Paul et al., http://www.jcb.org/cgi/content/full/jcb.201502040/DC1

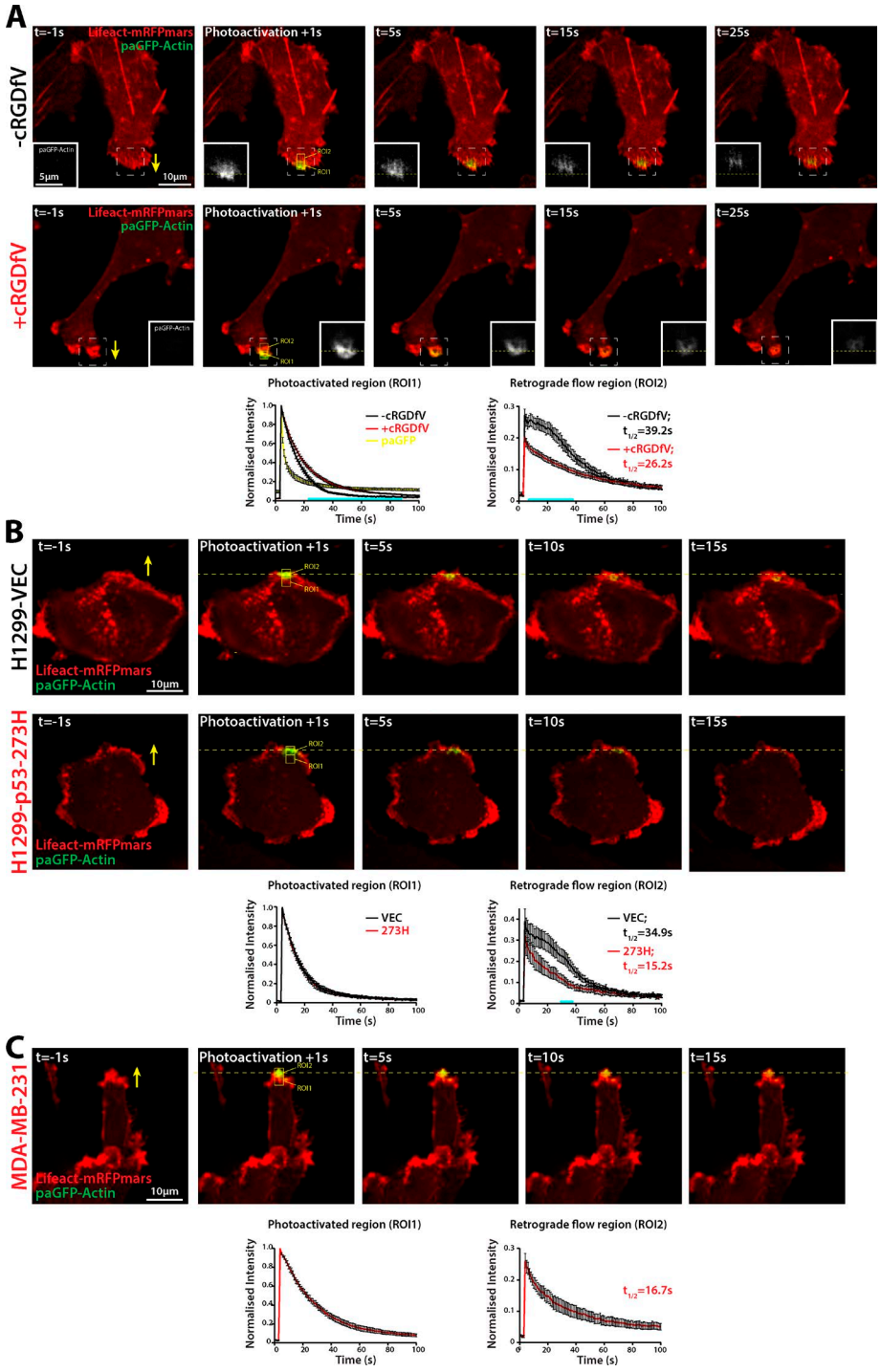


Figure S1. **Mutant p53-expressing cells display limited retrograde actin flow at the leading edge.** (A) A2780 cells transiently transfected with Lifeact-mRFP-mars and paGFP-Actin or paGFP were seeded onto glass-bottomed dishes 24 h before imaging and treated with cRGDfV (2.5 µM, 2 h) as indicated. ROI1 was photoactivated and cells were imaged every second for 100 s. Normalized intensity was analyzed for ROI1 and ROI2 (–cRGDfV, *n* = 21; +cRGDfV, *n* = 20). Insets show enlarged views of the boxed regions. (B) H1299-Vec and H1299-p53-273H cells transfected as in A were seeded onto glass-bottomed dishes 24 h before imaging. Photoactivation was performed as in A (VEC, *n* = 15; p53-273H, *n* = 10). (C) MDA-MB-231 cells transfected as in A were seeded onto CDM-coated glass-bottomed dishes 4 h before imaging. Photoactivation was performed as in A (*n* = 10). Yellow arrows indicate the direction of protrusion. Yellow broken lines mark the center of the photoactivated region. Error bars represent SEM. All data represent at least three independent experiments. Blue bars indicate time points at which – or +cRGDfV or mutant p53 datasets are significantly different ($P < 0.01$; Student's *t* test).

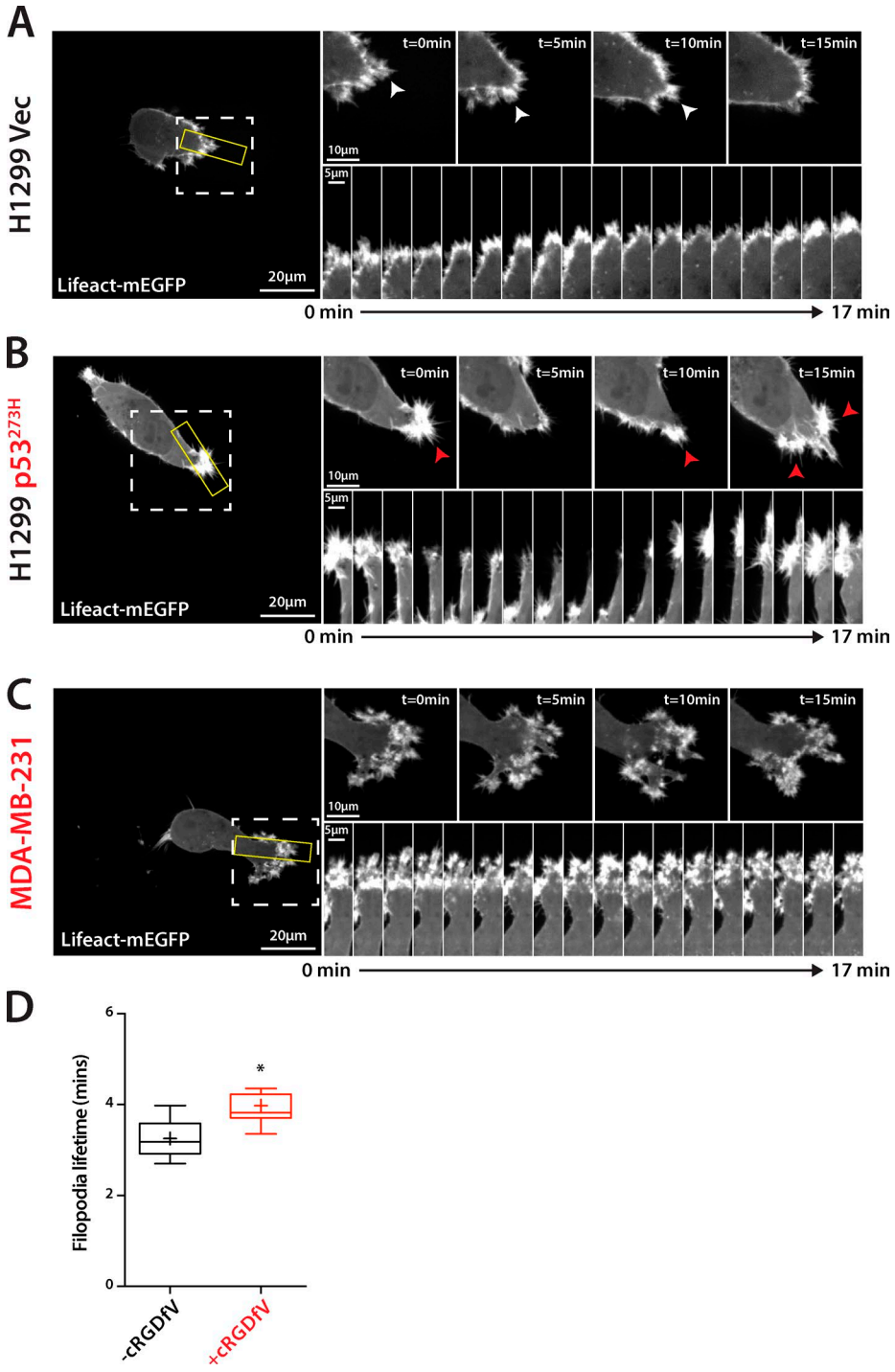


Figure S2. **Mutant p53-expressing cells generate bursts of actin spikes.** (A–C) H1299-Vec (A), H1299-p53-273H (B), or MDA-MB-231 (C) cells transiently transfected with Lifeact-mEGFP were seeded onto CDMs for 4 h and imaged using a spinning disk confocal microscope every minute for≥25 min. Z projections are shown. White arrowheads indicate lamellipodial protrusions; red arrowheads indicate actin spikes. The white boxed regions are enlarged on the right, and the kymograph images are taken from the yellow boxed regions. (D) A2780 cells transfected with Lifeact-mEGFP were seeded onto CDMs for 4 h and imaged using a spinning disk confocal microscope every minute for 15 min, and filopodia lifetime was analyzed. n = 8 cells per condition. All data represent at least three independent experiments. Statistical significance was evaluated using a Student's t test with Welch's correction. +, mean; *, $P < 0.05$.

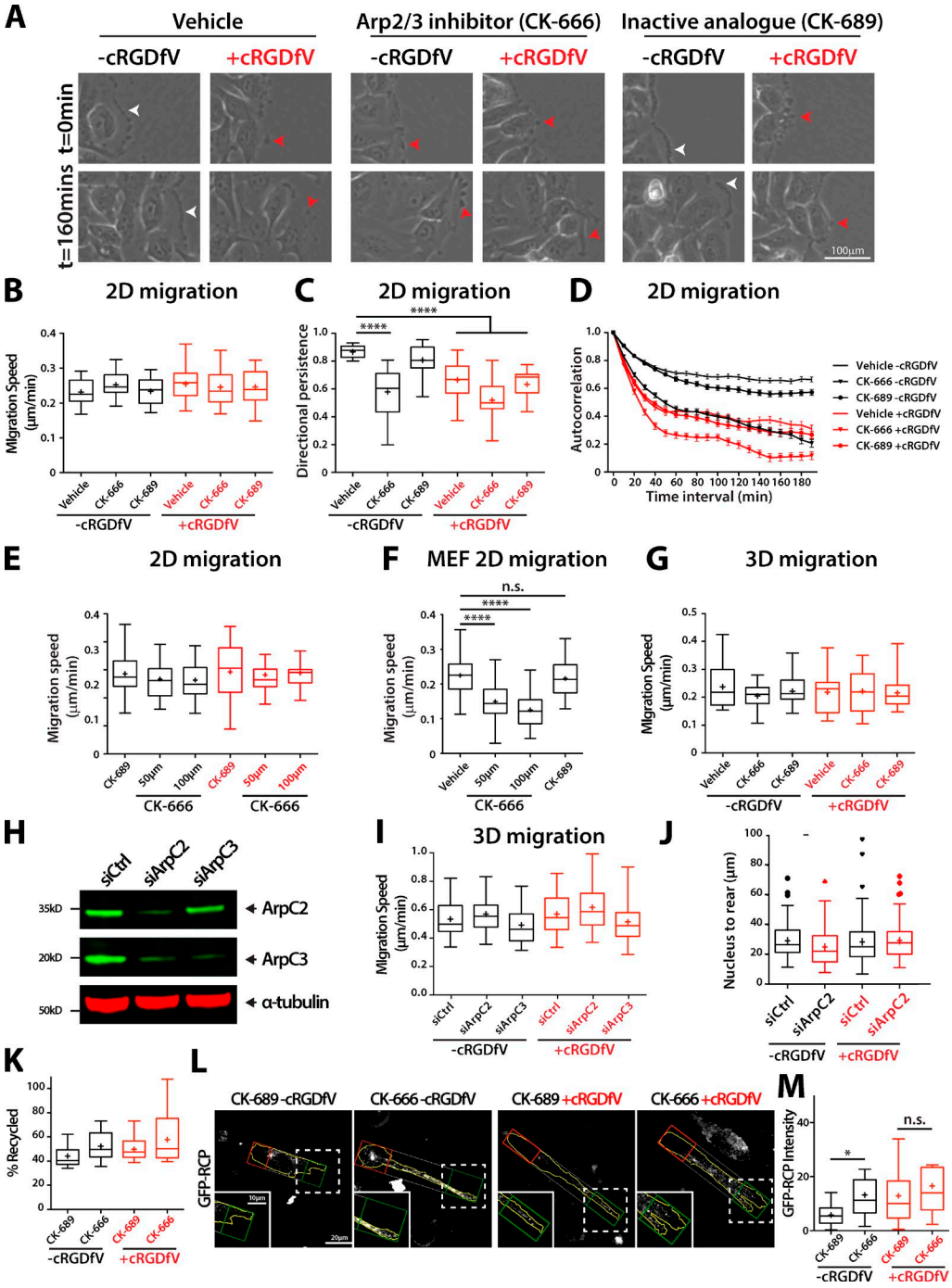


Figure S3. **Arp2/3 is not required for RCP–α5β1-driven migration in 2D and 3D.** (A) A2780 cells were seeded onto tissue culture plates 24 h before wounding. Cells were treated with cRGDfV (2.5 µM), DMSO, CK-666, or CK-689 (50 µM) as indicated before time-lapse imaging. White arrows indicate lamellipodia; red arrows indicate nonlamellipodial ruffling protrusions. (B–D) Migration speed (B), directional persistence (C), and autocorrelation (D) of cells migrating in 2D treated as in A. n = 20 cells per condition. Error bars represent the SEM. (E) A2780 cells were prepared as in A and treated with cRGDfV (2.5 µM), DMSO, or CK-666 (50 µM or 100 µM) as indicated before time-lapse imaging. 2D migration speed was analyzed. n = 35 cells per condition. (F) Mouse embryonic fibroblasts (MEF) were seeded onto tissue culture plates 24 h before wounding. Cells were treated with DMSO, CK-689 (100 µM), or CK-666 (50 µM or 100 µM) as indicated before time-lapse imaging. 2D migration speed was analyzed. n = 75 cells per condition. (G) A2780 cells were seeded onto CDM for 4 h and treated with cRGDfV (2.5 µM) and DMSO, CK-666, or CK-689 (50 µM) as indicated before time-lapse imaging. 3D migration speed was analyzed. n = 20 cells per condition. (H) siRNA SMARTpool knockdown of ArpC2 and ArpC3 in A2780 cells. (I) 3D migration speed of siCtrl, siArpC2, and siArpC3 A2780 cells migrating in CDM (as in Fig. 3 C). $n \geq 99$ cells per condition. (J) Distance from the rear of the cell to the center of the nucleus of siCtrl and siArpC2 A2780 cells migrating in CDM. n = 100 cells per condition. (K) α5 integrin recycling in A2780 cells was measured upon treatment with CK-666 or CK-689 (50 µM). n = 24 wells over three experiments. (L and M) GFP-RCP–transfected A2780 cells were seeded onto CDMs for 4 h before treatment with cRGDfV (2.5 µM), CK-666, or CK-689 (50 µM) as indicated before confocal imaging. Bounding boxes around the cell perimeter (yellow) were used to identify the front and rear quarter (green or red, respectively) of the cell, and the percentage of GFP-RCP intensity at the front was measured (M). Insets show enlarged views of the white boxed regions. n = 15–28 cells per condition. All data represent at least three independent experiments. Statistical significance was evaluated using ANOVA/Tukey's multiple comparison test. +, mean; n.s., not significant; *, $P < 0.05$; **, $P < 0.01$; ***, $P < 0.001$; ****, $P < 0.0001$.

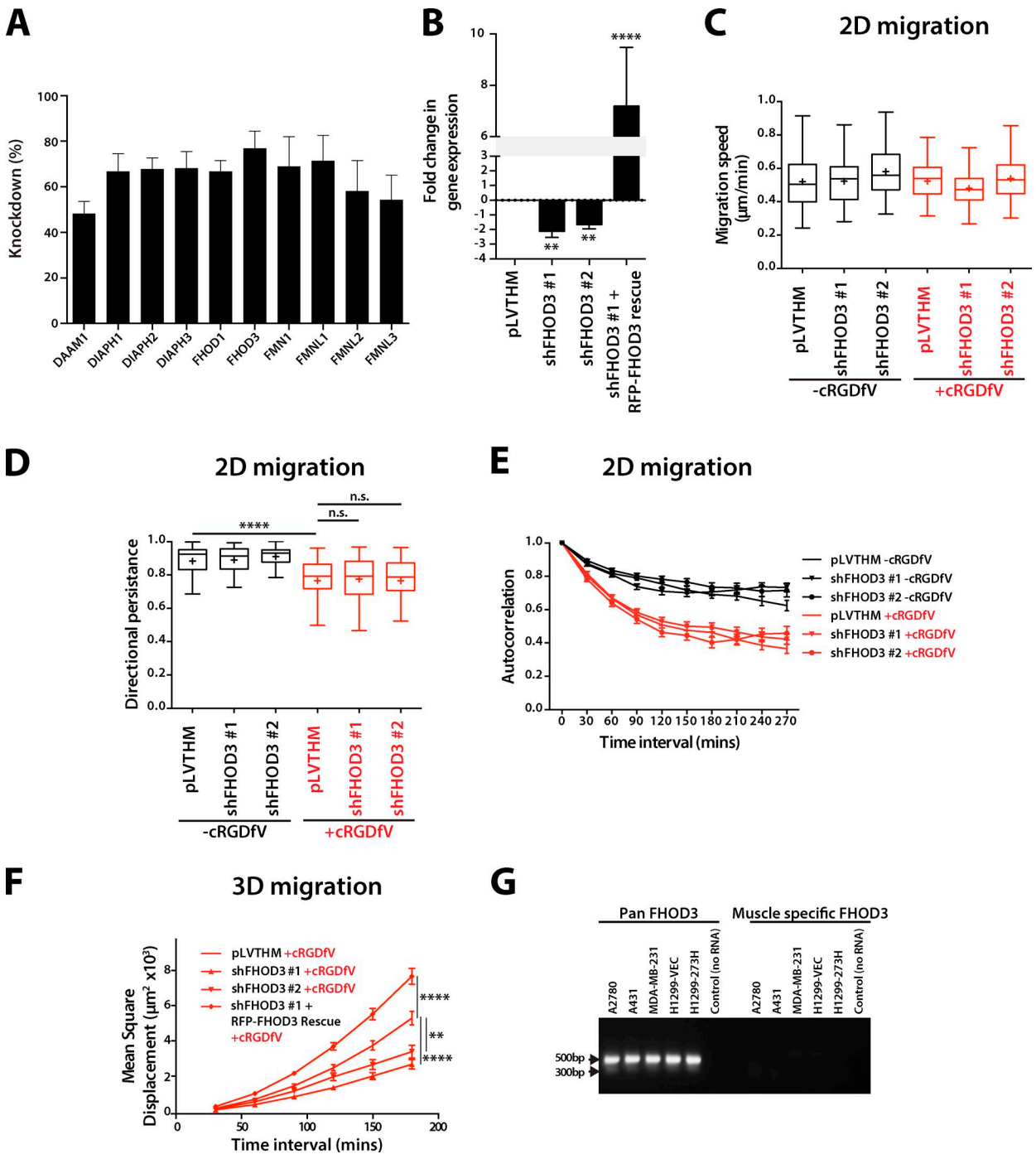


Figure S4. **FHOD3 is required for 3D (but not 2D) migration.** (A) Percentage knockdown of formins expressed in A2780 cells by siRNA, measured by qRT-PCR. *n* = 2–3 per condition. (B) A2780 cells were stably transfected with pLVTHM vector control, shFHOD3 #1, or shFHOD3 #2. shFHOD3 #1 was rescued with sh-resistant RFP-FHOD3. FHOD3 gene expression was measured by qRT-PCR. Fold change in gene expression is given relative to pLVTHM vector control. *n* = 3–6 independent experiments. (C) A2780 cells stably expressing pLVTHM vector control, shFHOD3 #1, or shFHOD3 #2 were seeded onto tissue culture plates 24 h before wounding. Cells were treated with cRGDfV (2.5 µM) as indicated before time-lapse imaging. (D and E) Quantification of 2D migration speed, directional persistence (D), and autocorrelation (E). *n* = 45–70 cells per condition. (F) A2780 cells stably expressing shFHOD3 #1, shFHOD3 #2, pLVTHM vector control, or RFP-FHOD3 rescue were seeded onto CDM and treated with cRGDfV (2.5 µM) where indicated before time-lapse imaging, and MSD was quantified. *n* = 96–134 cells per condition. (G) RNA extracted from cell lines as indicated was used as to determine FHOD3 isoforms expressed by RT-PCR, using primers specific for nonmuscle and muscle-specific isoforms of FHOD3 (pan FHOD3, to yield products at 478 bp and 502 bp, respectively) or muscle-specific FHOD3 primers (to yield a product at 316 bp). All data represent at least three independent experiments. Statistical significance was evaluated using ANOVA/Tukey's multiple comparison test. +, mean; n.s., not significant; **, $P < 0.01$; ****, $P < 0.0001$. Error bars represent the SEM.

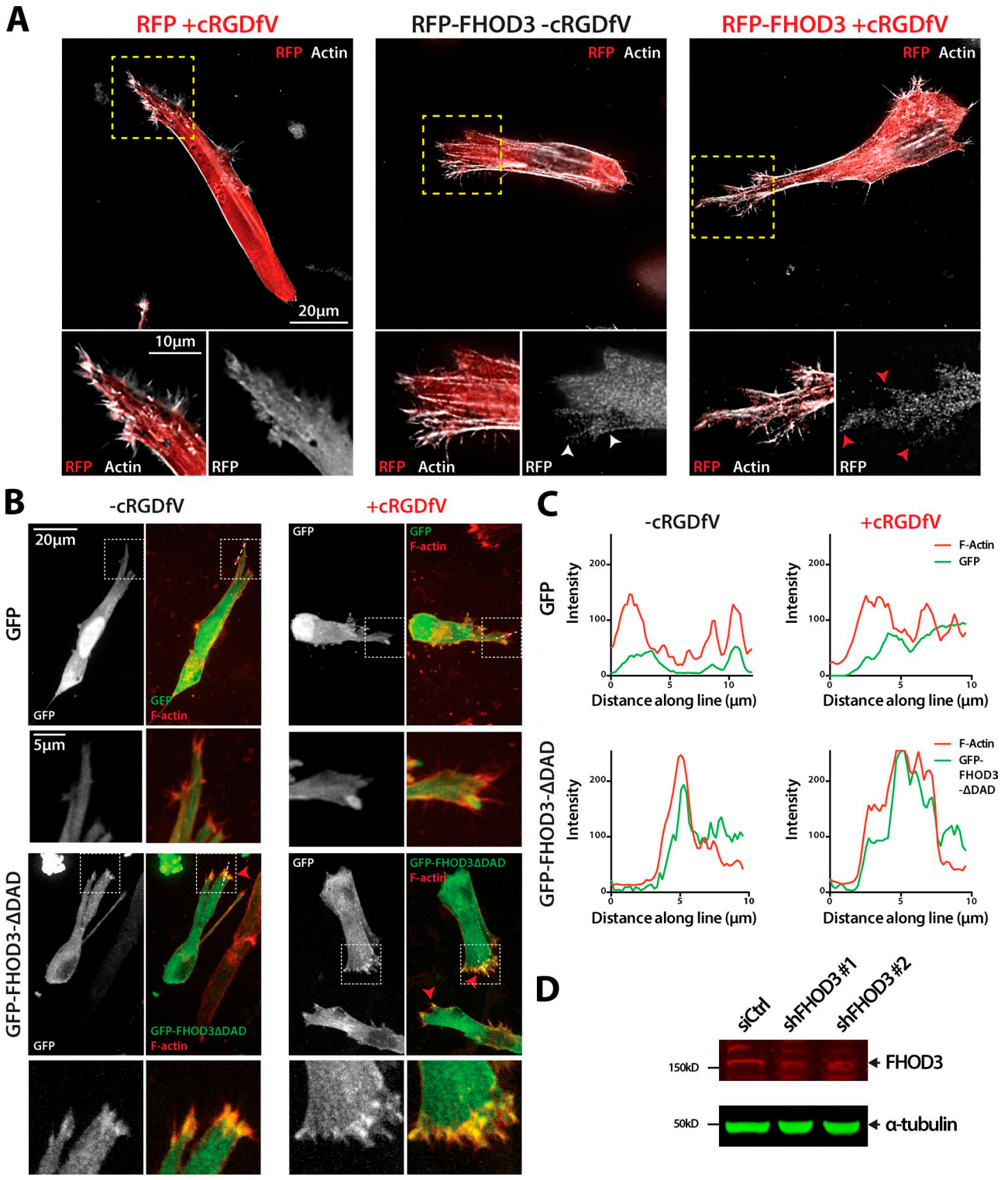
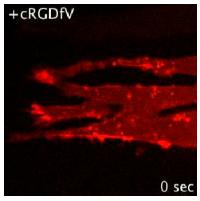
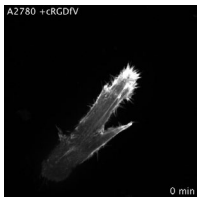


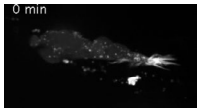
Figure S5. **Wild-type FHOD3 localizes to actin filaments in 3D CDM; active FHOD3 localizes to actin spikes in 3D CDM.** (A) A2780 cells stably expressing RFP or RFP-FHOD3 were seeded onto CDMs for 4 h and treated with cRGDfV (2.5 μM) where indicated. Cells were fixed and stained with anti-RFP antibody and Alexa Fluor 647–conjugated phalloidin before wide-field fluorescence imaging. Arrows indicate localization of RFP-FHOD3 on actin filaments. (B) A2780 cells were transiently transfected with GFP or GFP-FHOD3ΔDAD, seeded onto CDMs for 4 h, and treated with cRGDfV (2.5 μM) where indicated. Cells were fixed and stained with TRITC-phalloidin before spinning disk confocal imaging. Arrowheads indicate colocalization of actin spikes and GFP-FHOD3ΔDAD. Boxes with broken lines indicate the line profile intensity measurements in C. (D) H1299-273H cells were stably transfected with pLVTHM vector control, shFHOD3 #1, or shFHOD3 #2. Cells were lysed, and lysates were subjected to SDS-PAGE and Western blotting using antibodies specific for FHOD3 and α-tubulin. All data represent at least three independent experiments.



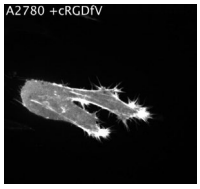
Video 1. **Retrograde actin flow is slowed within actin-based protrusions formed during RCP–α5β1-driven migration.** A2780 cells transiently transfected with Lifeact-mRFPmars and paGFP-actin were seeded onto 3D CDM 4 h before imaging and treated with cRGDfV (2.5 µM, 2 h) as indicated. ROI1 (Fig. 1 D) was photoactivated and cells were imaged every second for 100 s using a spinning disk confocal inverted microscope (Marianas; 3i). Frame rate is 7 frames per second (fps).



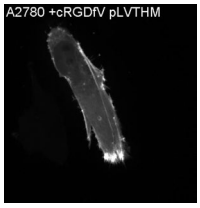
Video 2. **RCP–α5β1 integrin trafficking promotes actin spike formation.** A2780 cells transiently transfected with Lifeact-mEGFP were seeded onto 3D CDM for 4 h, and cRGDfV (2.5 µM, 2 h) was added as appropriate. Z stacks were captured (seven sections, 0.2 µm intervals) using a spinning disk confocal microscope (Marianas; 3i) every minute for 45 min. Z projection movies are shown. Frame rate, 7 fps.



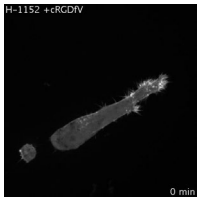
Video 3. **Dynamic actin spikes form protrusions in cells invading locally in vivo.** A2780 cells stably expressing Lifeact-mRFPMars were injected into the pericardial cavity of zebrafish embryos, and cRGDfV (2.5 µM) was added 1 dpi. Imaging was performed at 3 dpi using a spinning disk confocal microscope. Z stacks were captured (seven sections, 0.5-µm intervals) using a spinning disk confocal microscope (Marianas; 3i) every 15 s for 15 min. Z projection movies are shown. Frame rate, 7 fps.



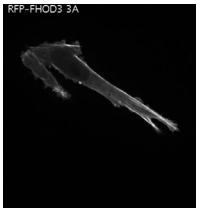
Video 4. **Arp2/3 activity is not required for RCP–α5β1-driven actin spike formation.** A2780 cells transiently transfected with Lifeact-mEGFP were seeded onto 3D CDM for 4 h, and treated with cRGDfV (2.5 µM, >2 h). Cells were treated with the indicated inhibitors (50 µM) or vehicle control immediately before imaging. Z stacks were captured (seven sections, 0.2 µm intervals) on a spinning disk confocal microscope (Marianas; 3i) every minute for 15 min. Z projection movies are shown. Frame rate, 7 fps.



Video 5. **FHOD3 is required for RCP–α5β1-driven actin spike formation.** A2780 cells stably expressing shFHOD3 #1, shFHOD3 #2, pLVTHM vector control, or RFP-FHOD3 rescue were transiently transfected with Lifeact-mTFP1 and seeded onto CDM for 4 h and treated with cRGDfV (2.5 µM). Z stacks were captured (seven sections, 0.2 µm intervals) on a spinning disk confocal microscope (Marianas; 3i) every minute for 15 min. Z-projection movies are shown. Frame rate, 7 fps.



Video 6. **ROCK is required for RCP–α5β1-driven actin spike formation.** A2780 cells were transiently transfected with Lifeact-mTFP1 and seeded onto CDM for 4 h and treated with cRGDfV (2.5 µM) in the presence or absence of the ROCK inhibitor Gly-H-1152. Z stacks were captured (seven sections, 0.2-µm intervals) on a spinning disk confocal microscope (Marianas; 3i) every minute for 15 min. Z projection movies are shown. Frame rate, 7 fps.



Video 7. **RFP-FHOD3-3A does not support actin spike formation.** A2780 cells stably expressing shFHOD3 #1 along with RFP or RFP-FHOD3 3A/3D rescue were transiently transfected with Lifeact-mTFP1 and seeded onto CDM for 4 h and treated with cRGDfV (2.5 µM). Z stacks were captured (seven sections, 0.2 µm intervals) on a spinning disk confocal microscope (Marianas; 3i) every minute for 15 min. Z projection movies are shown. Frame rate, 7 fps.