Sugiyama et al., http://www.jcb.org/cgi/content/full/jcb.201205176/DC1

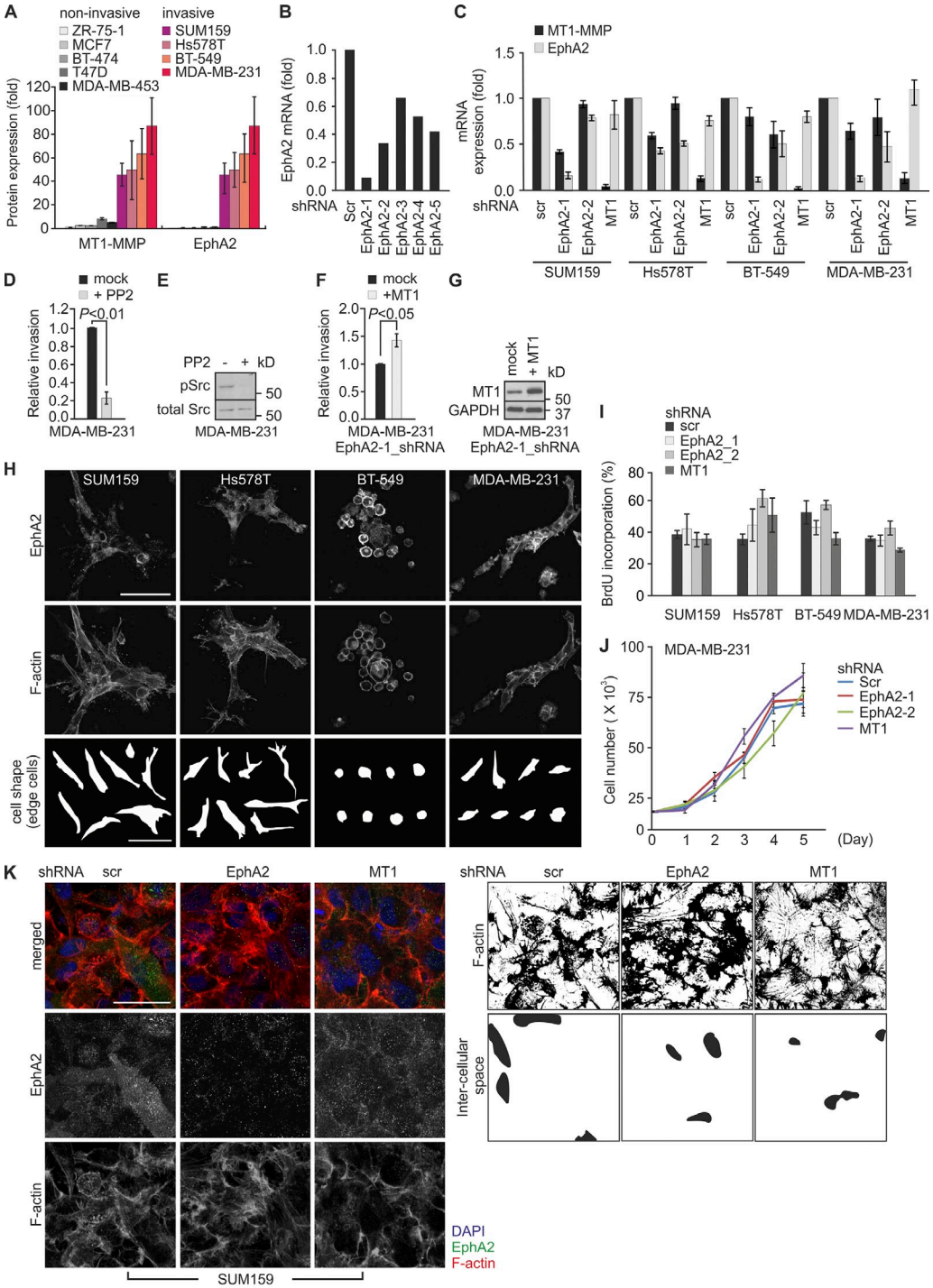


Figure S1. **EphA2 is a positive regulator of MT1-MMP–driven tumor cell invasion into collagen.** (A) Relative MT1-MMP and EphA2 protein expression was assessed by immunoblotting in nine human breast carcinoma cell lines (error bars indicate mean ± SEM, *n* = 3). (B) Relative EphA2 mRNA expression was assessed by qPCR after stable gene silencing by five lentiviral shRNAs against EphA2 (*n* = 2). (C) Relative expression of mRNAs for MT1-MMP and EphA2 were detected by qPCR after stable knockdown of EphA2 or MT1-MMP by lentiviral shRNAs in the four invasive cell lines as indicated (error bars indicate mean ± SEM; *n* = 3). (D) The Src kinase inhibitor PP2 reduces MDA-MB-231 cell invasion. The cells were incubated with the Src kinase inhibitor PP2 (5 µM) for 2 h, and relative invasion was quantified (error bars indicate mean ± SEM, *n* = 3). Relative invasion of the control cells was set to one. (E) Immunoblotting for phosphorylated (pSrc) and total Src after PP2 treatment (*n* = 3). (F) MT1-MMP overexpression rescues invasion of EphA2 knockdown MDA-MB-231 cells (error bars indicate mean ± SEM, *n* = 3). Relative invasion of the control cells was set to one. P-values were determined with a Mann–Whitney *U* test. (G) Immunoblotting for MT1-MMP in EphA2 knockdown cells (*n* = 3). GAPDH served as a loading control. (H) Single-channel images from merged images in Fig. 2 A. Representative morphologies of cells located at the edge of colonies of the indicated cell lines are shown in the bottom row as white shapes. (I and J) Stable knockdown of MT1-MMP and EphA2 did not affect cell growth in monolayer. Cell proliferation was assessed by BrdU incorporation (I) and by generating a cell growth curve (J; mean ± SEM; *n* = 3). (K, left) Representative confocal micrographs show EphA2 and filamentous actin (phalloidin) in control, EphA2, or MT1-MMP knockdown SUM159 cells. (K, right) Actin cytoskeleton (top) and intercellular spaces (bottom) are visualized as black-and-white images. Bars, 50 µm.

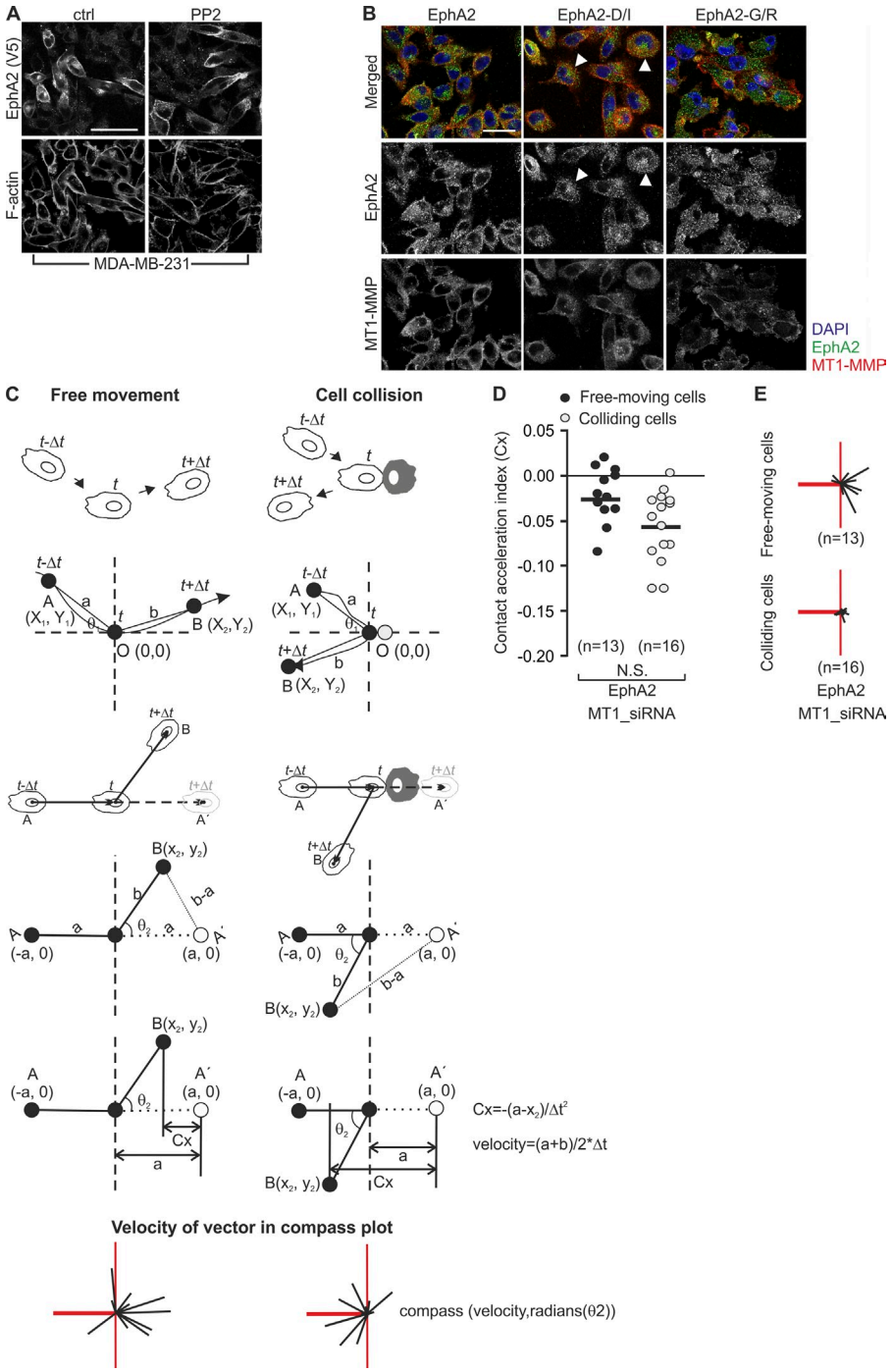


Figure S2. **Repulsive cell movement is quantified by comparing the contact acceleration index (Cx) of free-moving cells and colliding cells.** (A) Single-channel images from merged images in Fig. 3 F. MDA-MB-231 cells were incubated with 5 μM PP2 for 2 h. Bar, 50 μm. (B) Representative confocal micrographs show EphA2 and MT1-MMP in MDA-MB-231 cells stably overexpressing EphA2, EphA2-D/I, and EphA2-G/R. Arrowheads indicate prominent intracellular EphA2. Bar, 50 μm. (C) Analysis of the motile cell behavior was performed to verify how the motion of a cell changes as a result of a collision compared with the spontaneous changes of free-moving cells as previously described (Paddock and Dunn, 1986). The path of colliding cells is tracked, by ImageJ manual Track PlugIn, at time point $t - \Delta t$ (position A), t (contact point), and $t + \Delta t$ (position B). Free-moving cells are also tracked for the same time period. The path obtained is then plotted within a Cartesian plane with its origin O (0,0), coincident with the contact point, the position A and position B having coordinates (X_1,Y_1) and (X_2,Y_2), respectively. The displacements of a cell during the interval from $t - \Delta t$ to t and from t to $t + \Delta t$ are represented as vectors "a" ($\overline{AO}$) and "b" ($\overline{OB}$) , respectively. By concomitantly rotating vectors *a* and *b* anticlockwise through an angle θ_1 about the origin, as shown in the figure, the displacement of each cell before collision is set as *a* vector with known components (−a,0). The vector *b-a* represents the change in motion of the cell and the component Cx of vector *b-a* represents the difference between how far the cell has progressed in the direction of A′ and how far it would have gone had there been no collision. Given the fact that each cell moves with a certain speed, its change in direction can be defined as "contact acceleration index (Cx)" and it is calculated as $Cx = -(a - x_2)/\Delta t^2$. The component Cx of the vector *b-a* is obtained as a negative value because it lies in a direction opposite to that of A′, indicating that a more negative Cx value implies a greater response of contact-mediated repulsion. The statistical significance between the mean Cx values of free-moving cells and colliding cells is validated using a Mann–Whitney *U* test. Furthermore, the mean velocity over the double interval ($t - \Delta t$, $t + \Delta t$) is obtained simply from the vector sum $v = (a + b)/2 \times \Delta t$, and plotted on a compass plot together with the moving direction of vector B using MATLAB software. (D) Contact acceleration indices (Cx) of free-moving cells and colliding cells (see C). P-values were determined with a Mann–Whitney *U* test. (E) Representation of velocity vectors of free-moving cells and colliding cells in compass plots. The heavy red line represents the scaled displacement of all cells before collision and the thin black lines show the scaled displacements of each cell after collision. Thin red line is a reference line that marks the angle of 90° relative to the displacement before contact (heavy red line).

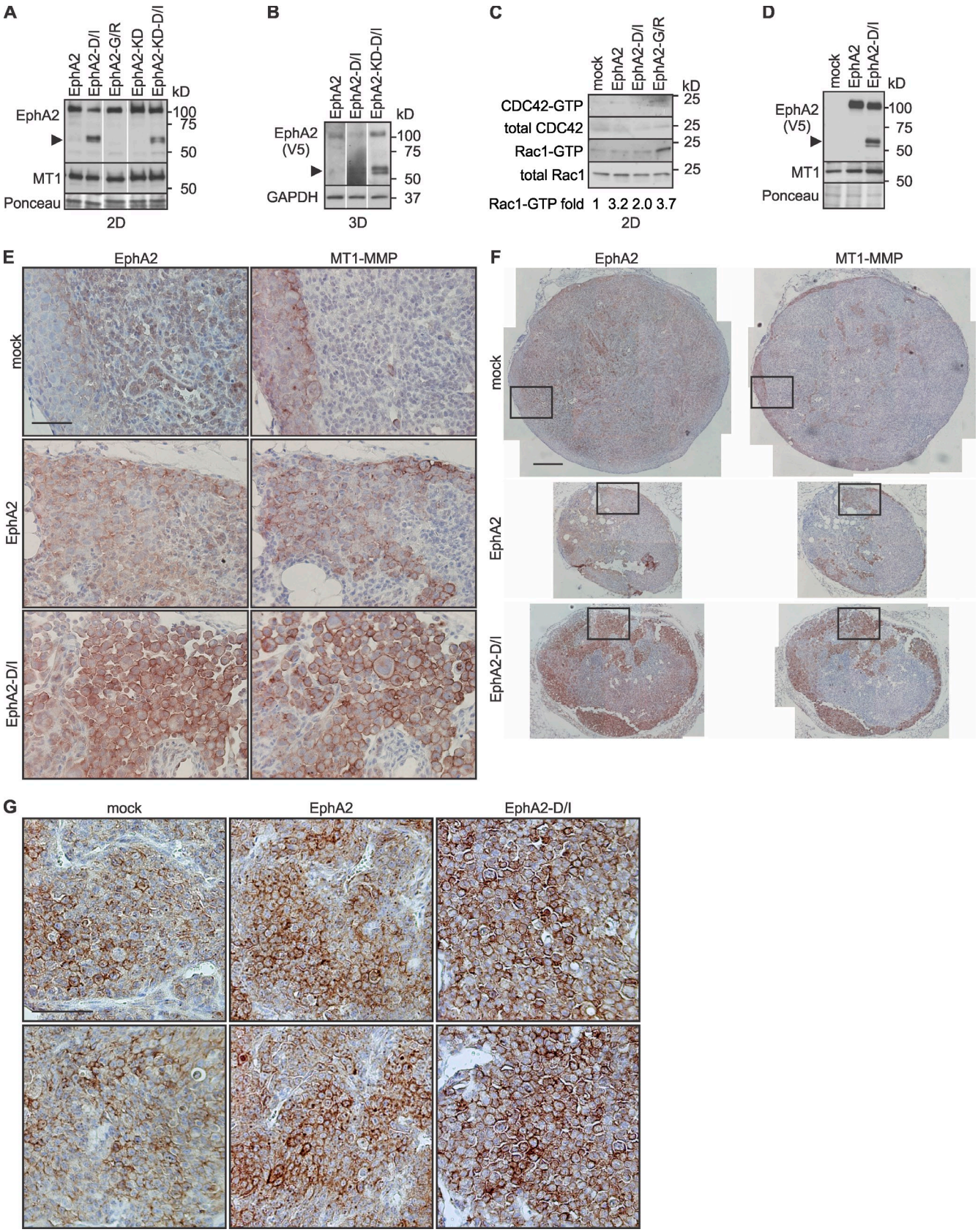


Figure S3. **EphA2 cleavage promotes single-cell invasion in MDA-MB-231 cells in vitro and in vivo.** (A) Control cells and EphA2-, EphA2-D/I–, EphA2-G/R–, EphA2-KD–, and EphA2-KD-D/I–expressing MDA-MB-231 cells were subjected to immunoblotting as indicated (n = 3). (B) The EphA2 processing also occurs within a 3D collagen matrix. EphA2-, EphA2-D/I–, and EphA2-KD-D/I–expressing cells were subjected to immunoblotting as indicated (n = 2). (C) Soluble lysates of EphA2 or the mutant protein–expressing cells were incubated with PAK-1 PDB-conjugated agarose beads followed by immunoblotting as indicated (n = 3). Mean values of Rac1-GTP normalized with total Rac1 are indicated below each blot. (D) Control, EphA2, and EphA2-D/I stably overexpressing cells were subjected to immunoblotting as indicated (n = 3). Arrowheads indicate an ~60-kD EphA2 fragment. Ponceau red and GAPDH staining served as loading controls. (E and F) Representative immunohistochemistry of metastatic tumor cell colonies in lymph nodes. Boxed regions (F) indicate the areas magnified in E. (G) Example images of representative light micrographs of MT1-MMP immunohistochemistry of mouse primary tumors used for intercellular space quantification. Bars: (E) 50 μm; (F and G) 200 μm.

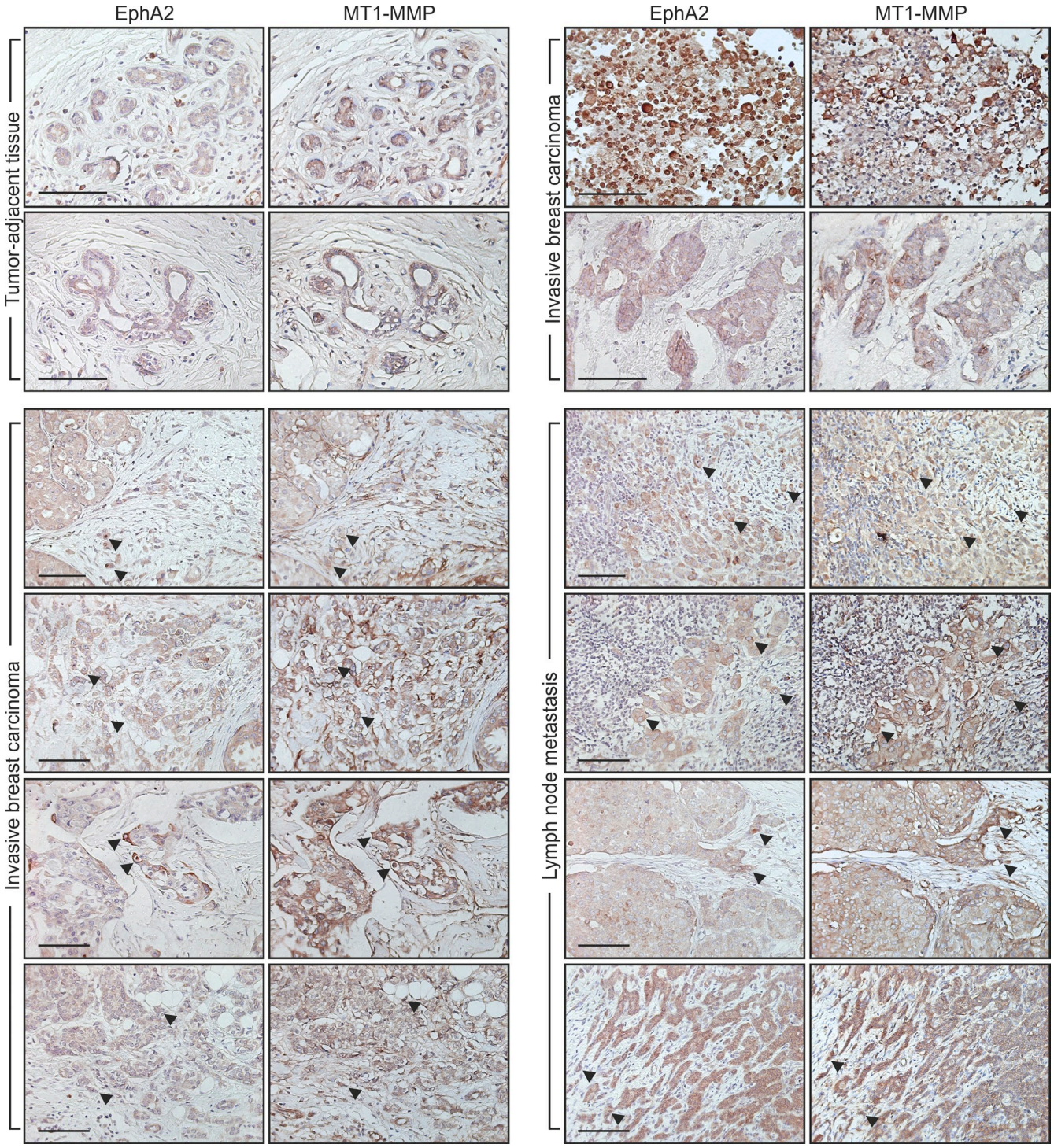
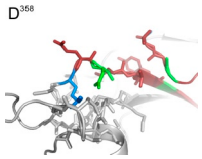
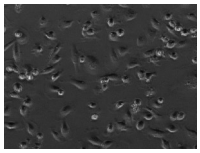


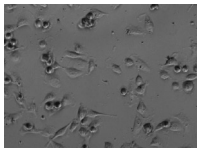
Figure S4. **EphA2 and MT1-MMP are coexpressed in invasive breast carcinoma cells.** EphA2 and MT1-MMP immunohistochemistry of pairs of tumor-adjacent tissue and invasive breast carcinoma, and invasive breast carcinomas and corresponding lymph node metastases from the same patient. Arrowheads indicate intracellular EphA2 in highly MT1-MMP–expressing carcinoma cells. Bars, 100 µm.



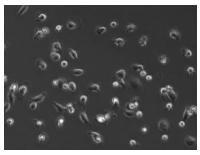
Video 1. **Animation visualizing sequences of all possible rotamers of EphA2-D/I and -G/R mutant proteins.** The rotamers at the mutation point are ordered according to their frequencies of occurrence in proteins. The cleavage area is shown in red, R^{537} is shown in blue, and D^{359} and G^{391} are shown in green. The altered amino acids at the mutation points, I^{359} or R^{391}, are shown in dark blue. The frequency of each rotamer is also indicated. Short green lines and disks indicate atoms in contact or slightly overlapping, and large red disks indicate significant van der Waals overlap.



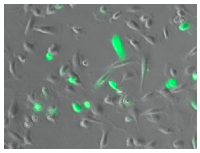
Video 2. **Time-lapse video of control MDA-MB-231 cell migration shown in Fig. 5 A.** The cells were transduced with scrambled control shRNA-containing lentiviral particles. Cell movement was recorded by time-lapse microscopy using a Stallion HIS microscope (Carl Zeiss). Frames taken every 5 min for 4 h are displayed at 1 fps.



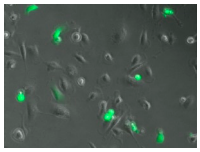
Video 3. **Time-lapse video of EphA2 knockdown MDA-MB-231 cell migration, shown in Fig. 5 A.** The cells were transduced with EphA2 shRNA-containing lentiviral particles. Cell movement was recorded by time-lapse microscopy using a Stallion HIS microscope (Carl Zeiss). Frames taken every 5 min for 4 h are displayed at 1 fps.



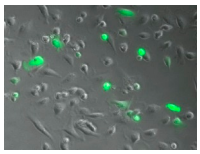
Video 4. **Time-lapse video of MT1-MMP knockdown MDA-MB-231 cell migration, shown in Fig. 5 A.** The cells were transduced with MT1-MMP shRNA-containing lentiviral particles. Cell movement was recorded by time-lapse microscopy using a Stallion HIS microscope (Carl Zeiss). Frames taken every 5 min for 4 h are displayed at 1 fps.



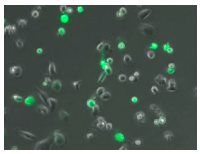
Video 5. **Time-lapse video of control GFP expressing MDA-MB-231 cell contacts and migration, shown in Fig. 5, C–F.** The cells were transfected with pcDNA3.1 and pEGFP-N1 control vectors. Movement of the EGFP-expressing (green) cells was recorded by time-lapse microscopy using a Stallion HIS microscope with (Carl Zeiss). Frames taken every 5 min for 5 h are displayed at 1 fps.



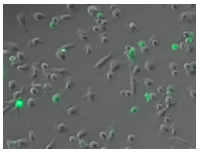
Video 6. **Time-lapse video of EphA2- and GFP-coexpressing MDA-MB-231 cell contacts and migration, shown in Fig. 5, C–F.** The cells were transfected with vectors encoding EphA2 cDNA and pEGFP-N1. Movement of the EGFP-expressing (green) cells was recorded by time-lapse microscopy using a Stallion HIS microscope (Carl Zeiss). Frames taken every 5 min for 5 h are displayed at 1 fps.



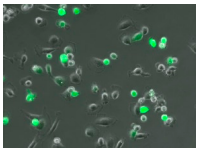
Video 7. **Time-lapse video of EphA2-D/I– and GFP-coexpressing MDA-MB-231 cell contacts and migration, shown in Fig. 5, C–F.** The cells were transfected with vectors encoding EphA2-D/I cDNA and pEGFP-N1. Movement of the EGFP-expressing (green) cells was recorded by time-lapse microscopy using a Stallion HIS microscope (Carl Zeiss). Frames taken every 5 min for 5 h are displayed at 1 fps.



Video 8. **Time-lapse video of a GFP-expressing MT1-MMP knockdown MDA-MB-231 cell expressing control contacts and migration, shown in Fig. 5 C.** Stable MT1-MMP knockdown MDA-MB-231 cells were transfected with pcDNA3.1 and pEGFP-N1 control vectors. Movement of the EGFP-expressing (green) cells was recorded by time-lapse microscopy using a Stallion HIS microscope (Carl Zeiss). Frames taken every 5 min for 5 h are displayed at 1 fps.



Video 9. **Time-lapse video of EphA2 and GFP-coexpressing MT1-MMP knockdown MDA-MB-231 cell contacts and migration, shown in Fig. 5 C and Fig. S2, D and E.** Stable MT1-MMP knockdown MDA-MB-231 cells were transfected with vectors encoding EphA2 cDNA and pEGFP-N1. Movement of the EGFP-expressing (green) cells was recorded by time-lapse microscopy using a Stallion HIS microscope (Carl Zeiss). Frames taken every 5 min for 5 h are displayed at 1 fps.



Video 10. **Time-lapse video of EphA2-D/I and GFP-expressing MT1-MMP knockdown MDA-MB-231 cell contacts and migration, shown in Fig. 5 (C–E) and Fig. S2 (D–E).** Stable MT1-MMP knockdown MDA-MB-231 cells were transfected with vectors encoding EphA2-D/I cDNA and pEGFP-N1. Movement of the EGFP-expressing (green) cells was recorded by time-lapse microscopy using a Stallion HIS microscope (Carl Zeiss). Frames taken every 5 min for 5 h are displayed at 1 fps.

Reference

Paddock, S.W., and G.A. Dunn. 1986. Analysing collisions between fibroblasts and fibrosarcoma cells: fibrosarcoma cells show an active invasionary response. *J. Cell Sci.* 81:163–187.

Tables S1 and S2 show Mascot search engine output for EphA2 fragments 1 and 2, respectively. Both tables are available for download as Excel files.