

Figure S1. **Growth of protrusions during branching morphogenesis and effects of inhibitors.** (A) MDCK acini plated in collagen gels were stimulated with 20 ng/ml HGF for the durations indicated. Acini were fixed and stained with phalloidin. (B) Box plot showing lengths of protrusions shown in Fig. 1 (B and C). (C) MDCK acini were stimulated with HGF with or without 100 μM CK666 for 48 h, fixed, and stained with phalloidin. (D) Plot of protrusion type and number by acini treated with or without 100 μM CK666. (E) Box plot of protrusion length. Box plots show the 25th and 75th percentiles and the median, circles indicate means, and whiskers mark 1.5 SDs. **, $P < 0.01$ by a Student's two-tailed t test assuming unequal variance.

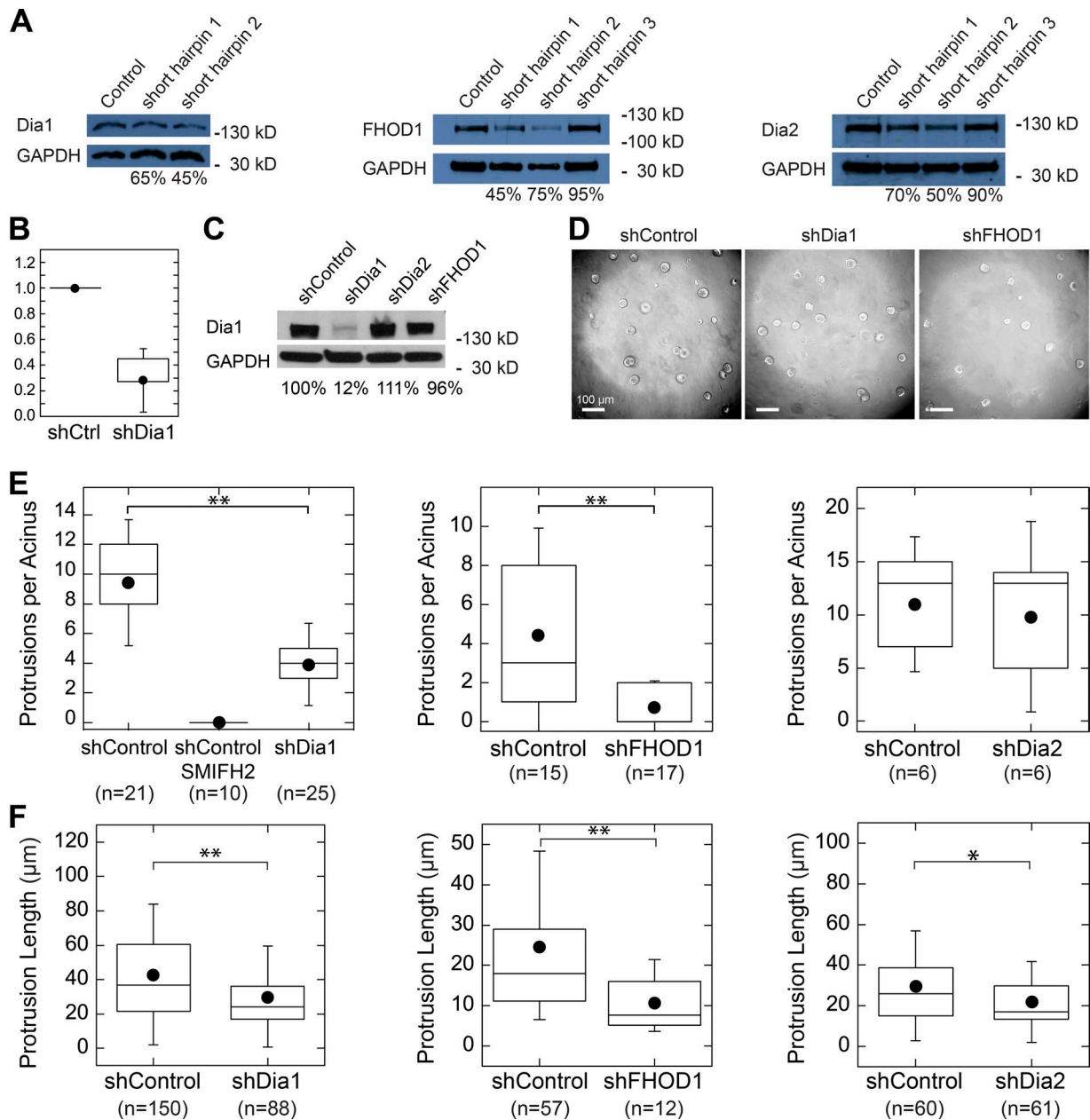


Figure S2. **Knockdown of formin genes by shRNA and branching morphogenesis.** (A) Western blots for the indicated proteins after knockdown using different short hairpin sequences for each target. Percentages indicate protein remaining relative to control calculated by densitometry. (B) Box plot showing Dia1 protein levels from three separate Western blots performed on the shDia1 cell line. (C) Western blot for Dia1 expression in the indicated knockdown cell lines. Percentages indicate protein remaining relative to shControl calculated by densitometry. (D) Images depicting acinar growth of the indicated genotypes after 6 d in Matrigel. (E) Box plots showing protrusions per acinus for each knockdown and its matched control after treatment with 20 ng/ml HGF for 48 h. Protrusions were scored as cell extensions $>5 \mu\text{m}$. (F) Box plots of protrusion length for each knockdown and its matched control. Box plots show the 25th and 75th percentiles and the median, circles indicate means, and whiskers mark 1.5 SDs. *, $P < 0.05$; **, $P < 0.01$ by a Student's two-tailed t test assuming unequal variance.

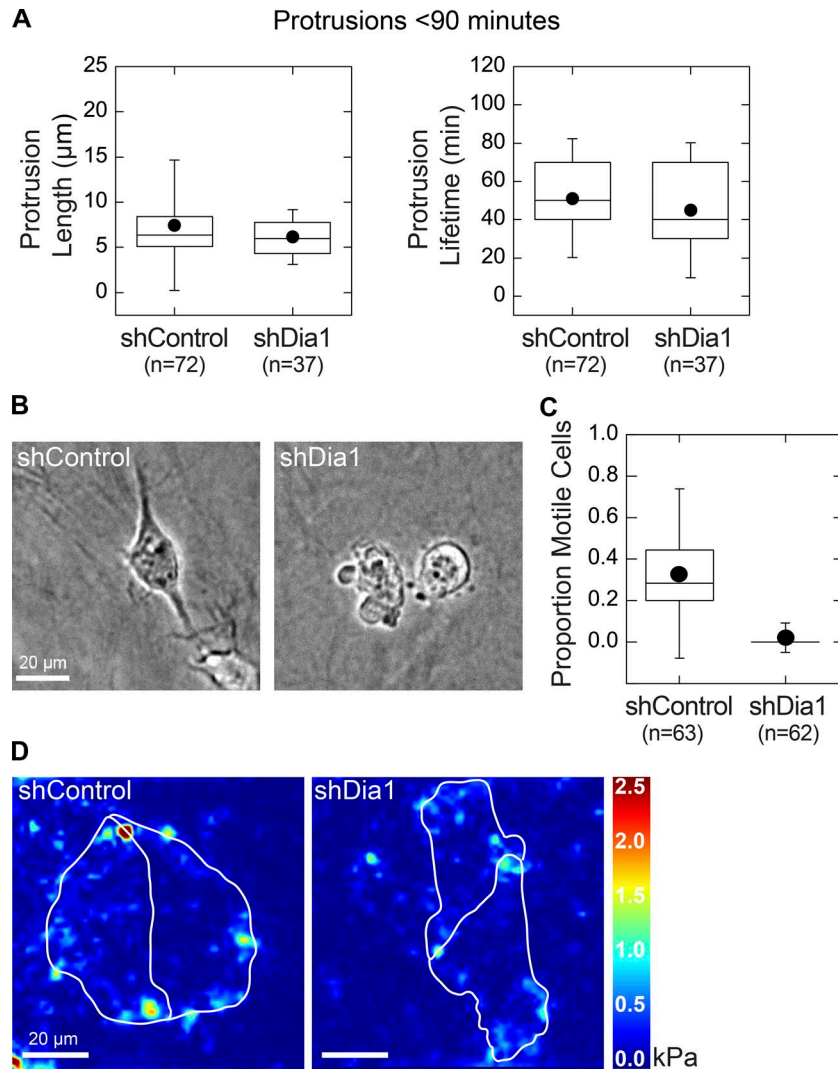


Figure S3. **Protrusions in collagen matrices and cell traction forces.** (A) Box plots comparing only protrusions lasting <90 min in shControl and shDia1 acini taken from the data shown in Fig. 4 (B–D). (B) Brightfield images showing single MDCK cells in collagen gels stimulated with 20 ng/ml HGF (see also Video 5). Images were obtained 5 h after addition of HGF. (C) Box plot of cell motility over 8 h with cell displacements >20 μm scored as motile. (D) Example traction stress heatmaps of cells plated on collagen-coated PA gels. Heatmaps are scaled to include lower stress values still present in shDia1 cells. See also Fig. 6 I. Box plots show the 25th and 75th percentiles and the median, circles indicate means, and whiskers mark 1.5 SDs.

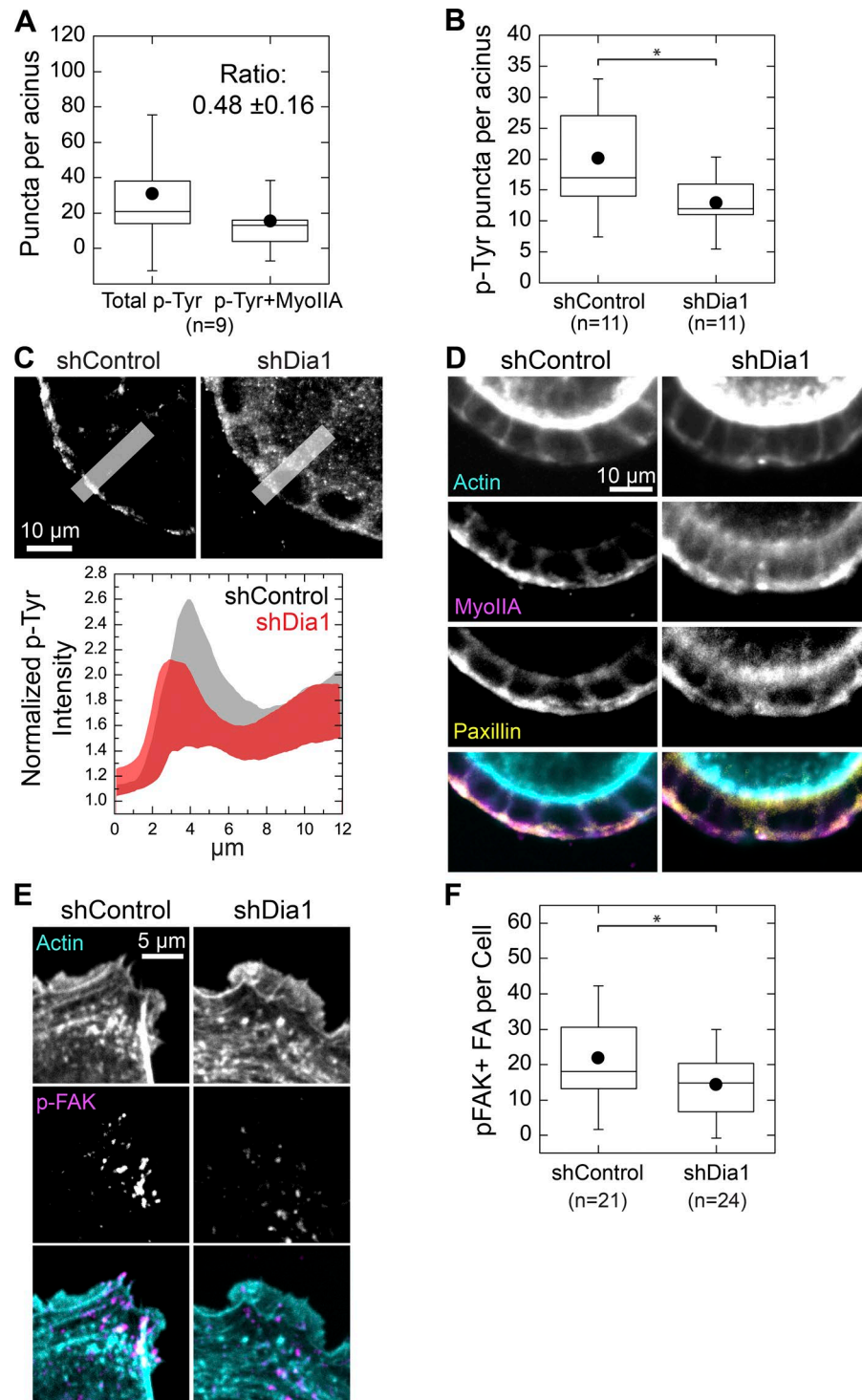
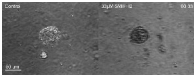
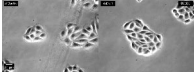


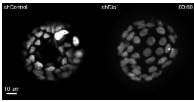
Figure S4. **Analysis of myosin IIA and focal adhesion components during branching morphogenesis.** (A) Box plots of total and myosin IIA–positive focal adhesions (FAs) in $n = 9$ acini 48 h after addition of 20 ng/ml HGF. (B) Box plots comparing phosphotyrosine (p-Tyr)-positive focal adhesions in shControl and shDia1 acini 4 h after addition of HGF. Numbers of acini scored are indicated in the legend. (C) Line scan analysis of phosphotyrosine in shControl and shDia1 acini 4 h after addition of HGF. Gray lines indicate the regions measured by line scans in example immunofluorescence images. The plot encompasses the minima and maxima of 77 line scans in 11 acini per condition. (D) Immunofluorescence images of shControl and shDia1 acini stained for F-actin (cyan), myosin IIA (magenta), and paxillin (yellow) at equatorial sections in shControl and shDia1 acini 4 h after addition of HGF. (E) Immunofluorescence images of shControl and shDia1 cells on coverslips undergoing scattering 6 h after addition of HGF stained for F-actin (cyan) and phospho-FAK (pFAK; magenta). (F) Box plot of phospho-FAK–positive focal adhesions per cell calculated from thresholded images of the cells from E. Numbers of cells analyzed are indicated in the legend. Box plots show the 25th and 75th percentiles and the median, circles indicate means, and whiskers mark 1.5 SDs. *, $P < 0.05$ by Student's two-tailed t test assuming unequal variance.



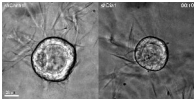
Video 1. **Mouse tumor explants invading into collagen gels.** Tumor explants from the PyMT-MMTV mouse strain were harvested and plated in 2 mg/ml collagen 1 gels. Media containing serum with 30 μ M DMSO or SMIFH2 was added before imaging in brightfield at 30-min intervals for 65 h. Time is indicated in h:min. See also Fig. 1 F. Frame rate, seven frames per second; playback, 210 min/s.



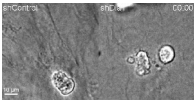
Video 2. **MDCK islands scattering in response to HGF.** MDCK cells were plated in plastic imaging chambers and serum starved for 12 h. Cells were imaged in brightfield immediately after the addition of 20 ng/ml HGF for 9 h at 10-min intervals. Time is indicated in h:min. See also Fig. 3 A. Frame rate, seven frames per second; playback, 70 min/s.



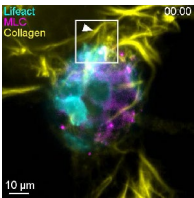
Video 3. **Tracking cell nuclei during branching morphogenesis.** shControl and shDia1 acini expressing the nuclear marker H2B-mCherry were placed in collagen gels in imaging chambers. After the addition of 20 ng/ml HGF, z stacks of nuclei were collected every 10 min for 10 h. The lower one third of each z stack was combined in maximum projections to capture cell movements in the x/y plane. Cell trajectories were tracked manually. Time is indicated in h:min. See also Fig. 3 D. Frame rate, seven frames per second; playback, 70 min/s.



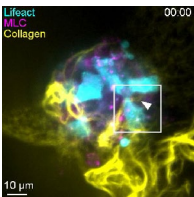
Video 4. **Transmitted light imaging of branching morphogenesis.** Acini of shControl or shDia1 cells were plated in collagen gels in imaging chambers. Brightfield images were obtained starting 3 h after the addition of 20 ng/ml HGF. Acini were imaged for 12 h at 10-min intervals. For clarity, select frames with low illumination were excluded. Time is indicated in h:min. See also Fig. 4 A. Frame rate, seven frames per second; playback, 70 min/s.



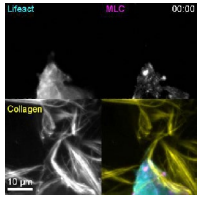
Video 5. **HGF-stimulated motility of single cells in 3D.** Single shControl and shDia1 cells were plated in collagen gels and cultured for 12 h. Immediately after addition of 20 ng/ml HGF, cells were imaged at 10-min intervals in transmitted light for 12 h. Time is indicated in h:min. See also Fig. S3 B. Frame rate, seven frames per second; playback, 70 min/s.



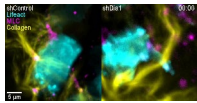
Video 6. **shControl cell deforming individual collagen fibril.** Time-lapse fluorescence imaging at the basal acinar surface of GFP-LifeAct (cyan), mCherry-MLC (magenta), and Alexa Fluor 647-labeled collagen (yellow) in an shControl acinus obtained 4 h after addition of 20 ng/ml HGF. Acini and collagen were imaged at 3-min intervals for 3 h. The z planes imaged were limited to 18 μ m at the lower surface of acini and the juxtaposed collagen network. The video first shows the full acinus, with LifeAct, MLC, and collagen overlaid. The video loops to show the boxed region in each channel separately. Arrowheads indicate acute deformation of a collagen fibril. Time is indicated in h:min. See also Fig. 5 A. Frame rate, seven frames per second; playback, 21 min/s.



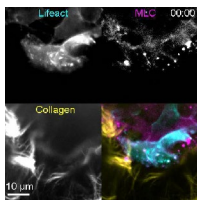
Video 7. **shDia1 cells unable to acutely deform individual collagen fibril.** Time-lapse fluorescence imaging at the basal acinar surface of GFP-LifeAct (cyan), mCherry-MLC (magenta), and Alexa Fluor 647-labeled collagen (yellow) in an shDia1 acinus obtained 4 h after addition of 20 ng/ml HGF. Acini and collagen were imaged at 3-min intervals for 3 h. The z planes imaged were limited to 18 μ m at the lower surface of acini and the juxtaposed collagen network. The video first shows the full acinus with LifeAct, MLC, and collagen overlaid. The video loops to show the boxed region in each channel separately. Arrowheads indicate a collagen fibril in contact with but not deformed by a cell moving within the acinus. Time is indicated in h:min. See also Fig. 5 B. Frame rate, seven frames per second; playback, 21 min/s.



Video 8. **Cell front migrating into labeled collagen during branching morphogenesis.** Leader cell protruding into the surrounding collagen matrix from an shControl acinus stimulated with 20 ng/ml HGF for 24 h. GFP-LifeAct (cyan), mCherry-MLC (magenta), and Alexa Fluor 647–labeled collagen (yellow) were imaged at 3-min intervals for 3 h. Time is indicated in h:min. See also Fig. 7 A. Frame rate, seven frames per second; playback, 21 min/s.



Video 9. **Myosin-rich adhesions in shControl and shDia1 acini.** Time-lapse fluorescence imaging at the basal acinar surface of GFP-LifeAct (cyan) and mCherry-MLC (magenta) obtained 4 h after addition of 20 ng/ml HGF. Acini were imaged at 3-min intervals for 3 h. The z planes imaged were limited to 18 μ m at the lower surface of acini. Arrows indicate locations of puncta rich in MLC and LifeAct as cells move within acini. Time is indicated in h:min. See also Fig. 8 C. Frame rate, seven frames per second; playback, 21 min/s.



Video 10. **Adjacent cells assemble adhesions at the same collagen region.** Time-lapse fluorescence imaging at the basal acinar surface of GFP-LifeAct (cyan), mCherry-MLC (magenta), and Alexa Fluor 647–labeled collagen (yellow) in an shControl acinus obtained 4 h after addition of 20 ng/ml HGF. Acini and collagen were imaged at 3-min intervals for 3 h. The z planes imaged were limited to 18 μ m at the lower surface of acini and the juxtaposed collagen network. Time is indicated in h:min. See also Fig. 8 D. Frame rate, seven frames per second; playback, 21 min/s.