Genna et al., https://doi.org/10.1083/jcb.201702184

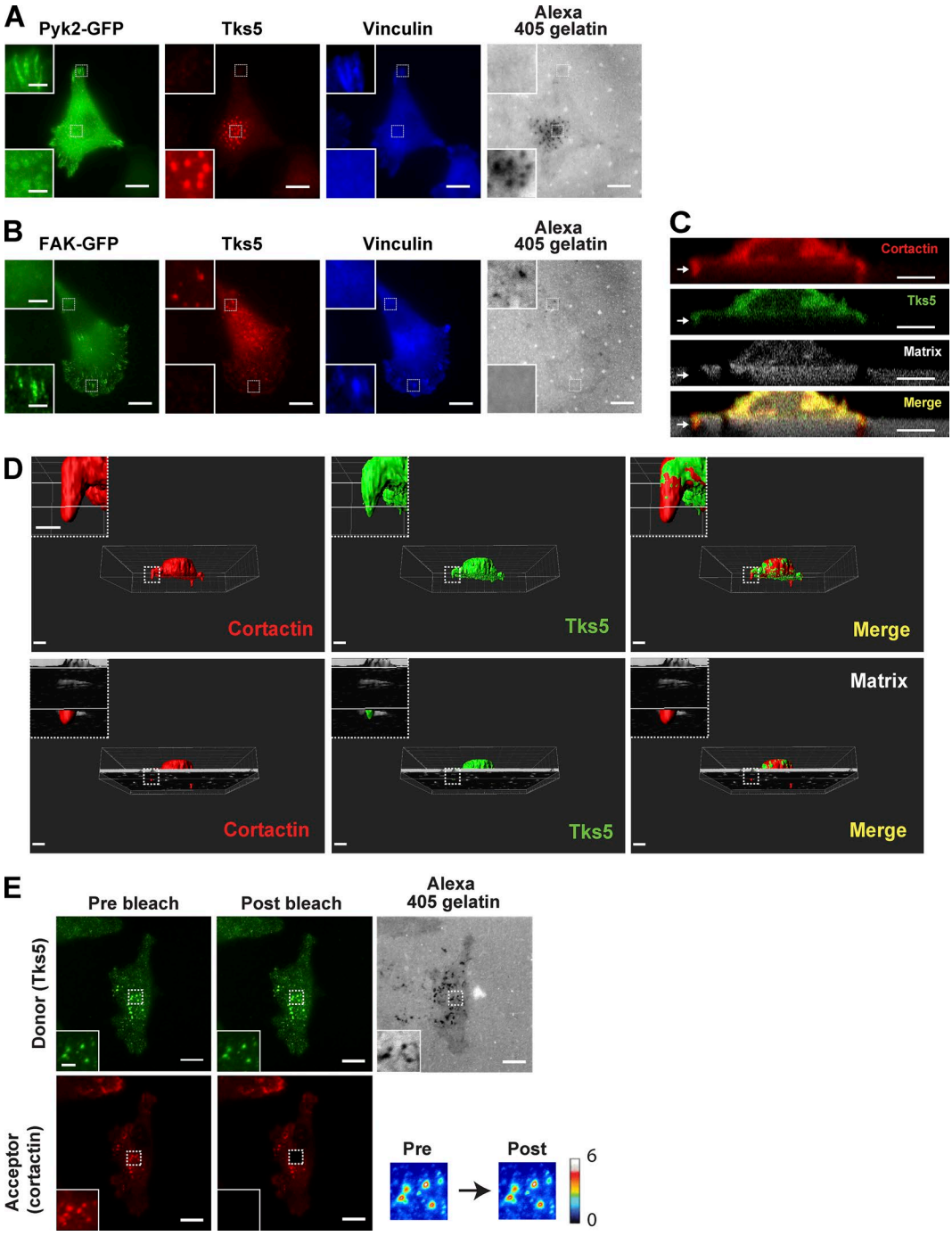

Figure S1. **Immunofluorescence controls for 2D, 2.5D, and FRET assays.** (A and B) MDA–MB-231 cells stably expressing WT Pyk2-GFP (A) or WT FAK-GFP (B) and treated with Pyk2 siRNA or FAK siRNA, respectively, were plated on Alexa Fluor 405 gelatin matrix, fixed, and fluorescently labeled for Tks5 and vinculin. Insets depict magnification of boxed areas showing either focal adhesions or invadopodia. Bars: (main images) 10 µm; (insets) 2 µm. (C) Representative X–Z confocal images showing colocalization of cortactin-TagRFP (red) and Tks5-GFP (green) to an invadopod (arrows) projecting into Matrigel/Alexa Fluor 405 gelatin matrix (gray) that was plated in the top chamber of a 1-µm transwell filter. Bar, 5 µm. (D) Representative 3D reconstruction images showing colocalization of cortactin-TagRFP (red) and Tks5-GFP (green) to an invadopod (box) projecting into the Matrigel/Alexa Fluor 405 gelatin matrix (white, bottom) that was plated in the top chamber of a 1-µm transwell filter. Bars: (main images) 5 µm; (inset) 2 µm. (E) Representative Tks5-cortactin FRET efficiency images of an MDA–MB-231 cell labeled for Tks5 (green) and cortactin (red). Insets (left) and box (right) indicate the bleached area. Bars: (main images) 10 µm; (insets) 2 µm.

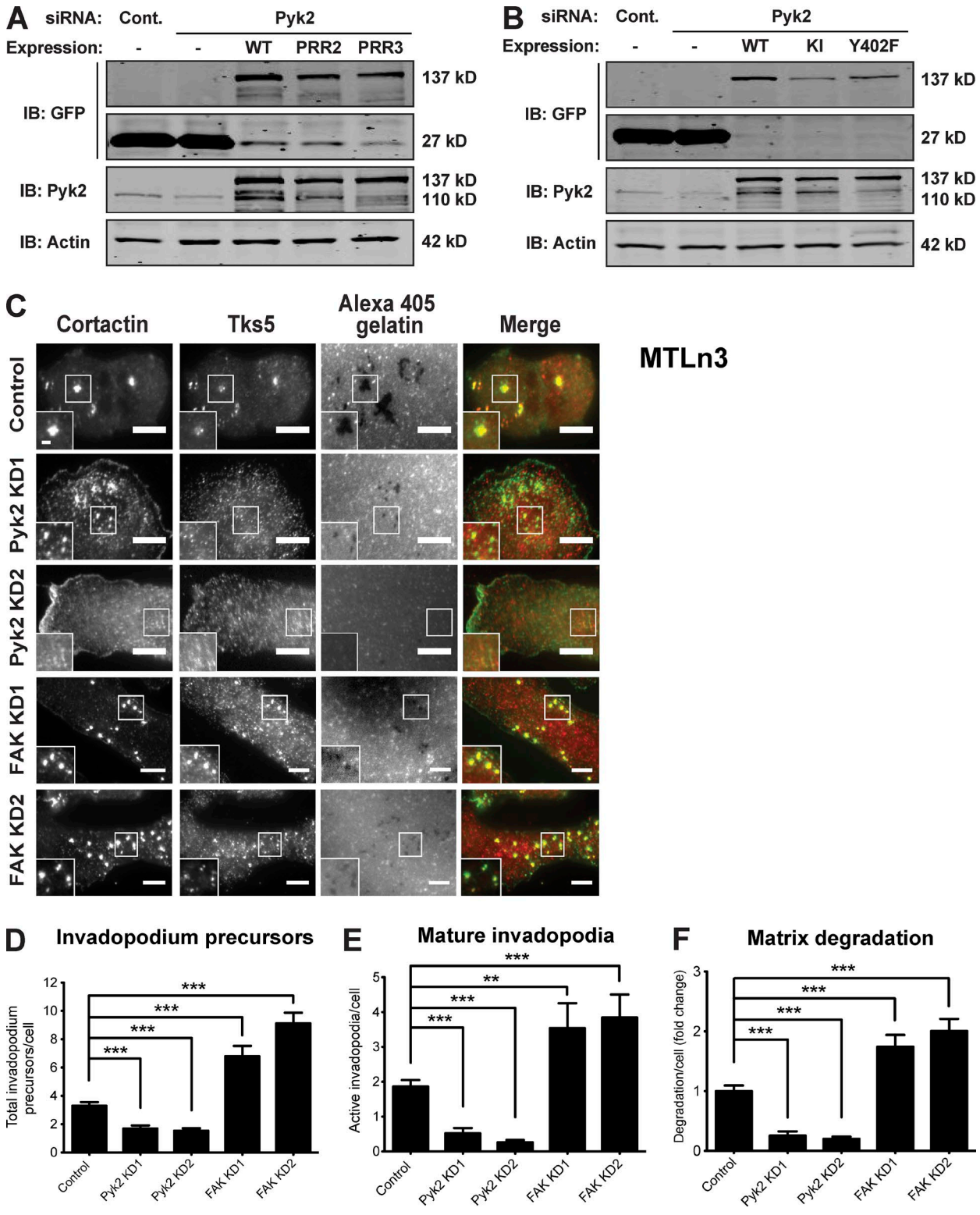

Figure S2. **Invadopodium precursor formation and matrix degradation are oppositely regulated by Pyk2 and FAK in MTLn3-knockdown cell lines.** (A) Western blot analysis of cell lysates from MDA–MB-231 cells stably expressing Pyk2-GFP WT, Pyk2-GFP PRR2, or Pyk2-GFP PRR3 and transiently knocked down for endogenous Pyk2 by siRNA. (B) Western blot analysis of cell lysates from MDA–MB-231 stably expressing Pyk2-GFP WT, Pyk2-GFP KI, or Pyk2-GFP Y402F and transiently knocked down for endogenous Pyk2 by siRNA. IB, immunoblot. (C) MTLn3 cells stably expressing control, Pyk2, or FAK shRNA were plated on Alexa Fluor 405 gelatin, fixed, and labeled for Tks5 and cortactin. Boxed regions and insets depict colocalization of Tks5 and cortactin as markers for invadopodium precursors, with Alexa Fluor 405 gelatin as a marker for mature invadopodia. Bars: (main images) 10 µm; (insets) 2 µm. (D) Quantification of invadopodium precursors, defined by colocalization of Tks5 and cortactin. *n* = 48–119 cells per group from three independent experiments. (E) Quantification of mature (active) invadopodia defined by colocalization of Tks5 and cortactin with degradation regions. *n* = 48–119 cells per group from three independent experiments. (F) Quantification of matrix degradation by control and knockdown cells. *n* = 39–50 fields per group from three independent experiments. **, P < 0.01; ***, P < 0.001. Error bars represent SEM.

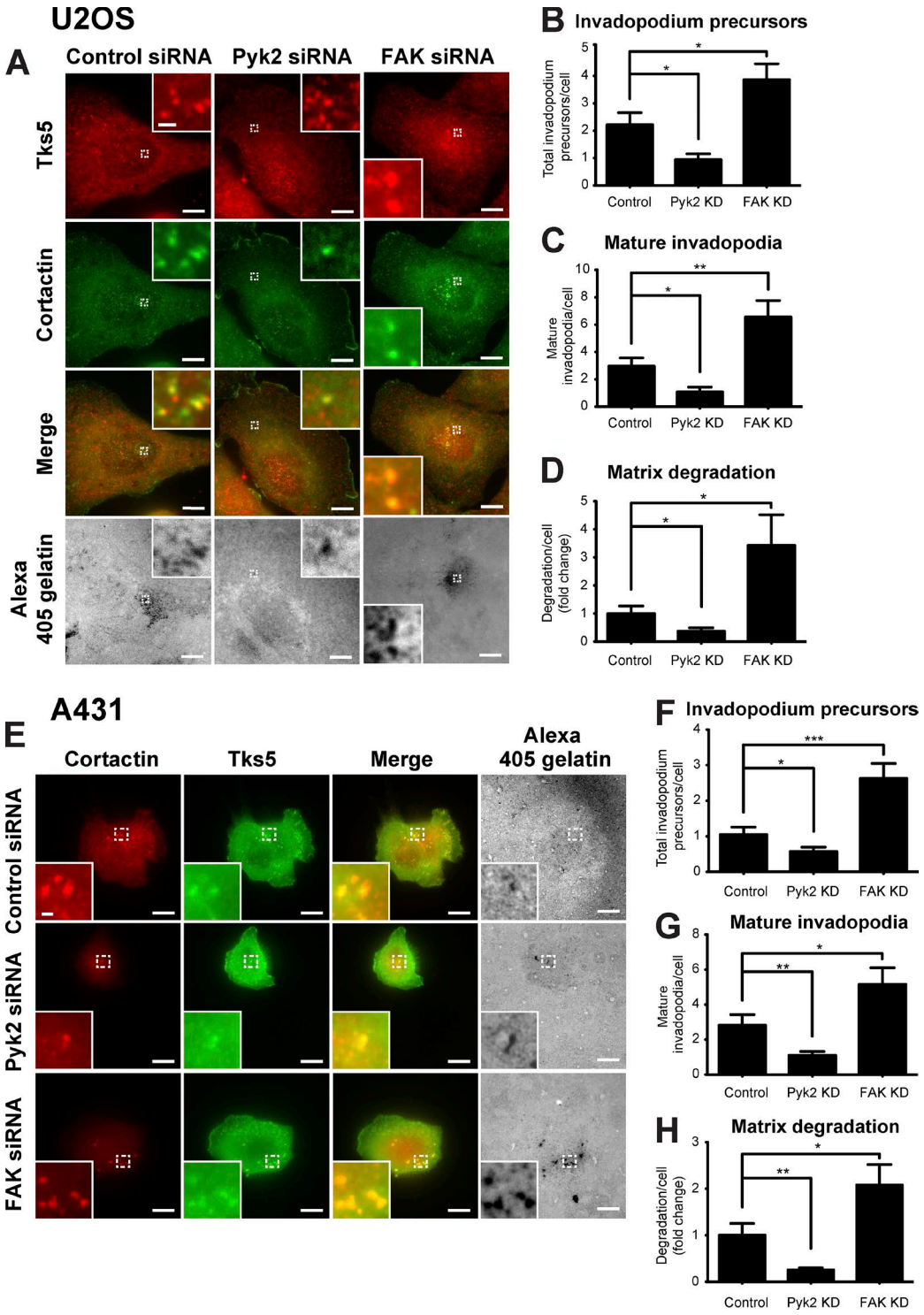

Figure S3. **Invadopodium precursor formation and matrix degradation are oppositely regulated by Pyk2 and FAK in osteosarcoma and epidermoid carcinoma knockdown cell lines.** (A) U2OS cells were transiently knocked down (KD) for control, Pyk2, or FAK, and then were plated on Alexa Fluor 405 gelatin, fixed, and labeled for Tks5 and cortactin as invadopodia markers. Boxed regions and insets depict colocalization of Tks5 and cortactin as markers for invadopodium precursors, with Alexa Fluor 405 gelatin as a marker for mature invadopodia. (B and C) Quantification of invadopodium precursors (B), defined by colocalization of Tks5 and cortactin, and quantification of mature (active) invadopodia (C), defined by colocalization of Tks5 and cortactin with degradation regions. n = 45–60 cells per group from three independent experiments. (D) Quantification of matrix degradation by control and knockdown cells. n = 18–19 fields per group from three independent experiments. (E) A431 cells were transiently knocked down for control, Pyk2, or FAK, plated on Alexa Fluor 405 gelatin, fixed, and labeled for Tks5 and cortactin as invadopodia markers. Boxed regions and insets depict colocalization of Tks5 and cortactin as markers for invadopodium precursors, with Alexa Fluor 405 gelatin as a marker for mature invadopodia. Bars: (main images) 10 µm; (insets) 1 µm. (F and G) Quantification of invadopodium precursors (F), defined by colocalization of Tks5 and cortactin, and quantification of mature (active) invadopodia (G), defined by colocalization of Tks5 and cortactin with degradation regions. n = 76–93 cells per group from two independent experiments. (H) Quantification of matrix degradation by control and knockdown cells. n = 24–28 fields per group from three independent experiments. *, $P < 0.05$; **, $P < 0.01$; ***, $P < 0.001$. Error bars represent SEM.

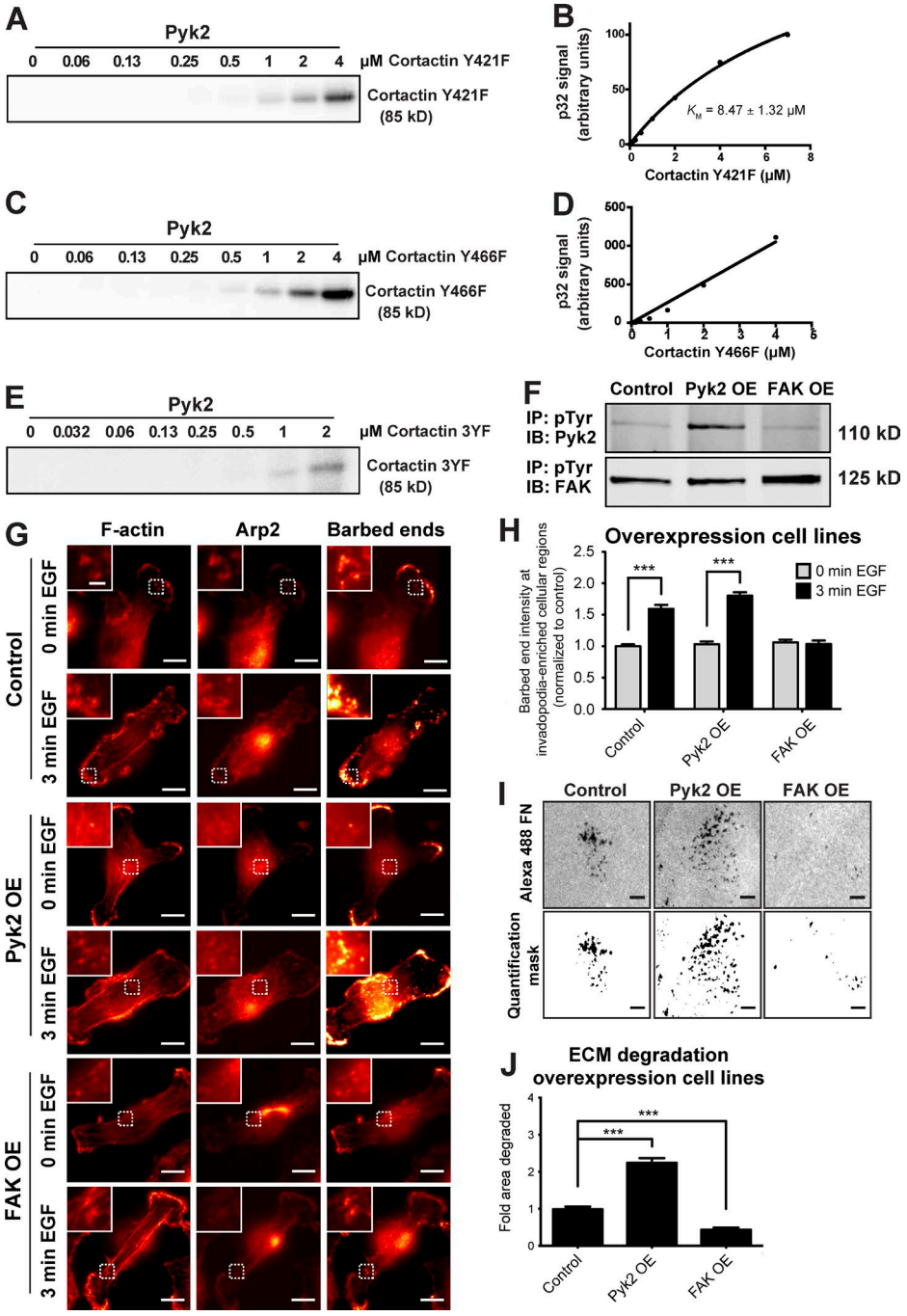

Figure S4. **Matrix degradation and barbed end formation are oppositely regulated in Pyk2- versus FAK-overexpressing cells.** (A–F) Increasing concentrations of recombinant His-tagged cortactin Y421F (0–4 µM; B and C), cortactin Y466F (0–4 µM; D and E), or cortactin 3YF (Y421F, Y466F, and Y482F; 0–2 µM; F) were incubated with a constant concentration of Pyk2 (10 nM) in the presence of [γ^{32}]P-ATP. Kinase reactions were permitted to proceed for 5 min and then were quenched by adding Laemmli sample buffer and boiling for 5 min. Reactions were run on an SDS-PAGE gel and exposed to a PhosphorImager screen. Representative phosphoimages (A, C, and E) and graphs (B and D) are shown. In three independent experiments (n = 3), kinase assays with cortactin Y466F did not reach saturation, and only residual phosphorylation of cortactin 3YF at cortactin concentrations >1 µM was observed. The Pyk2/cortactin Y421F kinase assays approached saturation (n = 2), but with a clearly reduced K_M of 8.47 ± 1.32 µM, compared with a K_M of 1.02 ± 0.304 µM for WT cortactin, indicating inefficient phosphorylation. (F) Representative immunoblots (IBs) of MDA–MB-231 cells overexpressing control, Pyk2 (Pyk2 overexpression [OE]), or FAK (FAK overexpression). Whole-cell lysates were immunoprecipitated with antiphosphotyrosine antibodies and probed for either Pyk2 (top) or FAK (bottom). IP, immunoprecipitation. (G) Control, Pyk2 overexpression, and FAK overexpression MDA–MB-231 cells were left untreated (0 min EGF) or were stimulated with EGF for 3 min (3 min EGF). Cells were fixed and labeled for F-actin and Arp2 as invadopodia markers and for biotin–actin as a marker for newly formed barbed ends. Bars: (main images) 10 µm; (insets) 2 µm. (H) Quantification of free barbed ends as measured by mean biotin–actin intensity at stimulated regions of cells rich in invadopodia containing F-actin and Arp2. n = 72–180 invadopods per group from three independent experiments. (I) Control, Pyk2 overexpression, and FAK overexpression MDA–MB-231 cells were plated on Alexa Fluor 488 FN/gelatin matrix and allowed to degrade for 24 h. Shown are representative images (left) and quantification masks (right) of degradation areas. Bars, 10 µm. (J) Quantification of matrix degradation by control, Pyk2 overexpression, and FAK overexpression cells. n = 130–474 fields per group from three independent experiments. ***, P < 0.001. Error bars represent SEM.

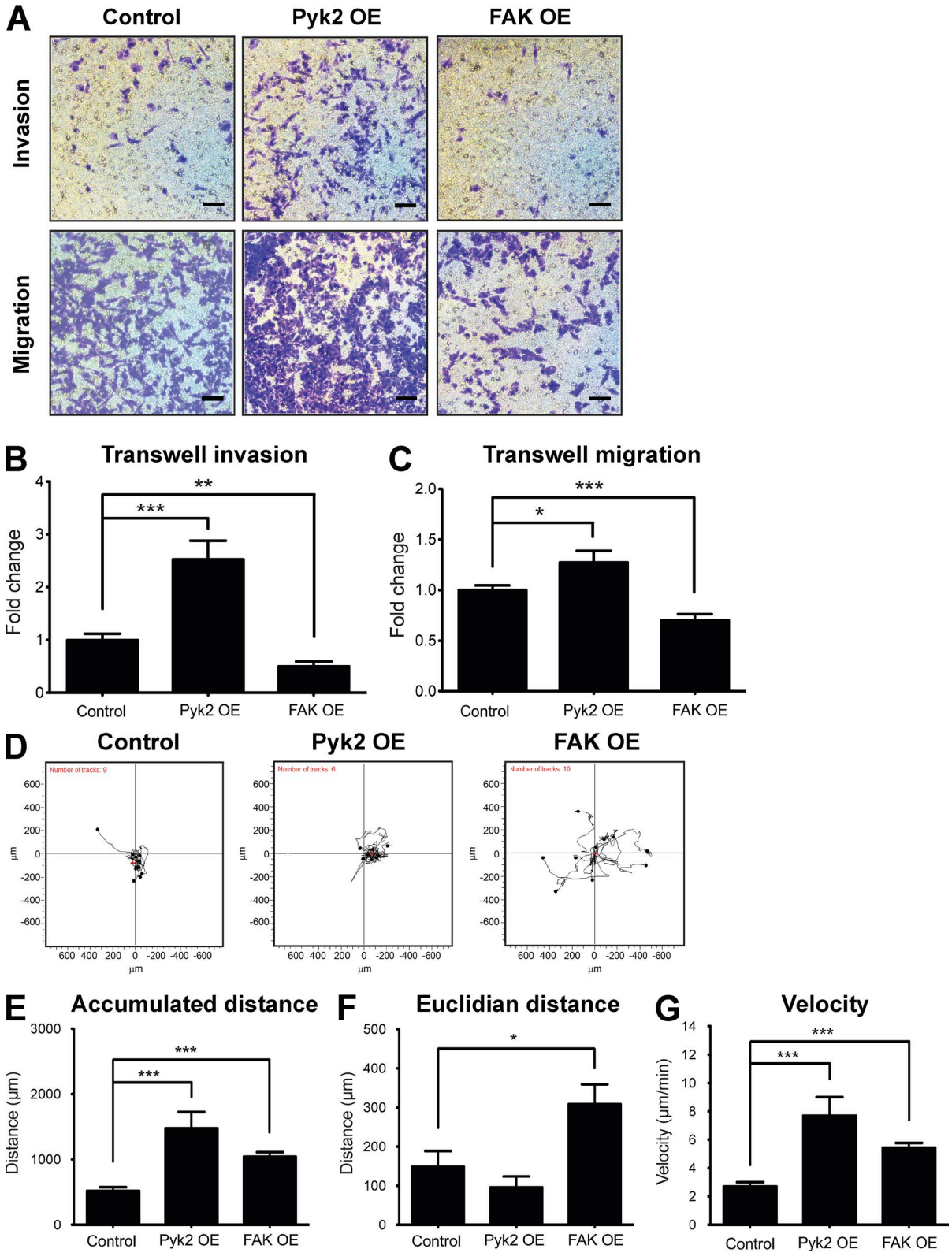

Figure S5. **ECM invasion and cell migration are oppositely regulated in Pyk2- versus FAK-overexpressing cells.** (A) Control, Pyk2 overexpression (OE), and FAK overexpression cells were plated on Matrigel-coated (top) or uncoated (bottom) membranes, allowed to invade or migrate for 24 h, fixed, and stained. Representative images of cells that migrated or invaded to the lower part of the membranes are shown. Bars, 5 µm. (B) Quantification of cell invasion through Matrigel-coated membranes. n = 24 (control, Pyk2 overexpression, and FAK overexpression) fields per group from three independent experiments. (C) Quantification of cell migration through uncoated membranes. n = 20–24 fields per group from three independent experiments. (D) Trajectory plots of single-cell migration experiments of control, Pyk2 overexpression, and FAK overexpression cell lines. (E–G) Quantification of accumulated distance (E), Euclidian distance (F), and velocity (G). n = 6–10 cells per group from two independent experiments. *, $P < 0.05$; **, $P < 0.01$; ***, $P < 0.001$. Error bars represent SEM.

Table S1 is a separate Excel document containing protein–protein interaction array data.
Table S2 is a separate Excel document containing literature mining information.