Supporting Information

Wang et al. 10.1073/pnas.1201141110

SI Materials and Methods

Reagents. Cytochalasin D (5μM; 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) 10× collagenase/hyaluronidase (STEMCELL Technologies; 7912) were resuspended in assay medium and diluted to 1× 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 IIReverse Transcriptase (Invitrogen) according to the manufacturer's instructions.

pcDNA3–H2B–Venus vector was obtained from Addgene (plasmid 20971) (1). H2B–Venus fusion was removed by KpnI/XbaI excision, blunted and subcloned in pMSCV–Blast opened with HpaI. For making pMSCV–Blast–GOLGA2–mCherry. The cDNA for human GOLGA2 was obtained from Open Biosystems (clone ID 6340972). GOLGA2 cDNA was amplified using primers containing NheI and AgeI and subcloned in pmCherry–N1. Resulting fusion was then excised using XhoI and NotI, blunted and subcloned in pMSCV–Blast opened with HpaI. LifeAct–TagRFP (Ibidi) was PCR amplified using primers containing Xho1 at both sides and inserted into the Xho1 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/11791019).

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 7×10^6 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 coverglass 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) CO_2, 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 20× 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 ($3 \times 3 \times 1$). 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 $15{,}000 \times g$ 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 define B1 type adult neural stem cells. *Cell Stem Cell* 5(5):515–26.

2. Ory DS, Neugeboren BA, Mulligan RC (1996) A stable human-derived packaging cell line for production of high titer retrovirus/vesicular stomatitis virus G pseudotypes. *Proc Natl Acad Sci USA* 93(21):11400–11406.

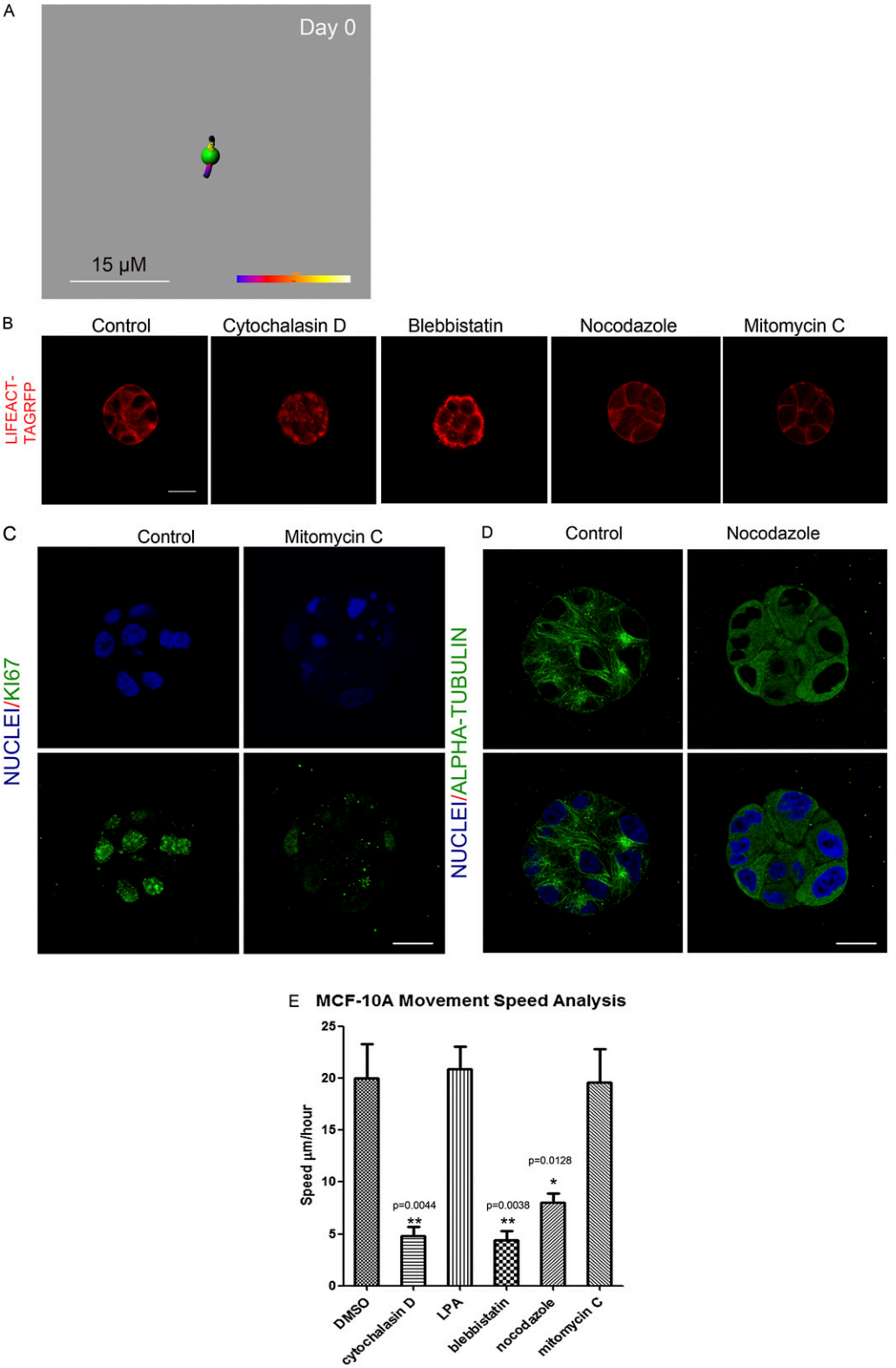


Fig. S1. MCF-10A cell movement analysis at day 0 of 3D morphogenesis and effects of cytoskeleton and proliferation inhibitors on coordinated rotational motion of MCF-10A acini. (*A*) Z stacks of MCF-10A cells expressing H2B–Venus were acquired every 10 min for 2.0 h at day 0 (6.0 h after plating) of 3D morphogenesis inside Matrigel. Blue end indicates t_0, 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.)

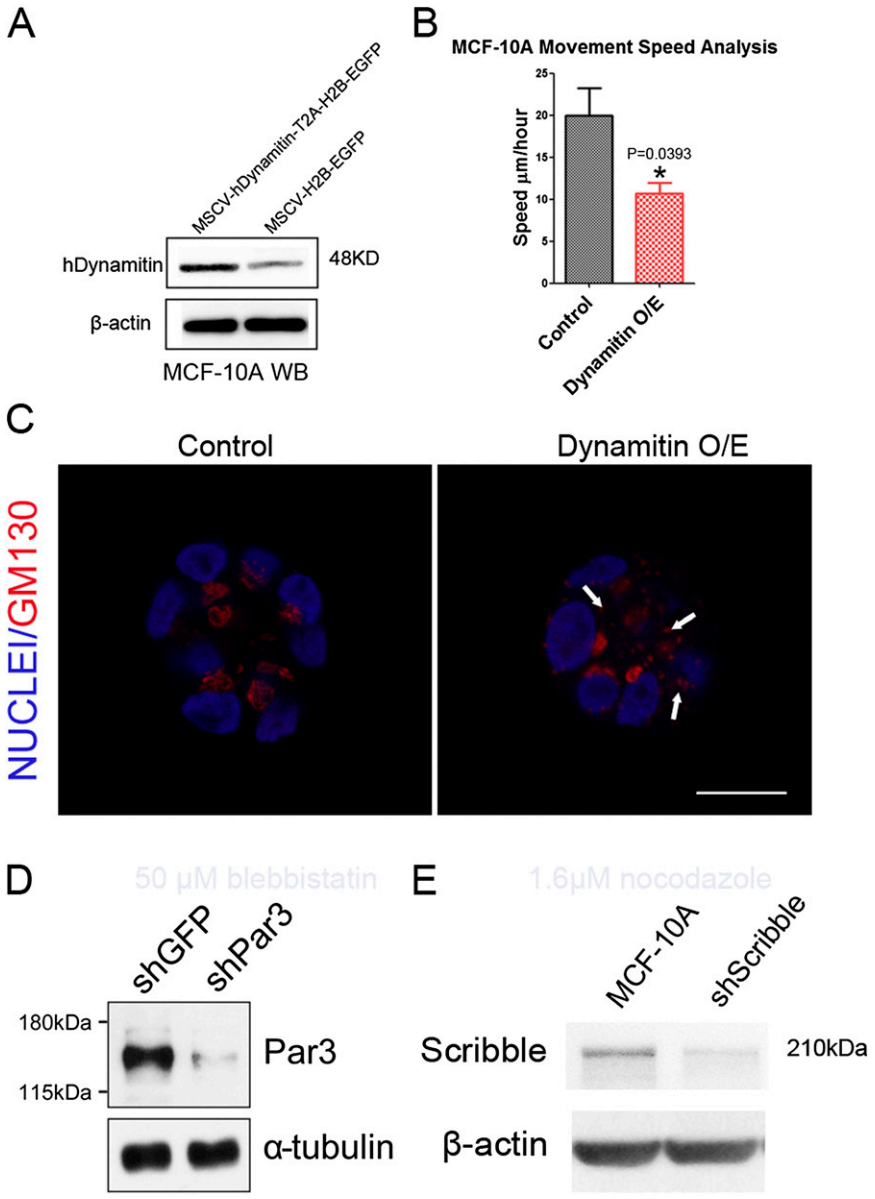


Fig. S2. Overexpression of hDynamitin and Pard3 and Scribble knockdown. (*A*) Representative Western blot analysis showing dynamitin protein levels. Lysates of MCF-10A cells infected with retroviruses expressing H2B–EGFP or hDynamitin–T2A–H2B–EGFP. Blots were probed with antibodies for dynamitin, and 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.)

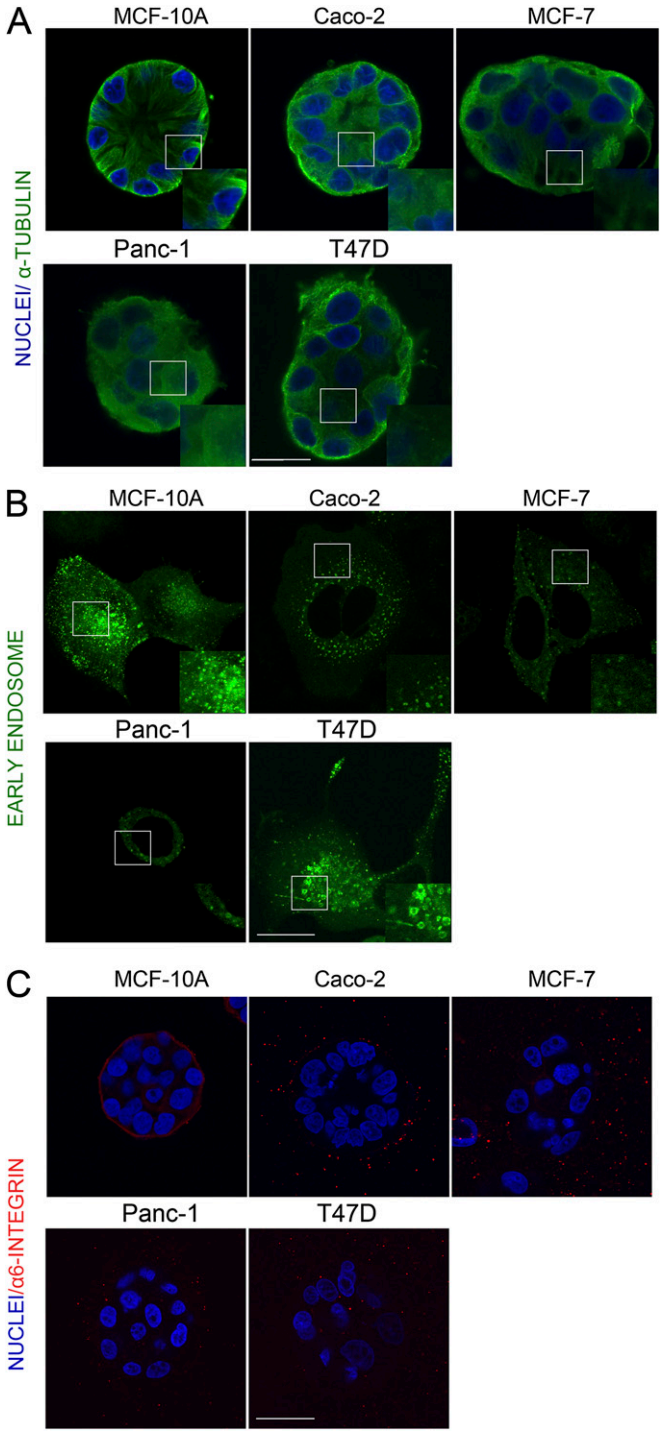


Fig. S3. Microtubule organization and vesicle trafficking. (*A*) Day4 MCF-10A and cancer-derived acini were fixed and immunostained for α-tubulin (green) and counterstained with Hoechst to show nuclei (blue). (*B*) Early endosomes were visualized in MCF-10A and cancer-derived 2D cell cultures by Rab5a targeting sequence fused with EGFP. (*C*) Day 4 structures of MCF-10A and cancer-derived acini were fixed and immunostained for α6 intergrin and counterstained with Hoechst to show nuclei (blue). (Scale bar, 20 μm.)

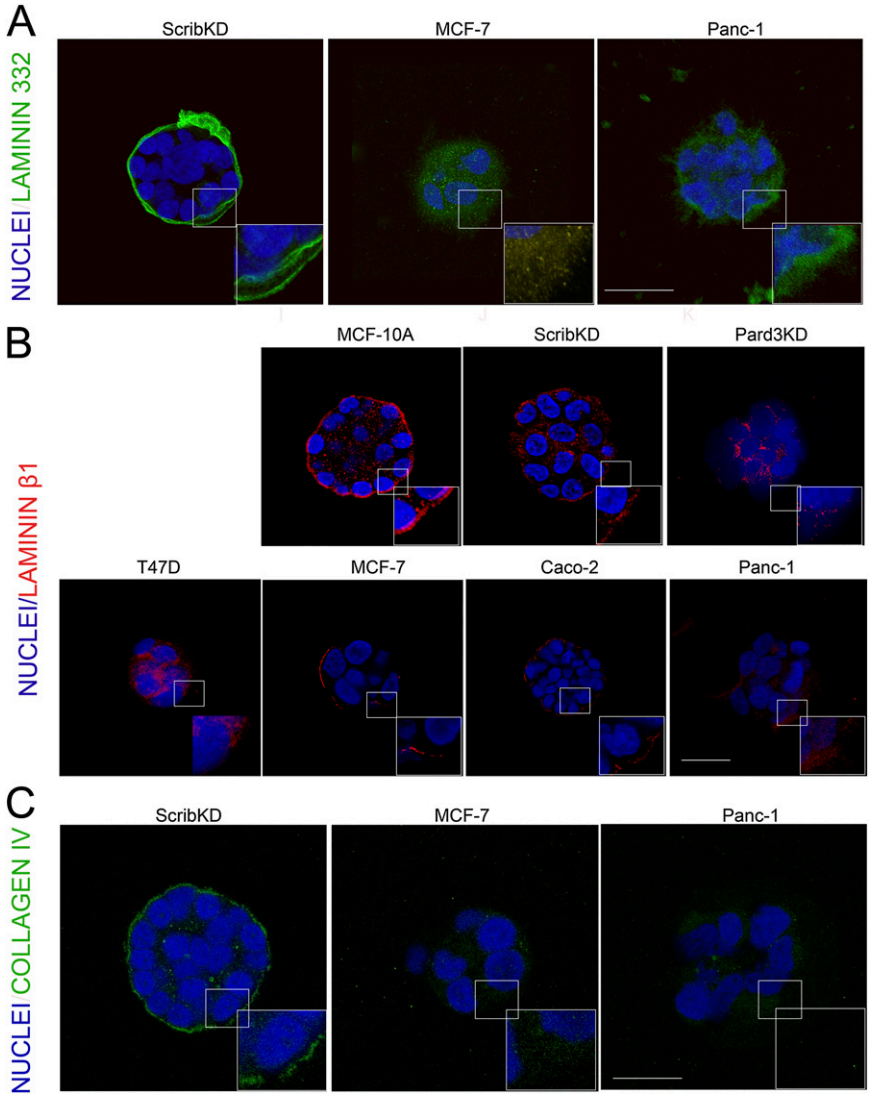


Fig. S4. Nonrotational acini have impaired assembly of laminins and collagen IV. Day 4 3D structures of indicated cell lines were fixed and immunostained for laminin-332 (*A*) or laminin β1 subunit (*B*) or collagen IV (*C*) by human specific antibodies and counterstained with Hoechst to show nuclei (blue). *Lower Right Inset* shows a magnification of the indicated white rectangular region. (Scale bar, 20 μm.)

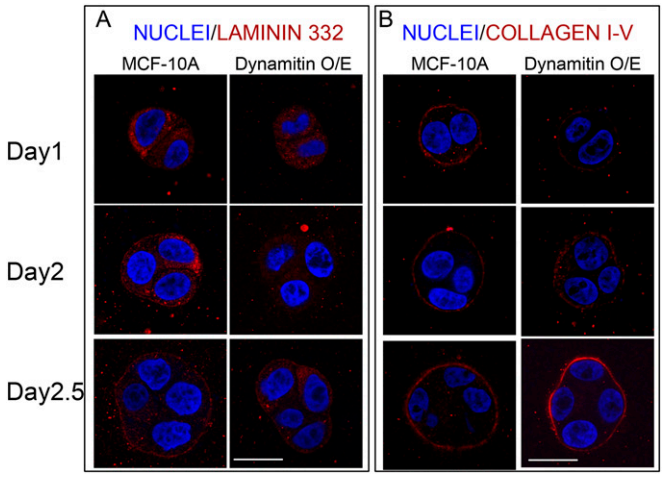


Fig. S5. Overexpression of hDynamitin causes a delay in laminin-332 and collagen IV assembly. Day 1 to day 2.5 acini of MCF-10A wild-type and dynamitin overexpression acini were fixed and immunostained for laminin-332 (*A*, red) and collagen IV (*B*, red) and counterstained with Hoechst to show nuclei (blue). (Scale bars, 20 μm.)

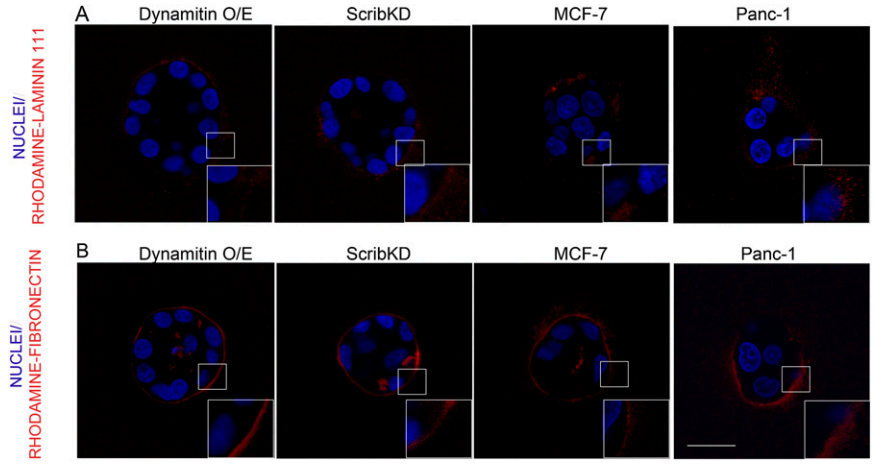
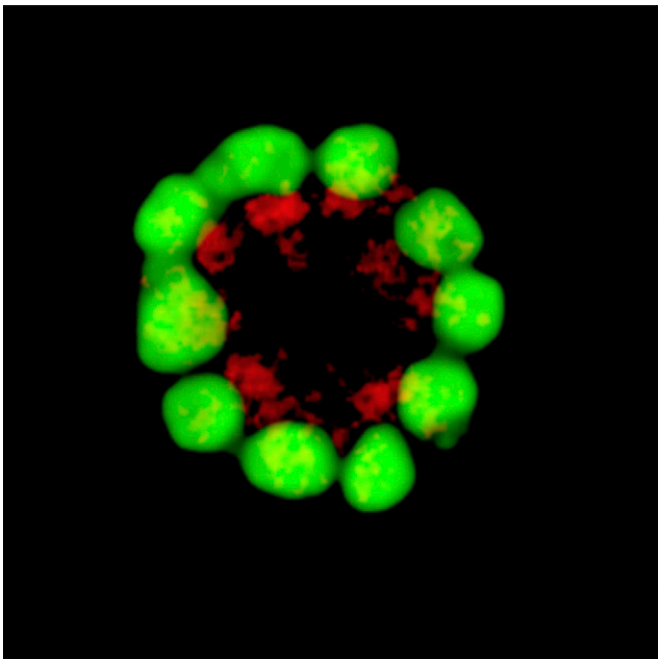


Fig. S6. Structures with impaired rotational motion do not assemble a proper basement membrane. Dynamitin overexpression and ScribKD MCF-10A, MCF-7, and Panc-1 cells were grown for 3 d in a Matrigel matrix, and then 50 μg/mL of rhodamine-labeled mouse laminin-111 (*A*) or fibronectin (*B*) was added 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

Days after seeding in Matrigel	Rotating structures/total no. of structures (%)
1	10/14 (71)
2	12/13 (92)
3	12/12 (100)
4	9/12 (75)
5	0/9 (0)
6	0/10 (0)
7	0/10 (0)
8	0/8 (0)
9	0/7 (0)
10	0/5 (0)
11	0/7 (0)
12	0/7 (0)



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 ($3 \times 3 \times 1$). 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