and no significant changes in movement speed were found in these structures (Fig. S1E). This is consistent with previous observations that hyperproliferation is not sufficient to induce noninvasive movement (9) and that mitotic inhibition does not block epithelial cell movement during submandibular gland morphogenesis (12).

Microtubules have been shown to mediate directional cell migration and Golgi positioning in cells (13). To test whether the microtubule cytoskeleton is involved in rotational motion, we disrupted the microtubule network using the microtubule depolymerizing drug, nocodazole (Fig. S1D). This drug blocked rotational movement of day 3 acini where only 8.3% rotated in treated groups compared with 100% of rotating structures in the control groups, without completely inhibiting random movements (Fig. 2C) and...
Fig. S1), demonstrating a selective role for microtubule dynamics during rotational movement. Lifeact-TagRFP images did not show a difference in the organization of cortical actin in nocodazole-treated cells (Fig. S1B), suggesting that the nocodazole effect was not due to indirect effects on actin. Dynemin motors play important roles during microtubule-mediated processes. To genetically interfere with microtubule function we overexpressed dynamitin and disrupted dynein function (Fig. S2A) (14,15). We observed a 75% decrease in the number of structures that rotated and a 50% decrease in the speed of movement (Fig. 2D and Fig. S2B). However, dynamitin overexpression did not block the random movement of cells within acini (Fig. 2D). Consistent with previous reports (14), overexpression of dynamitin disrupted Golgi organization and orientation (Fig. S2C), suggesting that organelle localization may play an important role during rotational motion. Thus, microtubule polymerization and dynein motors are required for coordinated rotational motion but are dispensable for random migration.

Rotational Motion Depends on Cell Polarity and Is Impaired in 3D Structures Generated from Cancer Cell Lines. Because loss of cell polarity proteins is known to interfere with morphogenesis (8), we investigated the effect of depleting expression of polarity proteins Scribble and Pard3 on rotational movement. We down-regulated expression of Scribble and Pard3 using RNA interference (Fig. S2D and E). To confirm that loss of these polarity proteins affects cell polarization in 3D acini, we monitored the orientation of the Golgi apparatus, which marks the apical pole of polarized epithelial cells. Whereas the Golgi was oriented toward the lumen in control acini (Fig. 3A), this orientation was disrupted in both Scribble knockdown (ScribKD) and Pard3 knockdown (Pard3KD) cells, with Pard3KD having a stronger effect than ScribKD (Fig. 3B and C). The ScribKD and Pard3KD day 4 acini failed to display any rotational motions (Fig. 3B and C); however, the cells retained the ability to undergo random movements. These observations demonstrate that the cell polarity proteins Scribble and Pard3 are required for rotational motion, whereas they are dispensable for the random movement in 3D acini.

Considering that morphogenetic programs are frequently altered in tumor-derived epithelial cells, we analyzed several human cancer-derived cell lines: breast cancer-derived MCF-7 and T47D, colon cancer-derived Caco-2, and pancreatic cancer-derived Panc-1 cells. We chose these cells to represent diverse epithelial duct-containing organs and due to their ability to grow as spheres when embedded within a 3D matrix, thus allowing for a direct comparison with MCF-10A acini. Whereas the spheres formed by MCF-7, T47D, and Panc-1 cells did not have a lumen (Fig. 3D, E, and G), Caco-2 cells formed cyst-like structures with a single epithelial cell layer surrounding a central lumen (Fig. 3F), consistent
with previous reports (16). Strikingly, none of the spheres formed by cancer-derived cell lines displayed rotational motion. The inability to undergo rotational movement was not due to differences in growth rates because these cancer cell lines developed into structures of similar or larger sizes and with similar cell numbers per structure, compared with MCF-10A acini. The lack of rotational motion was also not due to differences in lumen formation because structures developed from Caco-2 cells had a proper lumen (Fig. 3F). Interestingly, the orientation and/or organization of the Golgi apparatus was disrupted in all cancer cell-derived structures (Fig. 3 D–G), indicating that, like ScribKD and Pard3KD MCF-10A cells (Fig. 3 A and B), cancer-derived cells lack the ability to orient their apical pole within 3D structures. We note that, whereas Caco-2 cells retain the ability to establish apicobasal polarity (as defined by tight junctions and apical membrane proteins) (16), they fail to establish an apical pole (as defined by Golgi orientation), suggesting that establishment of apicobasal polarity and establishment of the apical pole are regulated by distinct mechanisms. Thus, we demonstrate that rotational motion is lost in cancer cells and identify a direct relationship between the ability of cells to orient their apical pole (as defined by Golgi localization) toward the lumen in 3D cysts and rotational motion.

Consistent with the role for microtubules during rotation of MCF-10A acini, microtubule organization was aberrant in all cancer cell lines analyzed compared with that observed in MCF-10A cells (Fig. S3A). Microtubule networks play critical roles during vesicle transport. To investigate whether there were gross differences in vesicle transport between MCF-10A and cancer cell lines, we monitored overall organization of early endosomes using a reporter that marks early endosomes using Rab5a–Venus. We noticed that MCF-10A cells showed a dense, sheet-like organization (Fig. 4D), whereas the cancer cell lines show a diffused signal throughout the cells, suggesting a defect in endosomal trafficking process (Fig. S3B). Consistent with the possible differences in trafficking processes, alpha6 integrin, failed to show basal localization in cancer cells compared with MCF-10A cells (Fig. S3C), suggesting that localization of matrix receptors may contribute to the defect in the ability of cancer cells to rotate.

**Rotation**

Rotation is a process by which cells change position in three-dimensional (3D) environments. It is crucial for the development and function of many tissues and organs. In the context of this study, rotation is observed in normal breast epithelial cells (MCF-10A) but is lost in cancer cells. The authors demonstrated that the loss of rotational motion in cancer cells is not due to differences in growth rates or lumen formation. They found that the inability to rotate was due to differences in microtubule organization and endosomal trafficking.

**Basement Membrane**

Basement membranes are specialized layers of extracellular matrix proteins that underlie the basal side of all epithelia and play a critical role during morphogenesis. In this study, the authors examined the assembly of matrix proteins such as laminins and collagen IV, which are key components of basement membranes. They found that while MCF-10A acini assembled a continuous, compact layer of laminin and collagen, cancer cell-derived structures showed clear defects in basement membrane assembly, particularly around the basal side. The authors demonstrated that rotational motion is lost in cancer cells and use this as a correlate to the defect in the ability of cancer cells to rotate.

**Cell Polarization**

Cell polarization is a fundamental process that is crucial for cell function and cell-cell interactions. The authors show that the loss of apical membrane proteins like GM130 and tight junction proteins like E-cadherin in cancer cells correlates with the loss of rotational motion. This suggests that the ability of cells to rotate is tightly coupled with their ability to maintain apicobasal polarity.

**ECM Assembly**

The extracellular matrix (ECM) is a dynamic network of proteins and glycosaminoglycans that provides structural support and guides cell behavior. The authors observed changes in ECM assembly in cancer cell-derived structures compared to normal breast epithelial cells. They found disruptions in the assembly of laminins and collagen IV, which are crucial components of basement membranes. These findings suggest that the loss of rotational motion is linked to defects in ECM assembly and function.

**Conclusions**

In conclusion, the authors demonstrate that rotational motion is lost in cancer cells, which correlates with defects in microtubule organization, endosomal trafficking, and ECM assembly. They propose that the inability to rotate may contribute to the defective behavior of cancer cells. This study provides insights into the molecular mechanisms underlying the loss of rotational motion and offers potential targets for the development of anti-cancer therapies.
In addition, we find that both cell polarity and microtubule dynamics are required for the rotational motion. Whereas inhibition of actomyosin contractility blocked both rotational and random cell movement, disruption of microtubules inhibited rotational motion without affecting random movement. We are intrigued by the specificity of the role played by microtubules because of the intimate relationship between the microtubule cytoskeleton and polarized cellular processes such as migration and vesicle trafficking (25). In polarized epithelia, microtubules align along the lateral membrane with minus ends facing the apical and plus ends facing the basal domains (26). Consistent with this notion, we find that the overall organization of microtubules was disrupted in 3D structures derived from cancer cell lines that failed to rotate (Fig. S3A). Whereas we observe defects in organization of Rab5a early endosome and basal localization of alpha6 integrin (Fig. S3 B and C), the mechanisms may also involve orientation of centrosomes, or Golgi or directed vesicle transport. It is also possible to establish polarity by regulating orientation of the apical pole by positioning organelles such as the Golgi or centrosomes along the axis of polarity. Golgi elements directly interact with microtubule motors, dynein and kinesin. The interaction of the Golgi with the dynein I complex is critical for both compaction of the Golgi and maintenance of Golgi stacks (27). Disruption of directional Golgi orientation by loss of golgins, golgin-160 and GMAP210, impairs directional cell migration and wound healing, demonstrating a direct relationship between polarization of Golgi stacks and directional migration (28). It is likely that the relationship between Golgi orientation and microtubule dynamics is required for the rotational motion.

Discussion

We report that 3D acini undergo rotational motion during the first 4 d of morphogenesis. Whereas our observation is consistent with recent studies on rotational motions (23, 24), we find an unexpected role for rotational motion during assembly of basement membrane matrix. Interestingly, assembly of fibronectin matrix did not require the rotational motion. Thus, we report a unique mechanophysical property associated with basement membrane assembly during 3D acinar morphogenesis.

Fig. 4. Structures with impaired rotational motion do not assemble a proper basement membrane. (A) Assembly of laminin-332 during 3D morphogenesis. MCF-10A cells grown inside Matrigel were immunostained for laminin-332 (green) at indicated days after plating. Confocal images through the equator of the structures are shown. (B and C) Day 4 structures of wild-type and Pard3KD MCF-10A, T47D, and Caco-2 cells were immunostained for laminin-332 or collagen IV. Lower Right Inset shows a magnification of the indicated white rectangular region. Scale bar, 20 μm. (D) Transmission electron microscopy images of basement membrane organization in MCF-10A, Pard3KD MCF-10A, T47D, and Caco-2 structures. Arrows indicate regions of densely organized basement membrane immediately adjacent to the basal cell surface of wild-type MCF-10A acini. Lower Left Inset shows the digital magnification of the indicated black rectangular region. Scale bar, 2.0 μm.

Fig. 5. Relationship between rotational motion and laminin-111 assembly. MCF-10A, Pard3KD MCF-10A, T47D, and Caco-2 cells were grown for 3 d in Matrigel, and then 50 μg/mL of rhodamine-labeled mouse laminin-111 (A) or fibronectin (B) was added into the culture medium. After 24 h (laminin) or 8 h (fibronectin), the structures were fixed and rhodamine-labeled matrix proteins were directly visualized in the 568-nm channel (red). Normal MCF-10A acini (C) or acini treated with collagenase/hyaluronidase treatment (D) were immunostained for collagen IV (Left) or incubated with rhodamine-laminin-111 (Right). Scale bars, 20 μm.
Materials and Methods

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collagen IV by the action of the linking proteins, nidogen, perle-

basement membrane assembly. The laminin matrix is linked to the

laminin-111, laminin-332, and collagen IV components of base-

membrane. In addition to collagen IV and laminin, base-

membrane contains nidogens, perlecann, and agrin. Whereas

the process of basement membrane assembly is not well un-
derstood, it is thought that interaction of laminin with cell sur-

face receptors integrin and dystroglycans initiate the process of

basement membrane assembly. The laminin matrix is linked to

the collagen IV by the action of the linking proteins, nidogen, perle-

can, and agrin during the maturation of the basement membrane

lattice (29). The role rotational motion plays during this process

is not known. Consistent with our observations, a recent study

highlight the need to place more emphasis on understanding the

mechanisms that regulate plasticity and dynamics of cellular inter-

actions where cell–cell and cell–matrix interactions are in a state

of constant flux.

Materials and Methods

Cell Culture and 3D Morphogenesis Assay. The culture condition of MCF-10A, Pard3KD, and ScribKD cells was described previously (33). T47D cells were cultured in RPMI medium 1640 supplemented with 10% (vol/vol) FBS and 10 μg/mL insulin. MCF-7 cells were cultured in Eagle’s Minimum Essential Medium (ATCC 30-2003) supplemented with 10% (vol/vol) FBS, 10 μg/mL insulin; Panc-1 cells were cultured in DMEM supplemented with 10% FBS. Caco-2 cells were cultured in EMEM medium supplemented with 10% FBS and 2 mM glutamine. The details of 3D morphogenesis assay were de-
scribed previously (33).

Electron Microscopy. Three-dimensional acini were fixed for transmission electron microscopy with 2% glutaraldehyde buffer (pH 7.4) for 1–2 h and processed subsequently by the University of Toronto EM facility (www.medresearch.utoronto.ca/mil.home.html).

Confocal Microscopy and Image Analysis. Day 3 and day 4 acini were fixed and processed for immunohistochemistry as previously described (33). The following primary antibodies were used. Anti-GM130 for visualizing the Golgi complex (1/100; BD), antiaminin-gama2 for laminin-332 (1/100; Milli-

pore), anticalcogen IV (1/100; DAKO), antialaminin (1/100; Millipore), antik-C67 (1/100; Zymed), anti-α-tubulin (1/100; Sigma), and anti-j- integrin (1/100; DSHB). After overnight incubation at 4 °C, secondary antibodies conjugated with Alexa 488 or 568 ( Molecular Probes) were applied for 2 h at room temperature. Slides were incubated for 15 min with Hoechst 33342 ( Molecular Probes) to label nuclei and examined using an Olympus LV1000 confocal microscope.

Detailed information on reagents, used molecular biology, production of stable cell lines, 4D confocal microscopy, immunoblotting, and statistical analysis can be found in SI Materials and Methods.

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Supporting Information

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SI Materials and Methods

Reagents. Cytochalasin D (5 μM; Sigma; C8273), nocodazole (1.6 μM; Sigma; M1404), blebbistatin (50 μM; Sigma; B0560), lysophosphatidic acid (LPA) (30 μM; Sigma; L7260), and mitomycin C (25 μg/mL; Sigma; M4287) were dissolved in DMSO or water and diluted to working concentrations in assay medium before live-cell imaging. Rhodamine-conjugated laminin-111 (50 μg/mL; Cytoskeleton; LMN01) and fibronectin (50 μg/mL; Cytoskeleton; FNR01-A) 10x collagenase/haeurodonidase (STEMCELL Technologies; 7912) were resuspended in assay medium and diluted to 1x working concentration.

Molecular Biology. Total RNA of MCF-10A cells growing on plastic dishes was isolated by TRIzol reagent (Invitrogen). First-strand cDNA synthesis from 2 μg of above total RNA was performed with the SuperScript III Reverse Transcriptase (Invitrogen) according to the manufacturer's instructions.

pDNA3–H2B–Venus vector was obtained from Addgene (plasmid 20971) (1). H2B–Venus fusion was removed by KpnI/XbaI excision, blunted and subcloned in pMSCV–Blunt opened with HpaI. For making pMSCV–Blunt–GOLGA2–mCherry. The cDNA for human GOLGA2 was obtained from Open Biosystems (clone ID 6340972). GOLGA2 cDNA was amplified using primers containing Nhel and AgeI and subcloned in pmCherry–N1. Resulting fusion was then excised using XhoI and NotI, blunted and subcloned in pMSCV–Blunt opened with HpaI. LifeAct–TagRFP (Ibidi) was PCR amplified using primers containing XhoI at both sides and inserted into the XhoI site of pMSCV–hygro. For making pMSCV–hygro–dynamitin–T2A–H2B–EGFP, dynamitin was obtained by reverse transcriptase PCR from MCF-10A total RNA as template. Dynamitin–T2A–H2B–EGFP cassette was obtained by overlapping PCR from fragments of dynamitin and T2A–H2B–EGFP and TOPO cloned into pCR8/GW/TOPO TA (Invitrogen) and transferred into GATEWAY (Invitrogen) compatible versions of pMSCV–hygro–RfB by the LR reaction as specified by the manufacturer (http://products.invitrogen.com/ivgn/product/11790109). 

Production of Stable Cell Lines. Replication-incompetent retrovirus-encoding fusion proteins were synthesized using the 293GPG, a human 293-derived retroviral packaging cell line capable of producing high titers of recombinant moloney murine leukemia virus particles that have incorporated the vesicular stomatitis virus G (VSV-G) protein (2). Briefly, 20 μg of retroviral vector was transfected into 7 × 10⁶ 293GPG cells cultured in a 10-cm dish using 60 μL of Lipofectamine (Invitrogen). Retroviruses were harvested on days 4 and 5 after transfection by filtering media from the 293GPG cell line with low protein binding 0.45-μm syringe filters. Fresh virus was used to infect the target cells for a period of 6 h in the appropriate medium in the presence of 8 μg/mL hexadimethrine bromide (Sigma; H9268) in target cell growth media.

Three-dimensional morphogenesis assays were performed using growth factor reduced Matrigel (BD Biosciences). Assays were carried out in eight-well chambers (Lab-Tek II chambered coverslips 1.5; Nunc 70378–81). Cells were seeded at a density of 5,000 cells per well. The culture consisted of two Matrigel layers fully embedding the cells. The first, underlying layer of 40 μL of Matrigel was established and allowed to solidify at 37 °C for 25 min. Cells were then plated on top of this layer and allowed to attach for 8 h. Top media was then collected and saved, and a second layer of 40 μL of Matrigel was applied, allowed to solidify as done previously, and the top media was returned on top of Matrigel. Top media consisted of assay media (DMEM/F12 + 2% horse serum, 0.5 μg/mL hydrocortisone, 0.1 μg/mL cholera toxin, 10 μg/mL insulin, and 1% penicillin/streptomycin) supplemented with 2% Matrigel and 5 ng/mL EGF. Regular growth media supplemented with 2% Matrigel was used for all other cell lines.

Four-dimensional Confocal Microscopy. Morphogenesis of 3D cultures was followed using a Nikon A1R confocal laser scanning microscope system coupled to an upright Eclipse TI microscope (Nikon). An integrated Tokai Hit incubation chamber was used to maintain optimal parameters for cell culture [5% (vol/vol) CO₂, 37 °C, and 100% humidity]. To follow H2B–Venus and GOLGA2–mCherry expression, we used 488 and 561 nm laser lines with laser power set to 2.5% for 488 nm and 5% for 561 nm. Gain was set between 130 and 180 depending on the structure. Structures were imaged through a CFI Plan Apo VC 20x NA 0.75 objective. X and y coordinates were acquired at 512 × 512. To visualize the 3D structures, we acquired z stacks at a resolution of 850 nm. Time-lapse imaging was done every 10 min or 120 min for the indicated period. Resulting raw data files (*.nd2) were then processed with NIS Element Advanced Research (Nikon). Data were deconvolved, and z and t intensities compensation were applied. Processed data were then imported into Imaris (Bitplane) and subjected to median filtering (3 × 3 × 1). Nuclei and the Golgi apparatus were then modeled following the "Spots" protocol of Imaris (detection diameter varying from 5,800 to 6,500 nm). Resulting spots were then tracked over time using the tracking function of Imaris with the following parameters: "max distance" was set to 2,500–20,000 nm (depending on the maximum displacement measured within the dataset) and "max gap size" was set to 1 for data having a Δt of 10 min.

Immunoblotting. Cells infected with retrovirus expressing T2A–H2B–EGFP or dynamitin–T2A–H2B–EGFP were trypsinized and rinsed with ice-cold PBS and lysed in RIPA buffer. After, the lysates were centrifuged at 15,000 × g for 15 min at 4 °C. Protein concentration was normalized using the Biorad protein assay. Dynamitin was visualized by antidynamitin antibody (1/1,000; Abcam). Add antibodies for beta-actin (1/3,000; Sigma), Pard3 (1/500; Millipore), and Scribble (1/200; Santa Cruz).

Statistical Analysis. Statistical analyses were performed using the software GraphPad Prism version 4.02. Quantitative data shown as histograms are expressed as means ± SEM. Results were assessed for statistical significance using Student’s t test (integrated in the software GraphPad Prism) and differences were considered statistically significant at P < 0.05. Asterisks in the histograms indicate the different P values: *P < 0.05; **P < 0.01.

1. Nam HS, Benezra R (2009) High levels of Id1 expression de
Fig. S1. MCF-10A cell movement analysis at day 0 of 3D morphogenesis and effects of cytoskeleton and proliferation inhibitors on coordinated rotational motion of MCF-10A acini. (A) Z stacks of MCF-10A cells expressing H2B–Venus were acquired every 10 min for 2.0 h at day 0 (6.0 h after plating) of 3D morphogenesis inside Matrigel. Blue end indicates t₀, progressing to purple, red, yellow, and ending with white. Only oscillation (speed < 5 μm·h⁻¹) but no rotational movement is observed. (B) MCF-10A acini from cells stably expressing LifeAct–TagRFP were treated with the indicated reagents on day 3 of morphogenesis. Acini were fixed and F-actin (red) was visualized by a 568-nm channel. (C) Day 3 MCF-10A acini were treated with mitomycin C for 7 h to block the cell cycle. Structures were fixed and immunostained for Ki67 (green) and counterstained with Hoechst to show nuclei (blue). (D) Day3 MCF-10A acini were treated with nocodazole for 2 h to depolymerize microtubules. Structures were fixed and immunostained for α-tubulin (green) and counterstained with Hoechst to show nuclei (blue). (E) MCF-10A cell movement speed analysis after treatment with indicated reagents. (Scale bar, 20 μm.)
Fig. S2. Overexpression of hDynamitin and Pard3 and Scribble knockdown. (A) Representative Western blot analysis showing dynamitin protein levels. Lysates of MCF-10A cells infected with retroviruses expressing H2B–EGFP or hDynamitin–T2A–H2B–EGFP. Blots were probed with antibodies for dynamitin, and β-actin as a loading control. (B) MCF-10A cell movement speed analysis at day 3 of 3D morphogenesis. (C) Day 3 MCF-10A acini overexpressing hDynamitin were fixed and immunostained for GM130 (Golgi apparatus marker, red; white arrows point to the fragmented Golgi apparatus) and counterstained with Hoechst to show nuclei (blue). (D and E) Representative Western blot analysis showing knockdown of the indicated cell polarity proteins. Lysates of MCF-10A cells infected with retroviruses expressing Pard3 or Scribble shRNA. Blots were probed with indicated antibodies, and β-actin or α-tubulin was used as a loading control. (Scale bar, 20 μm.)
Fig. S3. Microtubule organization and vesicle trafficking. (A) Day 4 MCF-10A and cancer-derived acini were fixed and immunostained for α-tubulin (green) and counterstained with Hoechst to show nuclei (blue). (B) Early endosomes were visualized in MCF-10A and cancer-derived 2D cell cultures by Rab5a targeting sequence fused with EGFP. (C) Day 4 structures of MCF-10A and cancer-derived acini were fixed and immunostained for α6 integrin and counterstained with Hoechst to show nuclei (blue). (Scale bar, 20 μm.)
Fig. S4. Nonrotational acini have impaired assembly of laminins and collagen IV. Day 4 3D structures of indicated cell lines were fixed and immunostained for laminin-332 (A) or laminin β1 subunit (B) or collagen IV (C) by human specific antibodies and counterstained with Hoechst to show nuclei (blue). Lower Right Inset shows a magnification of the indicated white rectangular region. (Scale bar, 20 μm.)
**Fig. S5.** Overexpression of hDynamitin causes a delay in laminin-332 and collagen IV assembly. Day 1 to day 2.5 acini of MCF-10A wild-type and dynamitin overexpression acini were fixed and immunostained for laminin-332 (A, red) and collagen IV (B, red) and counterstained with Hoechst to show nuclei (blue). (Scale bars, 20 μm.)

**Fig. S6.** Structures with impaired rotational motion do not assemble a proper basement membrane. Dynamitin overexpression and ScribKD MCF-10A, MCF-7, and Panc-1 cells were grown for 3 d in a Matrigel matrix, and then 50 μg/mL of rhodamine-labeled mouse laminin-111 (A) or fibronectin (B) was added into the culture medium. After 24 h (laminin) or 8 h (fibronectin), the structures were fixed and rhodamine-labeled matrix proteins were directly visualized in the 568-nm channel (red). Structures were counterstained with Hoechst to show nuclei (blue). (Scale bars, 20 μm.)

**Table S1.** Quantification of MCF-10A acini shown in Fig. 2 that display rotational motion

<table>
<thead>
<tr>
<th>Days after seeding in Matrigel</th>
<th>Rotating structures/total no. of structures (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>10/14 (71)</td>
</tr>
<tr>
<td>2</td>
<td>12/13 (92)</td>
</tr>
<tr>
<td>3</td>
<td>12/12 (100)</td>
</tr>
<tr>
<td>4</td>
<td>9/12 (75)</td>
</tr>
<tr>
<td>5</td>
<td>0/9 (0)</td>
</tr>
<tr>
<td>6</td>
<td>0/10 (0)</td>
</tr>
<tr>
<td>7</td>
<td>0/10 (0)</td>
</tr>
<tr>
<td>8</td>
<td>0/8 (0)</td>
</tr>
<tr>
<td>9</td>
<td>0/7 (0)</td>
</tr>
<tr>
<td>10</td>
<td>0/5 (0)</td>
</tr>
<tr>
<td>11</td>
<td>0/7 (0)</td>
</tr>
<tr>
<td>12</td>
<td>0/7 (0)</td>
</tr>
</tbody>
</table>
Movie S1. Live-imaging analysis of cell polarity during development of an MCF-10A acinus. An MCF-10A reporter cell line engineered to express fluorescent markers identifying the nuclei and the Golgi apparatus (H2B–Venus and GOLGA2–mCherry). Development of the 3D structure was followed by recording time-lapse images every 120 min for 10 d (from day 0 to day 9). Resulting raw data files were then deconvolved, intensity compensated in z and t, and subjected to median filtering (3 × 3 × 1). Resulting intensity signals for each time point were then assembled into a movie file. For clarity, only the equatorial cross-section of the acinus has been selected.

Movie S1