Jacquemet et al., http://www.jcb.org/cgi/content/full/jcb.201302041/DC1

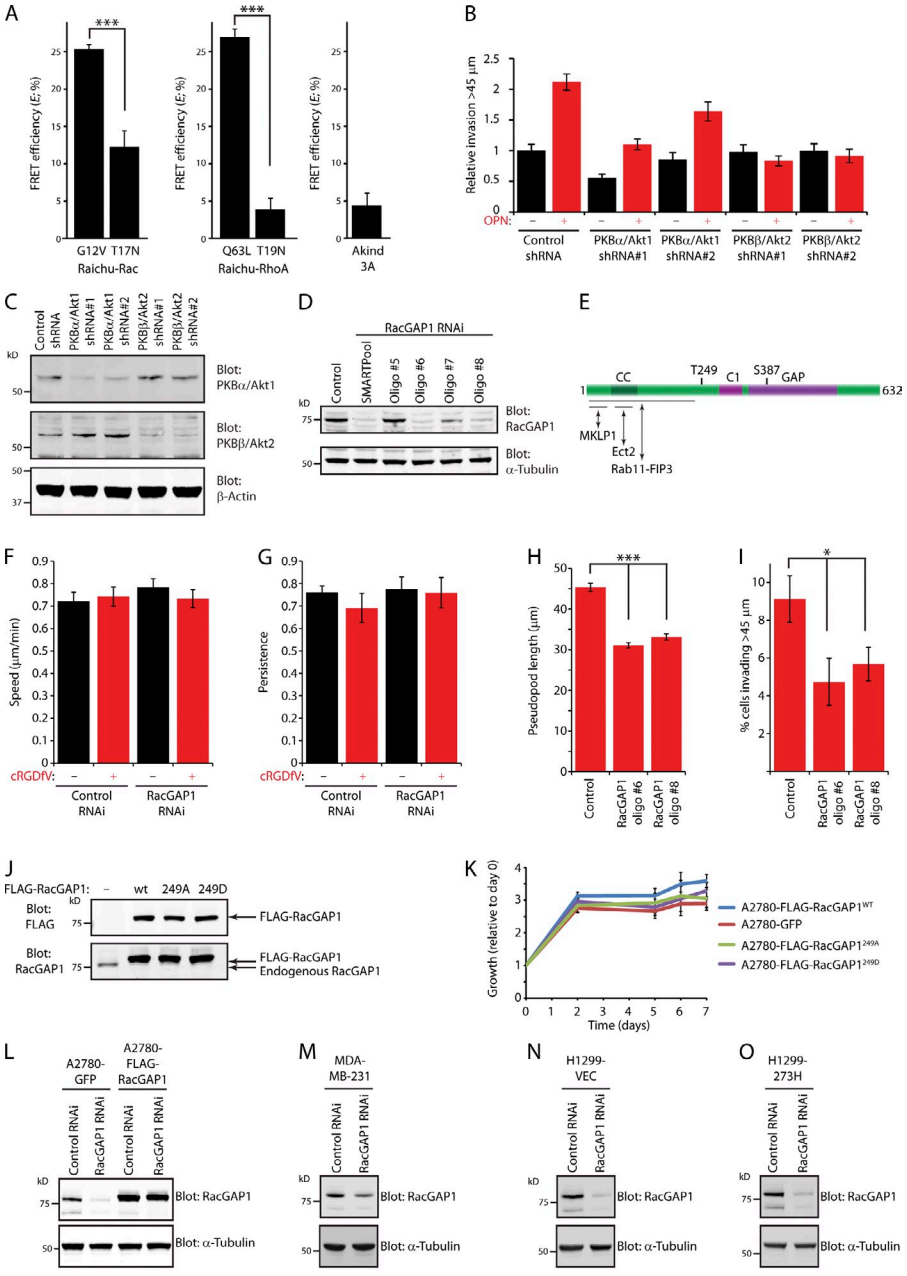


Figure S1. **Akt2 and RacGAP1 are required for RCP–α5β1-driven invasion.** (A) A2780 cells were transfected with Raichu-Rac G12V/T17N, Raichu-RhoA Q63L/T19N, or Akind-3A and seeded onto glass-bottom plates after 16–24 h. Fluorescence lifetime images were captured, and FRET efficiency was calculated for ROIs around the entire cell periphery from lifetime maps. (Raichu-Rac G12V, $n = 34$; Raichu-Rac T17N, $n = 45$; Raichu-RhoA Q63L, $n = 19$; Raichu-RhoA T19N, $n = 21$; Akind-3A, $n = 17$). (B) A2780 cells were transfected with control, PKB-α/Akt1–, or PKB-β/Akt2–specific shRNA constructs. After 16 h, cells were seeded into inverted invasion assays in the presence of FN and stimulated with osteopontin (OPN) as indicated. Cells were visualized with Calcein-AM 48 h later, and serial confocal sections were captured at 15-µm intervals using a 10× objective lens. Invasion was quantitated by measuring the fluorescence intensity of cells invading 45 µm or more and expressing this as a percentage of the fluorescence intensity of all cells within the plug. (C) A2780 cells transfected as in A were lysed after 48 h. Lysates were subjected to SDS-PAGE/Western blot analysis to assess relative levels of PKB-α/Akt1, PKB-β/Akt2, and β-actin. (D) A2780 cells were transfected with control, RacGAP1 SMARTpool, or individual RacGAP1 siRNA oligos and lysed after 48 h. Lysates were subjected to SDS-PAGE/Western blot analysis to assess relative levels of RacGAP1 and α-tubulin. (E) Schematic depicting domain structure of RacGAP1 and phosphorylation and known interaction sites. CC, coiled coil domain; C1, C1 domain; GAP, GAP domain. (F and G) A2780 cells were transfected with control or RacGAP1-specific siRNA oligonucleotides and seeded onto CDMs after 24–36 h. Cells were tracked using ImageJ, and speed (≥18 cells; F) and persistence (≥18 cells; G) of migration were analyzed using the manual tracking plug-in. (H) A2780 cells were transfected with control or RacGAP1-specific siRNA oligos and seeded onto CDMs after 24–36 h. Cells were stimulated with 2.5 µM cRGDfV, and images were captured at 10-min intervals using a 20× objective lens. Pseudopod length (>200 cells) was measured for all moving cells within the 20th frame using ImageJ. (I) A2780 cells were transfected with control or RacGAP1-specific siRNA oligos and seeded into inverted invasion assays after 16 h in the presence or absence of FN and cRGDfV. Cells were visualized, and invasion was quantified as in B. (J) A2780 cells were stably transfected by lentivirus to express GFP (control), FLAG-RacGAP1^{WT}, FLAG-RacGAP1^{249A}, or FLAG-RacGAP1^{249D}. Cells were lysed, and lysates were analyzed by SDS-PAGE/Western blotting with mouse anti-FLAG and mouse anti-RacGAP1 antibodies. (K) A2780 cells stably transfected as in J were seeded into 96-well plates, and cell viability was assayed over 7 d using the CellTiter 96 kit. (L) A2780 cells stably expressing GFP or FLAG-RacGAP1^{WT} were transfected with control oligo or RacGAP1 oligo #6 and lysed after 48 h. Lysates were subjected to SDS-PAGE/Western blot analysis to assess relative levels of RacGAP1 and α-tubulin. (M) MDA-MB-231 cells were transfected with control or RacGAP1 SMARTpool oligos and lysed after 48 h. Lysates were subjected to SDS-PAGE/Western blot analysis to assess relative levels of RacGAP1 and α-tubulin. (N and O) H1299 cells stably transfected with control plasmid (vector [VEC]; N) or mutant p53 (273H; O) cells were transfected with control or RacGAP1 SMARTpool oligos and lysed after 48 h. Lysates were subjected to SDS-PAGE/Western blot analysis to assess relative levels of RacGAP1 and α-tubulin. Data represent means ± SEM from at least three independent experiments. *, $P < 0.05$; ***, $P < 0.001$.

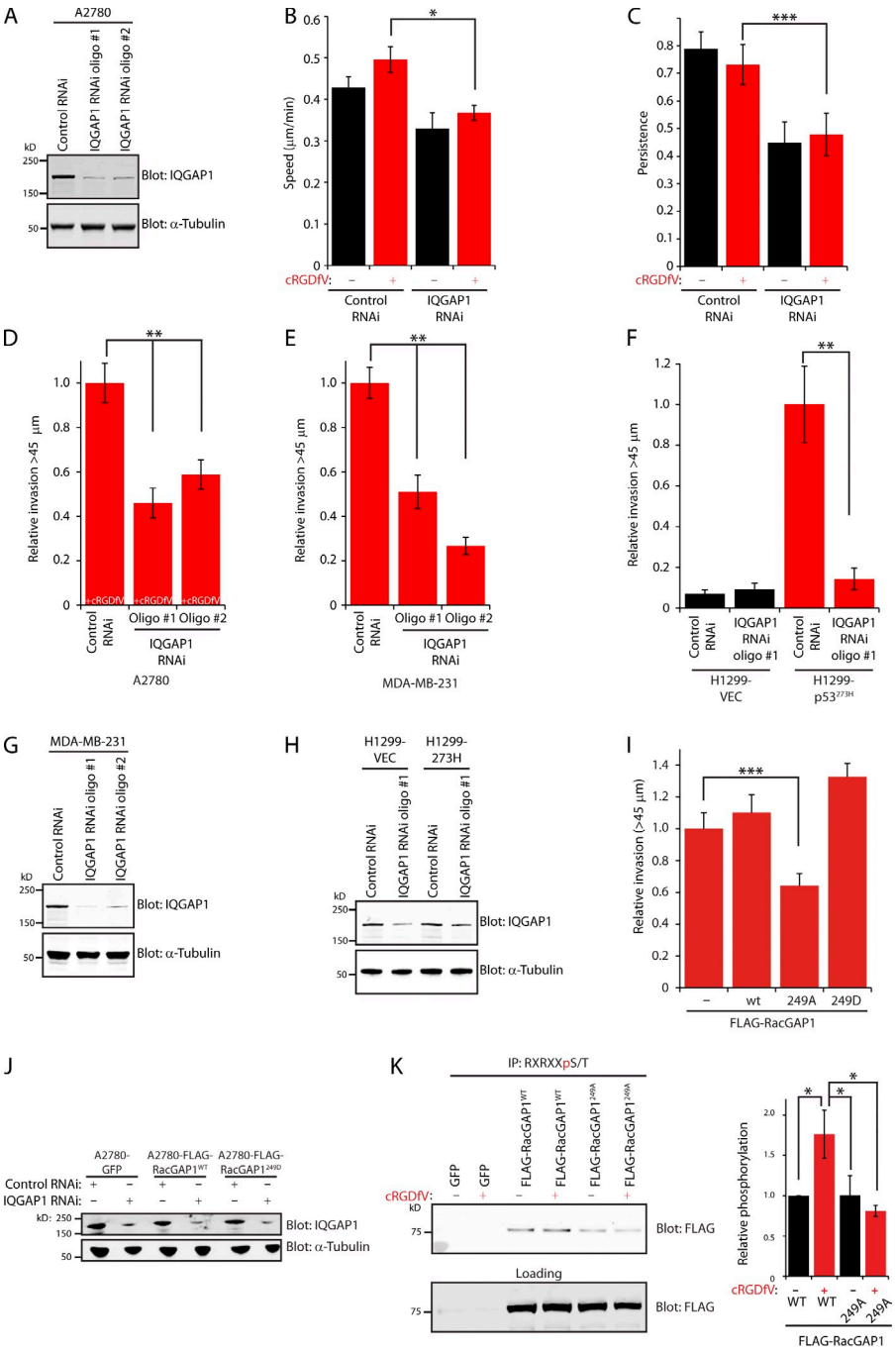


Figure S2. **IQGAP1 is required for migration on CDM and invasion into collagen/FN matrix.** (A) A2780 cells were subjected to two rounds of control or IQGAP1 RNAi and lysed 48 h after the second transfection. Lysates were subjected to SDS-PAGE/Western blot analysis to assess relative levels of IQGAP1 and α-tubulin. (B and C) A2780 cells were subjected to two rounds of control or IQGAP1 RNAi and seeded onto CDMs. Cells were stimulated with 2.5 μM cRGDfV as indicated, and images were captured at 10-min intervals using a 20× objective lens. Displacement of cells was tracked using ImageJ, and speed and persistence of migration were analyzed using the manual tracking plug-in. (D–F) A2780 (D), MDA-MB-231 (E), or H1299-vector [VEC]/273H (F) cells were transfected with control or IQGAP1-specific siRNA oligos and seeded into inverted invasion assays after 16 h in the presence or absence of FN and cRGDfV (A2780s) or FN (MDA-MB-231/H1299). Cells were visualized, and invasion was quantified as in Fig. 1 B. (G and H) MDA-MB-231 (G) or H1299-vector/273H (H) cells were transfected, and knockdown efficiency was established as in A. (I) MDA-MB-231 cells stably transfected with control vector, RacGAP1^{WT}, RacGAP1^{249A}, or RacGAP1^{249D} were seeded into inverted invasion assays in the presence of FN, cells were visualized, and invasion was quantified as in Fig. 1 B. (J) A2780 cells stably transfected with control vector, FLAG-RacGAP1^{WT}, or FLAG-RacGAP1^{249D} were subjected to two rounds of control or IQGAP1 RNAi and lysed 48 h after the second transfection. Lysates were subjected to SDS-PAGE/Western blot analysis to assess relative levels of IQGAP1 and α-tubulin. (K) A2780 cells stably transfected with control vector, FLAG-RacGAP1^{WT}, or FLAG-RacGAP1^{249A} were treated as in Fig. 1 B and immunoprecipitated using anti-RxRxxS*/T* or an isotype-matched control. IPs were analyzed by SDS-PAGE and Western blotting for RacGAP1 and quantified using the Odyssey system. Data represent means ± SEM from at least three independent experiments. *, P < 0.05; **, P < 0.01; ***, P < 0.001.

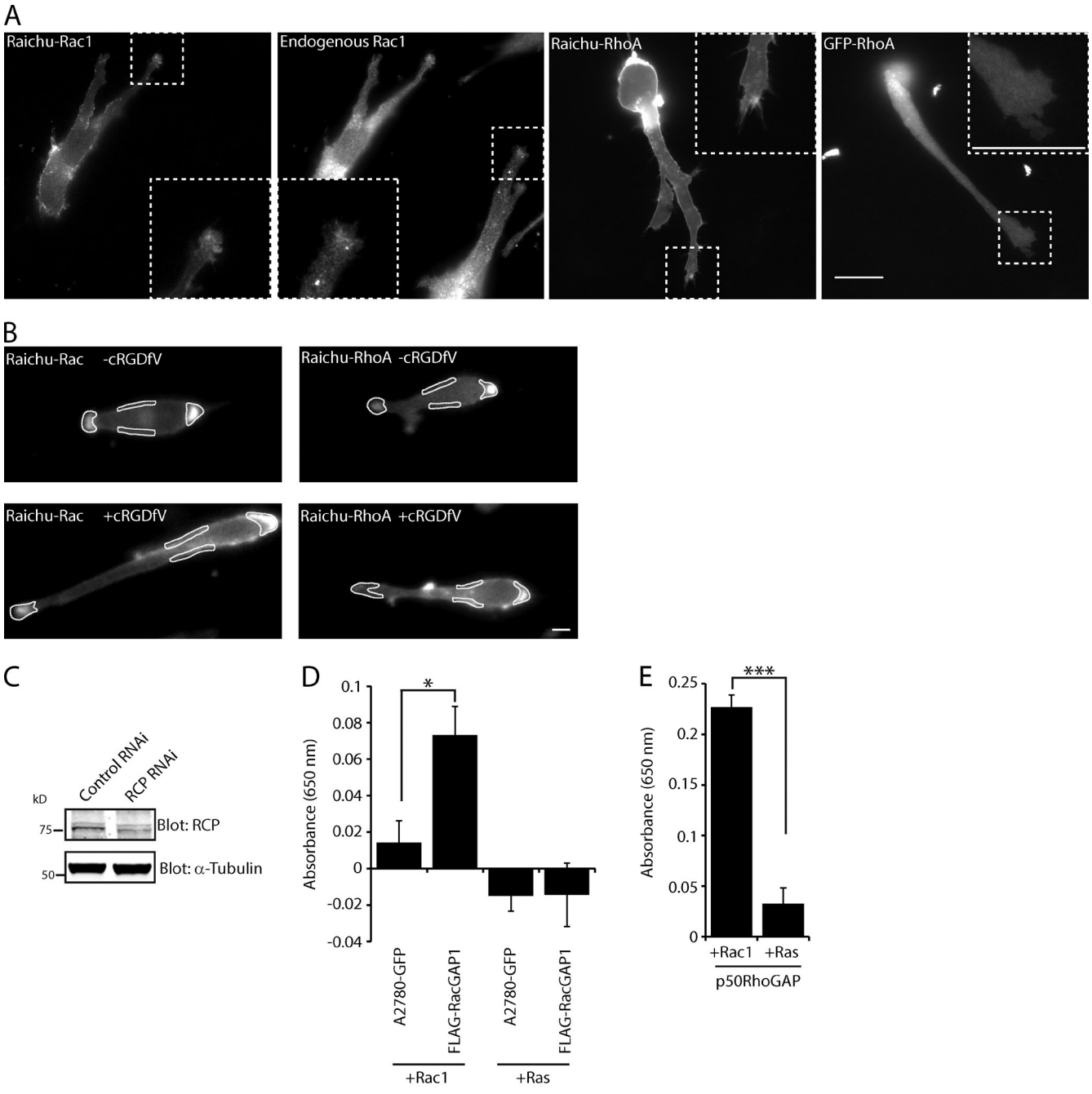


Figure S3. **Localization of Raichu-Rac and -RhoA and GAP activity of RacGAP1 in A2780 cells.** (A) A2780 cells were transfected with Raichu-Rac1 and fixed and stained for endogenous Rac1 or transfected with Raichu-RhoA or GFP-RhoA and fixed. Images were captured using a spinning-disk confocal microscope. Zoomed insets correspond to areas indicated by dotted ROIs. Bar, 20 µm. (B) A2780 cells were transfected with Raichu-Rac, seeded onto CDMs after 16–24 h, and stimulated with cRGDfV as indicated. Fluorescence lifetime images were captured at 1-min intervals, and intensity images are shown. Dotted lines indicate ROIs drawn for quantification of probe lifetime at the plasma membrane at the front, middle, and rear of the cell. Bar, 10 µm. (C) A2780 cells were transfected with control or RCP-specific siRNA and allowed to recover for 24 h. Cells were then retransfected with Raichu probes and lysed 24 h later. Lysates were subjected to SDS-PAGE/Western blot analysis to assess relative levels of RCP and α-tubulin. (D) A2780 cells stably expressing GFP or FLAG-RacGAP1^{WT} were lysed and immunoprecipitated with anti-FLAG antibodies. Immunoprecipitates were washed copiously and then applied to GAP assays against Rac1 or Ras. (E) GAP assays performed using recombinant p50RhoGAP against Rac1 or Ras (positive and negative control). Data represent means ± SEM from at least three independent experiments. *, $P < 0.05$; ***, $P < 0.001$.

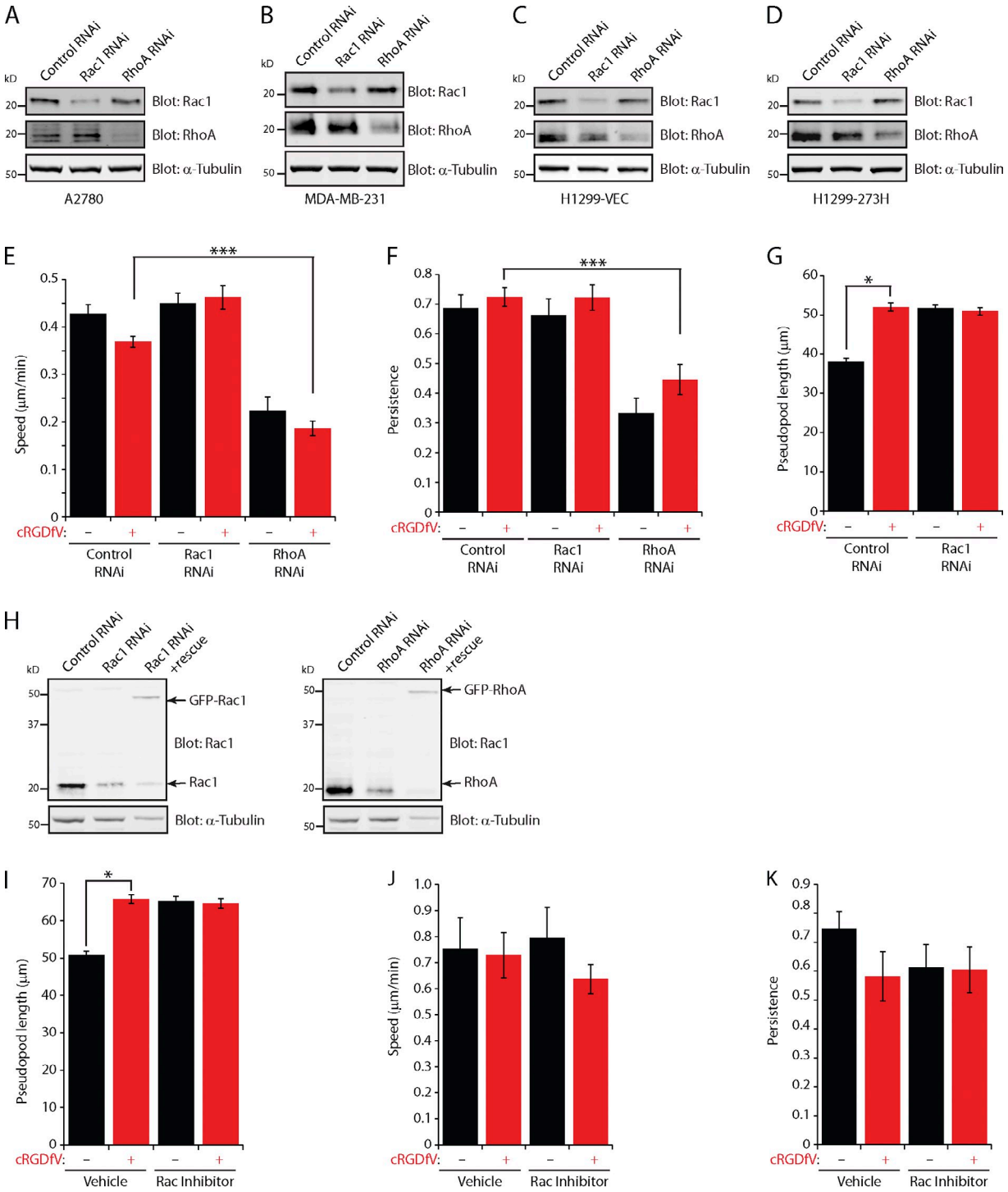
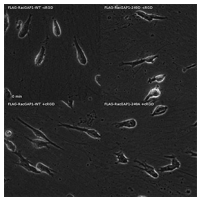
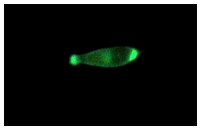


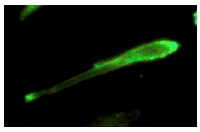
Figure S4. **Rac and RhoA differentially regulate migration on CDM.** (A–D) A2780 (A), MDA-MB-231 (B), or H1299-vector [VEC]/273H (C and D) cells were transfected with control, Rac1-, or RhoA-specific siRNA SMARTpool oligos and lysed after 48 h. Lysates were subjected to SDS-PAGE/Western blot analysis to assess relative levels of Rac1, RhoA, and α-tubulin. (E–G) A2780 cells were transfected as in A and seeded onto CDM, and cells migration was tracked in the presence or absence of cRGDfV using ImageJ. Speed and persistence (≥19 cells; E and F) of migration were analyzed using the manual tracking plug-in. Pseudopod length (>250 cells; G) was measured for all moving cells within the 20th frame. (H) A2780 cells were cotransfected with GFP-Rac1 or GFP-RhoA alongside control, Rac1 #1, or RhoA #1 RNAi oligos and lysed after 36 h. Lysates were subjected to SDS-PAGE/Western blot analysis to assess relative levels of Rac1, RhoA, and α-tubulin. Levels of Rac1 or RhoA were normalized against tubulin, and relative expression is shown. (I–K) A2780 cells were seeded onto the CDM and allowed to adhere and begin migrating. Cells were treated with or without 2.5 μM cRGDfV and 25 μM Rac inhibitor as indicated. Images were captured at 10-min intervals using a 20× objective lens. Pseudopod length (>250 cells; I) was measured for all moving cells within the 20th frame, and speed (≥15 cells; J) and persistence (≥15 cells; K) of migration were analyzed using the manual tracking plug-in in ImageJ. Graphs display means ± SEM from at least three independent experiments. *, $P < 0.05$; ***, $P < 0.001$.



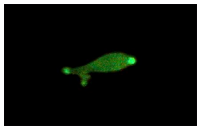
Video 1. **Phosphorylation of RacGAP1 on T249 is required for pseudopod extension.** A2780 cells stably expressing FLAG-RacGAP1^{WT}, FLAG-RacGAP1^{249A}, or FLAG-RacGAP1^{249D} were seeded onto CDMs and stimulated with or without 2.5 μM cRGDfV, and phase-contrast images were captured every 10 min on an inverted AS-MDW microscope system using a 20× objective lens.



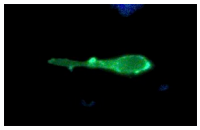
Video 2. **Rac activity is high at the cell front under basal conditions as cells migrate on CDM.** A2780 cells were transfected with Raichu-Rac and seeded onto CDMs after 16–24 h, and fluorescence lifetime images were captured at 1-min intervals using the Marianas inverted microscope system and a 63× objective lens. Videos of lifetime maps are presented, with colder colors representing low lifetime (and high activity) and warm colors representing high lifetime (and low activity).



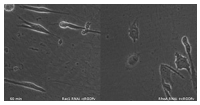
Video 3. **cRGDfV suppresses Rac activity at the cell front as cells migrate on CDM.** A2780 cells were transfected with Raichu-Rac, seeded onto CDMs after 16–24 h, and stimulated with 2.5 μM cRGDfV. Fluorescence lifetime images were captured at 1-min intervals using the Marianas inverted microscope system and a 63× objective lens. Videos of lifetime maps are presented, with colder colors representing low lifetime (and high activity) and warm colors representing high lifetime (and low activity).



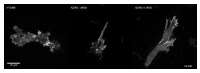
Video 4. **RhoA activity is low at the cell front under basal conditions as cells migrate on CDM.** A2780 cells were transfected with Raichu RhoA and seeded onto CDMs after 16–24 h, and fluorescence lifetime images were captured at 1-min intervals using the Marianas inverted microscope system and a 63× objective lens. Videos of lifetime maps are presented, with colder colors representing low lifetime (and high activity) and warm colors representing high lifetime (and low activity).



Video 5. **cRGDfV activates RhoA at the cell front as cells migrate on CDM.** A2780 cells were transfected with Raichu RhoA, seeded onto CDMs after 16–24 h, and stimulated with 2.5 μM cRGDfV. Fluorescence lifetime images were captured at 1-min intervals using the Marianas inverted microscope system and a 63× objective lens. Videos of lifetime maps are presented, with colder colors representing low lifetime (and high activity) and warm colors representing high lifetime (and low activity).



Video 6. **Rac knockdown promotes pseudopod extension; RhoA is required for migration on CDM.** A2780 cells were subjected to Rac or RhoA knockdown, seeded onto the CDM, and stimulated with (RhoA knockdown) or without (Rac1 knockdown) 2.5 μM cRGDfV, and phase-contrast images were captured every 10 min on an inverted AS-MDW microscope system using a 20× objective lens.



Video 7. **Dynamics of mEGFP-Lifeact in HT1080 cells and A2780 cells ± cRGDfV migrating on CDM.** HT1080 and A2780 cells were transfected with Lifeact-mEGFP and, after 16 h, plated onto CDM for 4 h before imaging. Actin dynamics were captured as cells move in 3D using a spinning-disk confocal inverted Marianas microscope system and a 63× objective lens.