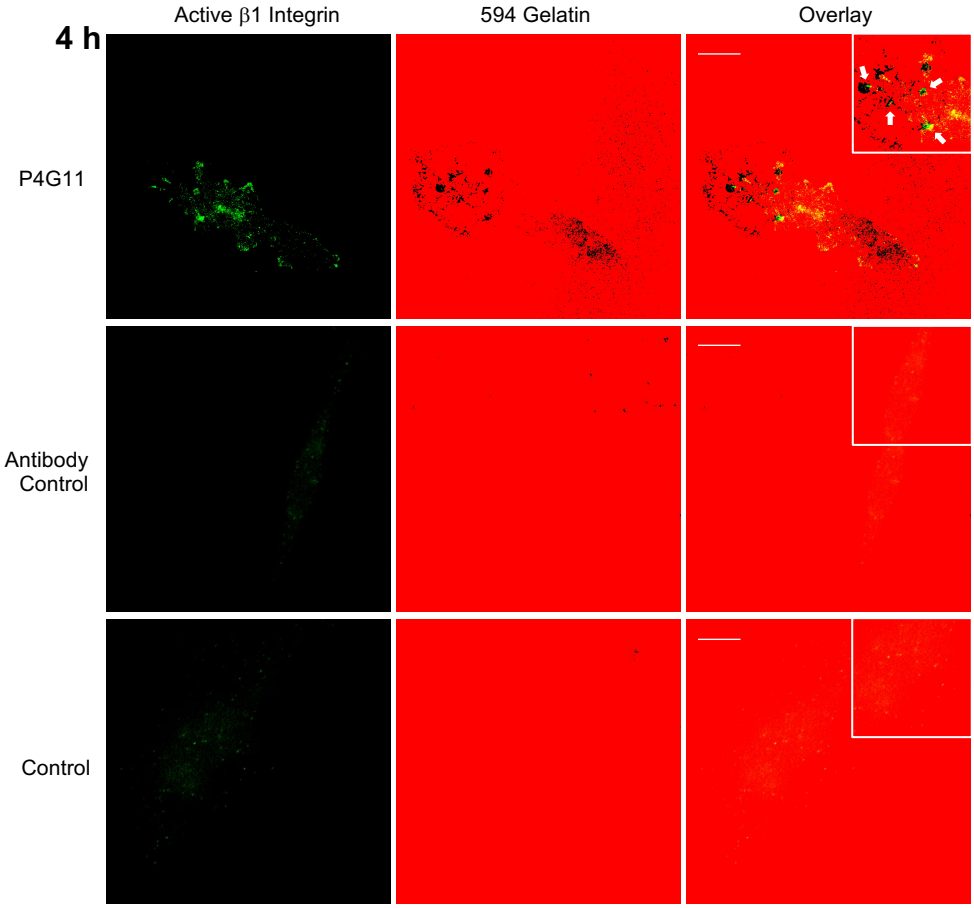

Figure S1. Cells Treated with P4G11 Display Colocalization of Active β1 Integrin and Areas of Gelatin Degradation. MDA-MB-231 cells were serum-starved for 24 hours, then plated onto fluorescent gelatin-coated coverslips for 4 hours with simultaneous antibody treatment. Cells were then fixed, permeabilized, and stained for active β1 integrin. Cells were analyzed by confocal microscopy. A representative section from the ventral region of a cell is shown. White arrows indicate areas of colocalization. All data represent three or more biological replicates with at least three technical replicates. Scale bar, 10 μm.

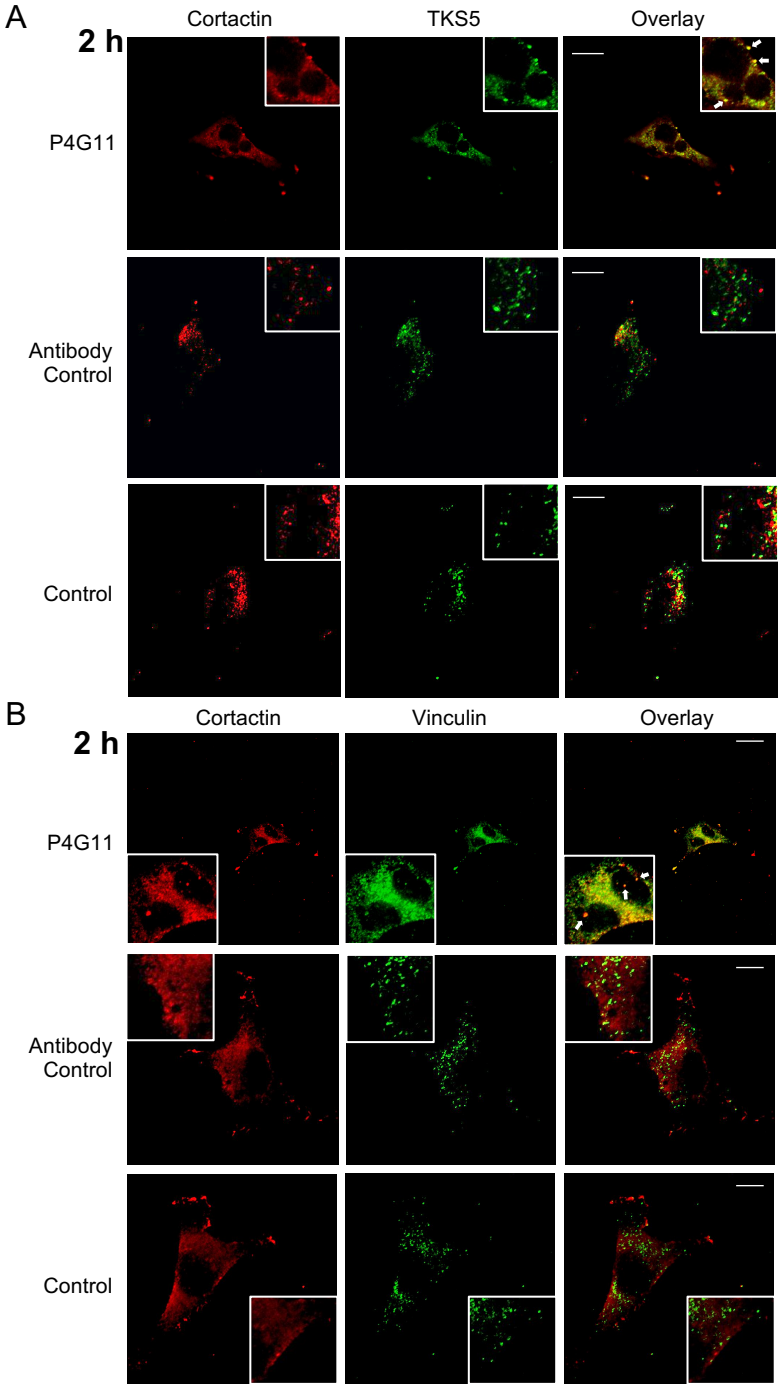

Figure S2. Cells Treated with P4G11 Display Colocalization of Invadopodia Precursor Markers. Cells were serum-starved for 24 hours, then seeded onto coverslips for 2 hours with antibody treatment, then fixed, permeabilized, and stained for (*A)* cortactin and TKS5, or *(B*) cortactin and vinculin. Cells were analyzed by confocal microscopy. A representative section from the ventral region of a cell is shown. White arrows indicate invadopodia precursors as defined by overlay of stained proteins. All data represent three or more biological replicates with at least three technical replicates. Scale bar, 10 µm.

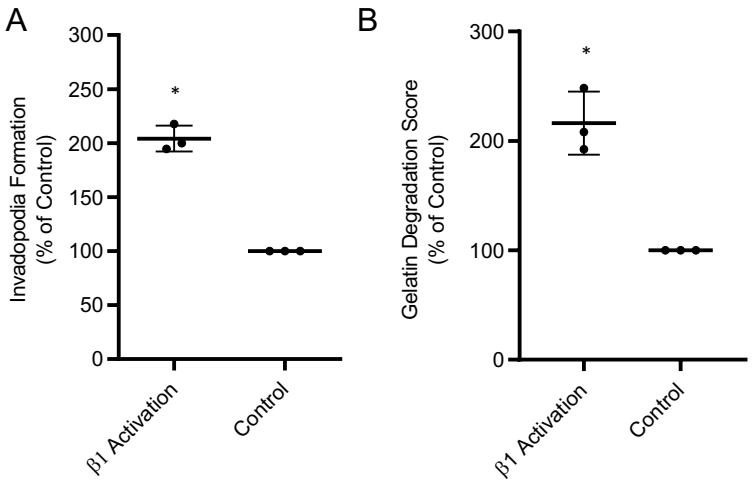

Figure S3. β1 Integrin Activation Increases Invadopodium Formation and Local Gelatin Degradation in HT-1080 Cells. Cells were serum-starved, treated with P4G11, and plated on fluorescently labelled gelatin-coated coverslips for 4 h (*A*) or 16 h (*B*). *A*, Quantification of invadopodium formation. Cells with F-actin puncta overlaying dark spots of gelatin degradation were counted as cells forming invadopodia. *B*, Quantification of gelatin degradation. Cells were analyzed for dark areas of degradation and scored as described under "Experimental Procedures." The percentages of cells are shown from experiments in which 50 cells/sample were analyzed and normalized to the control condition. All data are presented as percent of control ± S.D. All data represent three or more biological replicates with at least three technical replicates. Asterisks denote values significantly different from control (*, $p < 0.05$).

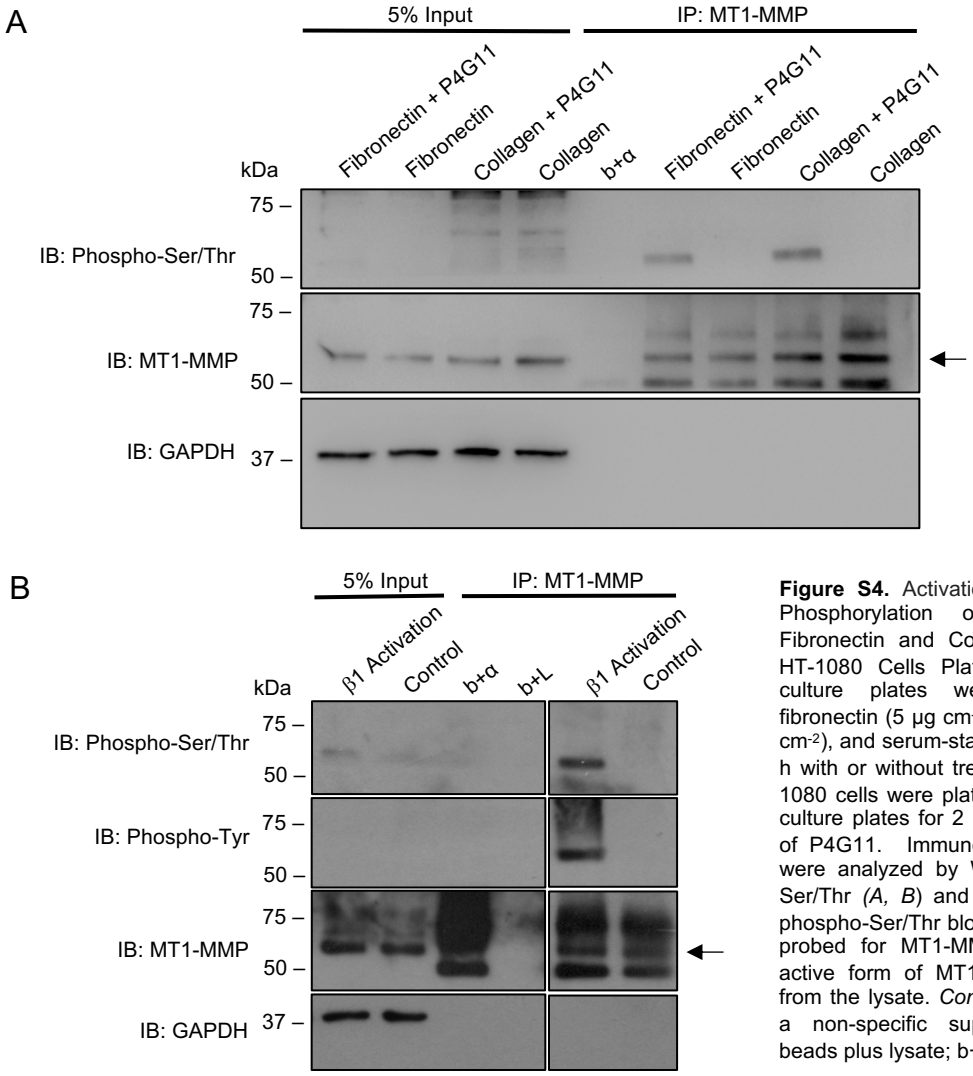

Figure S4. Activation of β1 Integrin Induces Phosphorylation of MT1-MMP on Both Fibronectin and Collagen Substrates, and in HT-1080 Cells Plated on Gelatin. *A*, Cell culture plates were coated with either fibronectin (5 μg cm^{-2}) or collagen type I (10 μg cm^{-2}), and serum-starved cells were plated for 2 h with or without treatment of P4G11. *B*, HT-1080 cells were plated onto gelatin-coated cell culture plates for 2 h with or without treatment of P4G11. Immunoprecipitates of MT1-MMP were analyzed by Western blot for phospho-Ser/Thr *(A, B)* and phospho-Tyr *(B)*, and the phospho-Ser/Thr blot was then stripped and re-probed for MT1-MMP. Arrow indicates the active form of MT1-MMP immunoprecipitated from the lysate. *Control* cells were treated with a non-specific supernatant antibody. b+L, beads plus lysate; b+α, beads plus antibody.

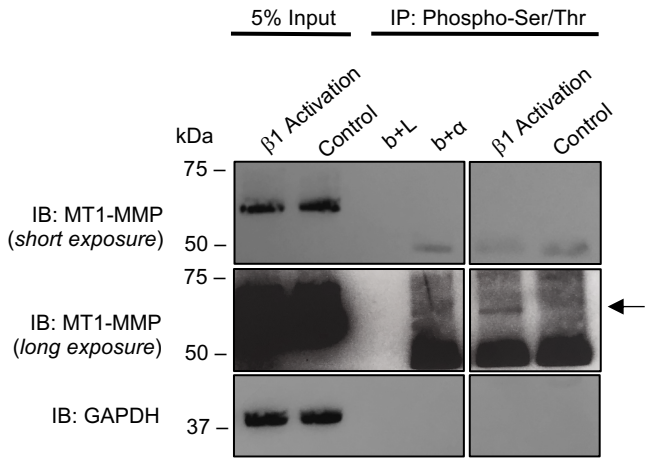

Figure S5. Activation of β1 Integrin Induces Phosphorylation of MT1-MMP. *A*, Immunoprecipitation of Phospho-Ser/Thr from MDA-MB-231 cells. Cells were plated onto gelatin-coated cell culture plates for 2 h, with or without treatment of P4G11, and immunoprecipitates of phospho-Ser/Thr were analyzed by Western blot for MT1-MMP. Arrow indicates immunoprecipitated MT1-MMP. *Control* cells were treated with a non-specific supernatant antibody. *b+L*, beads plus lysate; *b+α*, beads plus antibody. All data represent three or more biological replicates with at least three technical replicates.

A

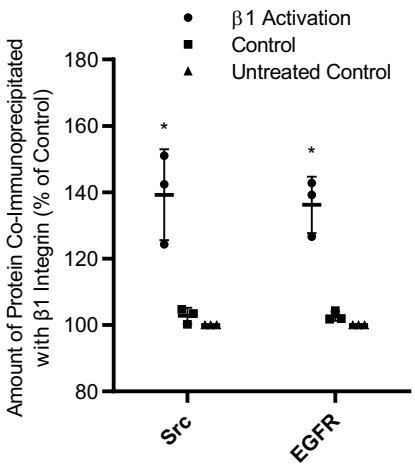

Figure S6. Treatment with P4G11 Increases the Colocalization and Association of Invadopodial Proteins. Serum-starved MDA-MB-231 cells were plated onto gelatin for 2 hours with or without treatment with P4G11. Immunoprecipitates of β1 integrin were analyzed for association with Src kinase and EGFR by Western blot and quantified. All data are presented as percent of control ± S.D. Asterisks denote values significantly different from the control condition (*, $p < 0.05$). All data represent three or more biological replicates with at least three technical replicates.

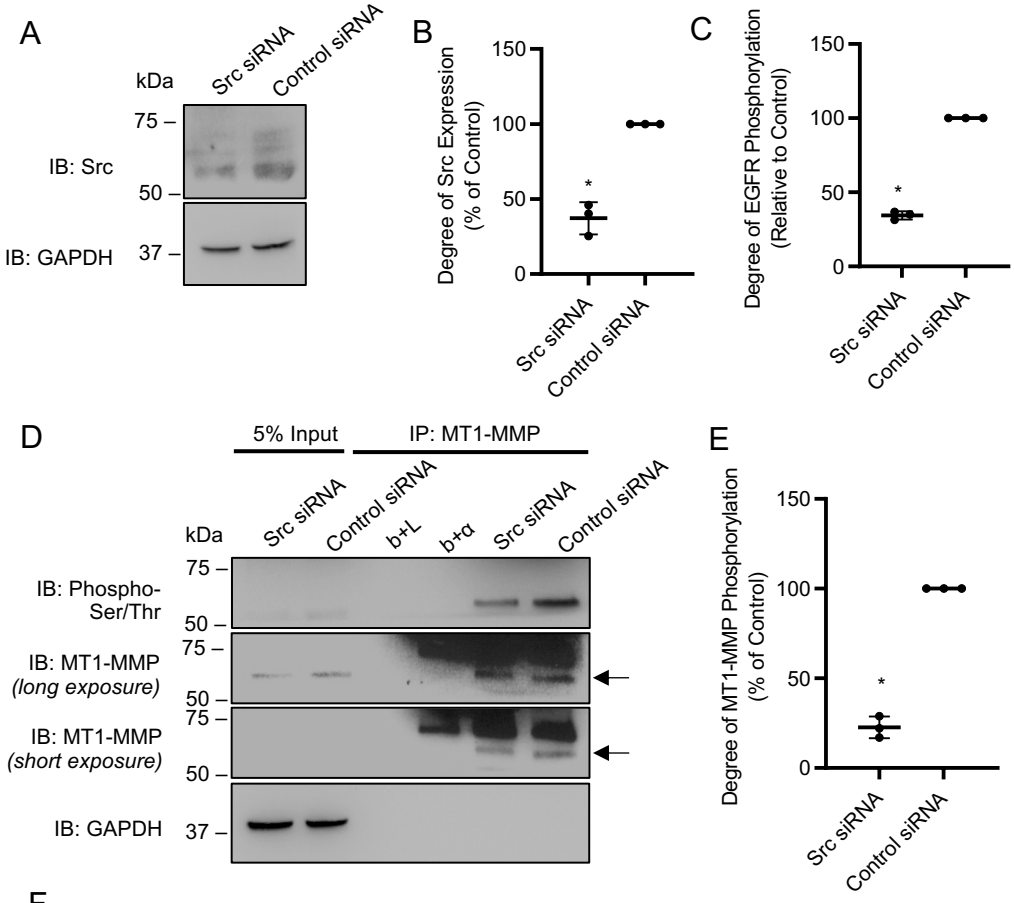

Figure S7. RNAi-mediated Knockdown of Src Kinase and FAK Decreases the Phosphorylation of MT1-MMP Downstream of β1 Integrin Activation. MDA-MB-231 cells were transiently transfected with either siRNA targeting Src, FAK, or nonspecific control siRNA for 24 hours, followed by incubation in serum-free media for 22 hours. *A*, Degree of Src knockdown assessed by Western blot. *B*, Densitometric analysis of the relative amount of Src expressed by cells as presented in *A*. *C*, Quantification of EGFR tyrosine phosphorylation following treatment with P4G11 for 2 hours as analyzed by Western Blot. *D*, Phosphorylation of MT1-MMP as analyzed by Western Blot in cells transiently transfected with Src siRNA or nonspecific control siRNA. Following incubation, cells were plated onto gelatin for 2 hours with simultaneous treatment with P4G11 prior to being lysed. Immunoprecipitates of MT1-MMP were analyzed for phospho-Ser/Thr, then stripped and re-probed for MT1-MMP by Western Blot. Arrow indicates the active form of MT1-MMP immunoprecipitated from the lysate. *E,* Quantification of the relative degree of MT1-MMP phosphorylation presented in *D F,* Phosphorylation of MT1-MMP as analyzed by Western Blot in cells transiently transfected with FAK siRNA or nonspecific control siRNA. Following incubation, cells were plated onto gelatin for 2 hours with simultaneous treatment with P4G11 prior to being lysed. Immunoprecipitates of MT1-MMP were analyzed for phospho-Ser/Thr, then stripped and re-probed for MT1-MMP by Western Blot. *b+L*, beads plus lysate; *b+α,* beads plus antibody. All data represent three or more biological replicates with at least three technical replicates.

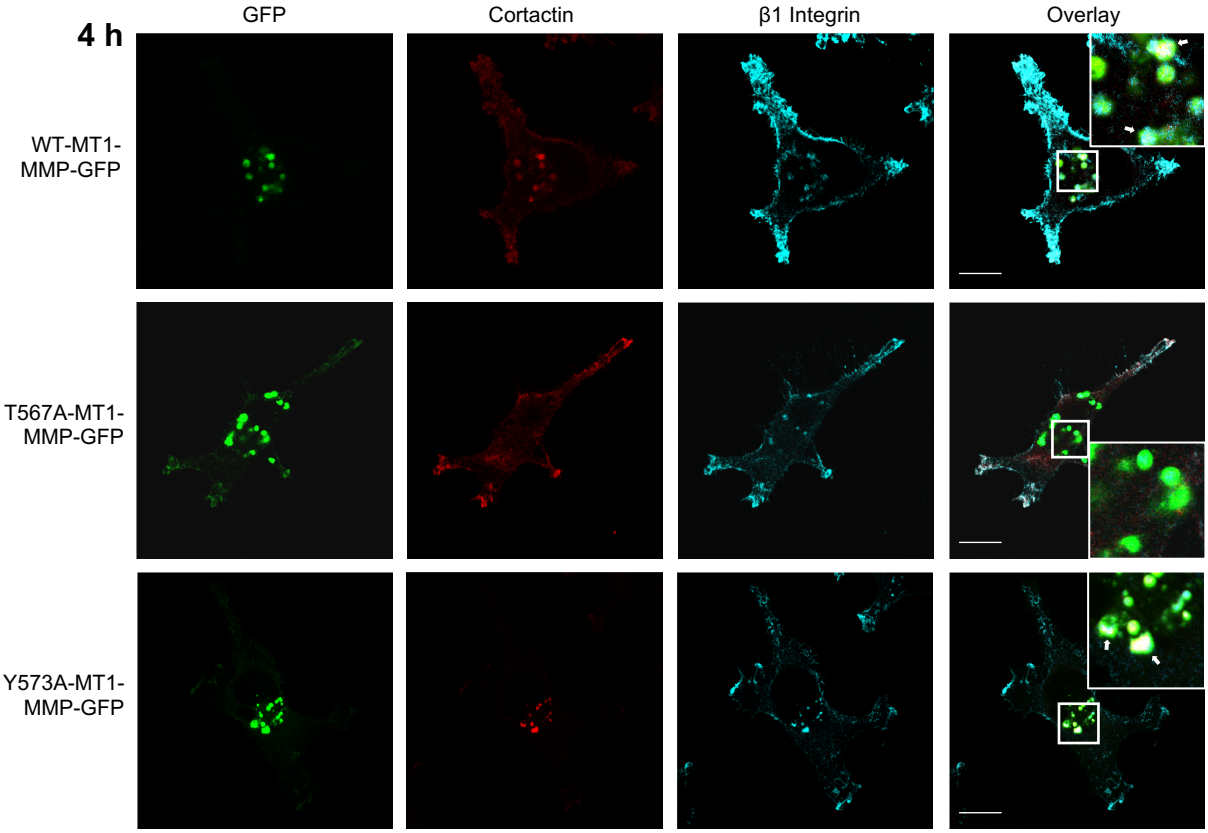

Figure S8. Cells Expressing an MT1-MMP Threonine-567 Mutant Demonstrate Decreased Association Between the GFP-tagged Construct, Cortactin, and β1 Integrin, and Decreased Invadopodium Formation. Cells transfected with WT-MT1-MMP-GFP, T567A-MT1-MMP-GFP, or Y573A-MT1-MMP-GFP were serum-starved and treated with P4G11 and plated onto gelatin-coated coverslips for 4 h. Cells were then fixed, permeabilized, and stained for cortactin and β1 integrin, and analyzed by confocal microscopy. White arrows in the zoomed region indicate overlap of all three proteins. All data represent three or more biological replicates with at least three technical replicates. Scale bar, 10 μm.