Dart et al., http://www.jcb.org/cgi/content/full/jcb.201501072

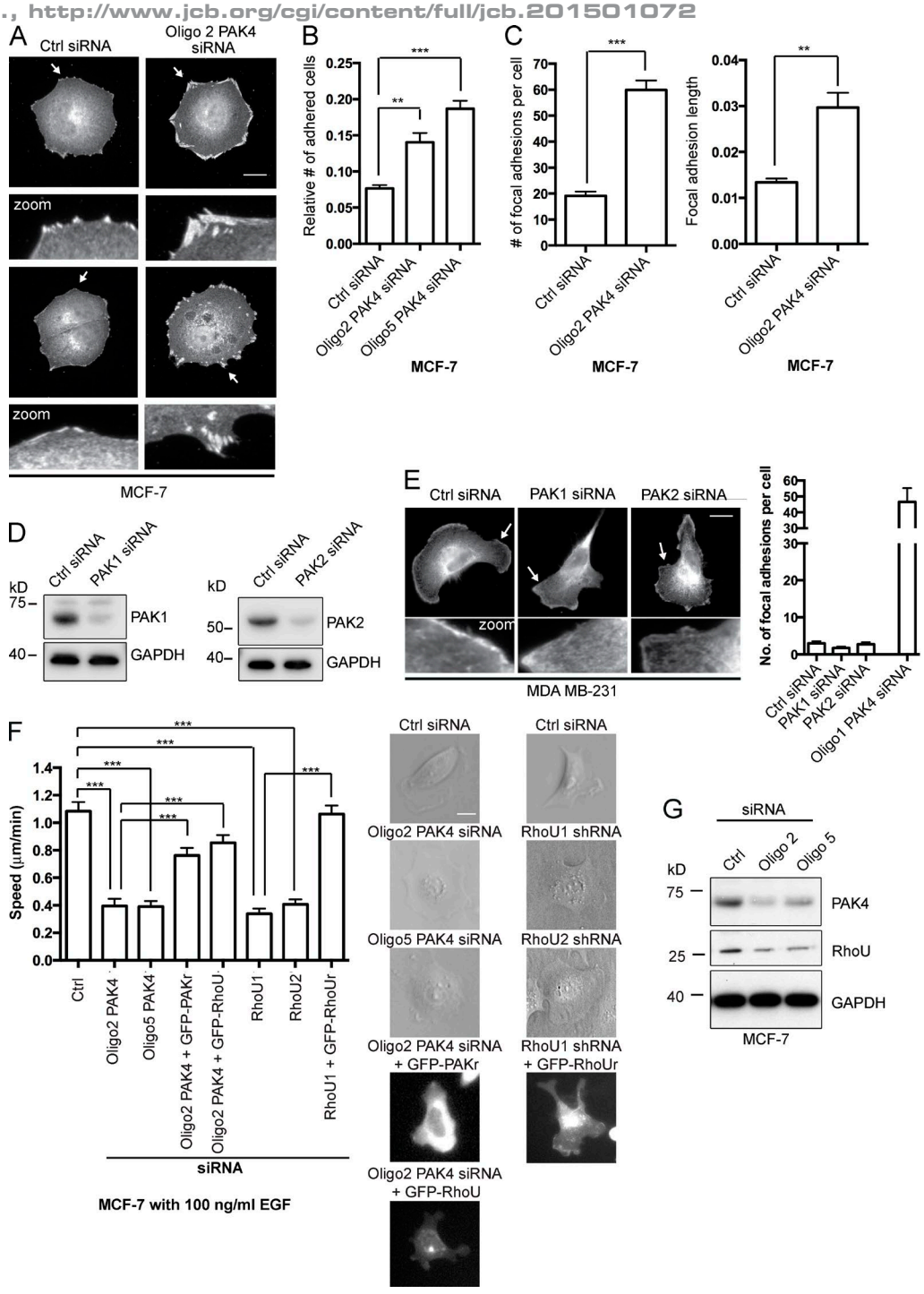

Figure S1. **Reduced PAK4 expression, but not reduced PAK1 or PAK2 expression, leads to an increase in cell adhesion.** (A) Paxillin labeling of MCF-7 cells transiently transfected with control or PAK4 siRNA. Areas highlighted with an arrow are enlarged as insets under each corresponding image. Bar, 20 µm. (B) Adhesion assay of control and PAK4 siRNA-expressing MCF-7 cells plated onto 10 µg/ml fibronectin for 60 min. The relative number of adhered cells is absorbance at 560 nm. **, $P < 0.01$; ***, $P < 0.001$. (C) The mean number of focal adhesions per cell and the mean length of focal adhesions for all cell populations were measured using ImageJ software. $n > 60$ cells per condition over three independent experiments. **, $P < 0.01$; ***, $P < 0.001$. (D) WT MDA-MB-231 cells were transiently transfected with control, PAK1, or PAK2 siRNA SMARTpools, and protein levels were determined after 48 h by immunoblotting. (E) Paxillin labeling of MDA-MB-231 cells transiently expressing control, PAK1, or PAK2 siRNA. The arrows highlight the areas in the magnified insets. Bar, 20 µm. The mean number of focal adhesions per cell for all cell populations was measured using ImageJ software. $n > 60$ cells per condition over three independent experiments. (F) MCF-7 cells were transiently transfected with control siRNA, PAK4 siRNA, or RhoU shRNA. 24 h after this transfection, Oligo2 PAK4 siRNA-expressing MCF-7 cells were also transiently transfected with GFP-PAK4r or GFP-RhoU, and RhoU 1 shRNA-expressing MCF-7 cells with GFP-RhoUr. All conditions were plated onto 10 µg/µl fibronectin and stimulated with 100 ng/ml EGF to induce motility. Cells were filmed for 18 h with time-lapse video microscopy. 60 individual cells per condition (n) were tracked over three separate experiments. The mean migration speed ± SEM was calculated for each condition. ***, $P < 0.001$. Image stills from the videos depict typical cell morphologies. Bar, 20 µm. (G) Lysates were made from control siRNA- and PAK4 siRNA-expressing MCF-7 cells and analyzed by immunoblotting with anti-PAK4 and RhoU antibodies. GAPDH was used as a loading control. Ctrl, control.

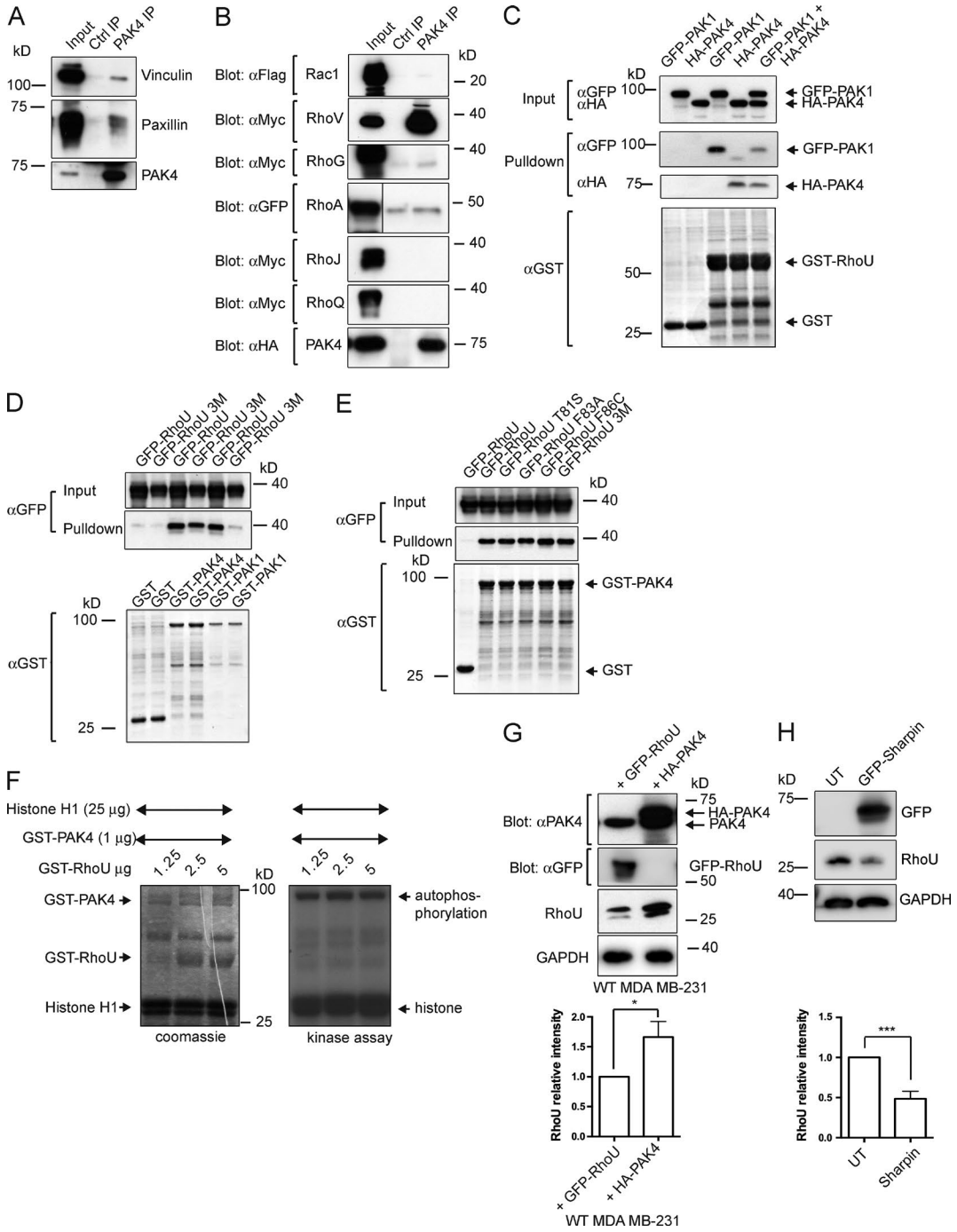

Figure S2. **RhoU–PAK interactions.** (A) Endogenous PAK4 was immunoprecipitated from WT MDA-MB-231 cell lysates using an in-house rabbit anti-PAK4 antibody, and the blot was probed for mouse antipaxillin and antivinculin antibodies. Control immunoprecipitation was a rabbit anti–VSV-G antibody. (B) Anti–in-house PAK4 antibody coimmunoprecipitation of RhoV but not Rac1, RhoG, RhoA, RhoJ, or RhoQ from HEK-293 cells expressing HA-PAK4 and GFP-, myc-, or flag-tagged fusions of the Rho GTPases. Control immunoprecipitation was a rabbit anti–VSV-G antibody. Black line indicates that intervening lanes have been spliced out. (C) GST or GST-RhoU beads were used to pull down overexpressed GFP-PAK1 or HA-PAK4 from HEK-293 cell lysates. Blot is representative of three separate experiments. (D) GST, GST-PAK4, or GST-PAK1 beads were used to pull down overexpressed GFP-tagged RhoU or the RhoU effector loop mutant from HEK-293 cell lysates. Triple point mutation was RhoU 3M. Representative of three independent experiments. (E) GST or GST-PAK4 beads were used to pull down overexpressed GFP-tagged RhoU effector loop mutants from HEK-293 cell lysates. Single point mutants were RhoU T81S, RhoU F83A, and RhoU F86C, and the triple point mutant was RhoU 3M. Representative of three independent experiments. (F) An in vitro kinase assay was performed using 1 μg purified GST-PAK4, 25 μg histone H1, and increasing amounts of GST-RhoU (1.25, 2.5, and 5 μg). (Left) Coomassie staining demonstrates purified protein input. (Right) Phosphorylation of histone H1 and autophosphorylation of PAK4 were detected by autoradiography. (G) Lysates were made from WT MDA-MB-231 cells overexpressing either GFP-RhoU or HA-PAK4 and analyzed by immunoblotting with anti-PAK4, GFP, and RhoU antibodies. GAPDH was used as a loading control. Autoradiographs were quantified using ImageJ software, and the relative intensity of the RhoU signal was normalized to the loading control. The results shown are the means ± SEM of at least three independent experiments. *, $P < 0.05$. (H) Lysates from HEK-293 cells expressing GFP-Sharpin blotted with anti-GFP and RhoU antibodies. GAPDH was used as a loading control. UT, untransfected. Autoradiographs were quantified using ImageJ software, and the relative intensity of the RhoU signal was normalized to the loading control. The results shown are the means ± SEM of at least three independent experiments. ***, $P < 0.001$. Ctrl, control.

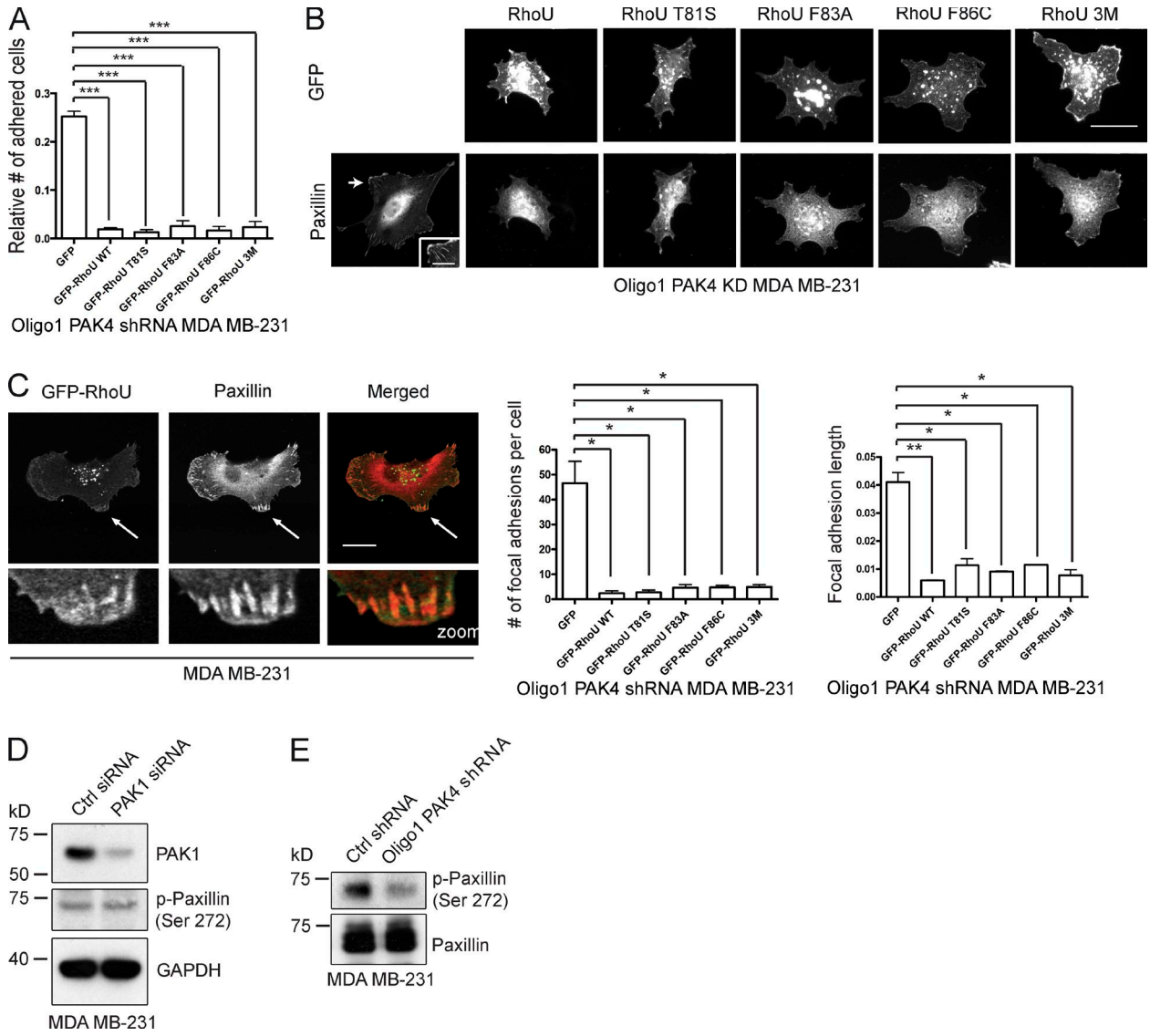

Figure S3. **RhoU effector loop mutants that bind PAK4 can rescue the adhesion phenotype of PAK4-depleted MDA-MB-231 cells.** (A) Adhesion assay of Oligo1 PAK4-depleted MDA-MB-231 cells expressing GFP-RhoU or GFP-tagged RhoU effector loop mutants plated onto 10 µg/ml fibronectin for 60 min. The relative number of adhered cells is absorbance at 560 nm. Error bars represent mean ± SEM. ***, $P < 0.001$. (B) Paxillin labeling of Oligo1 PAK4 shRNA-expressing MDA-MB-231 cells transiently expressing GFP-tagged RhoU effector loop mutants. Bars: (top) 20 µm; (inset) 5 µm. The mean number of focal adhesions per cell and the mean length of focal adhesions for all cell populations were measured using ImageJ software. $n > 60$ cells per condition over three independent experiments. *, $P < 0.05$; **, $P < 0.01$. (C) MDA-MB-231 cells were transfected with GFP-RhoU and then fixed and labeled for paxillin. Representative images taken by confocal microscopy are shown. Bar, 20 µm. Areas highlighted with an arrow are enlarged as insets under each corresponding image. (D) Blot showing the levels of paxillin (S272) in control and PAK1 siRNA-expressing MDA-MB-231 cells plated onto 10 µg/ml fibronectin substrate. (E) Blot showing the levels of paxillin (S272) and total paxillin in control and PAK4 shRNA-expressing MDA-MB-231 cells plated onto 10 µg/ml fibronectin substrate. Ctrl, control.

Table S1 is provided as an Excel file and lists the RhoU-interacting proteins identified from the protein microarray.