JCB

Supplemental material

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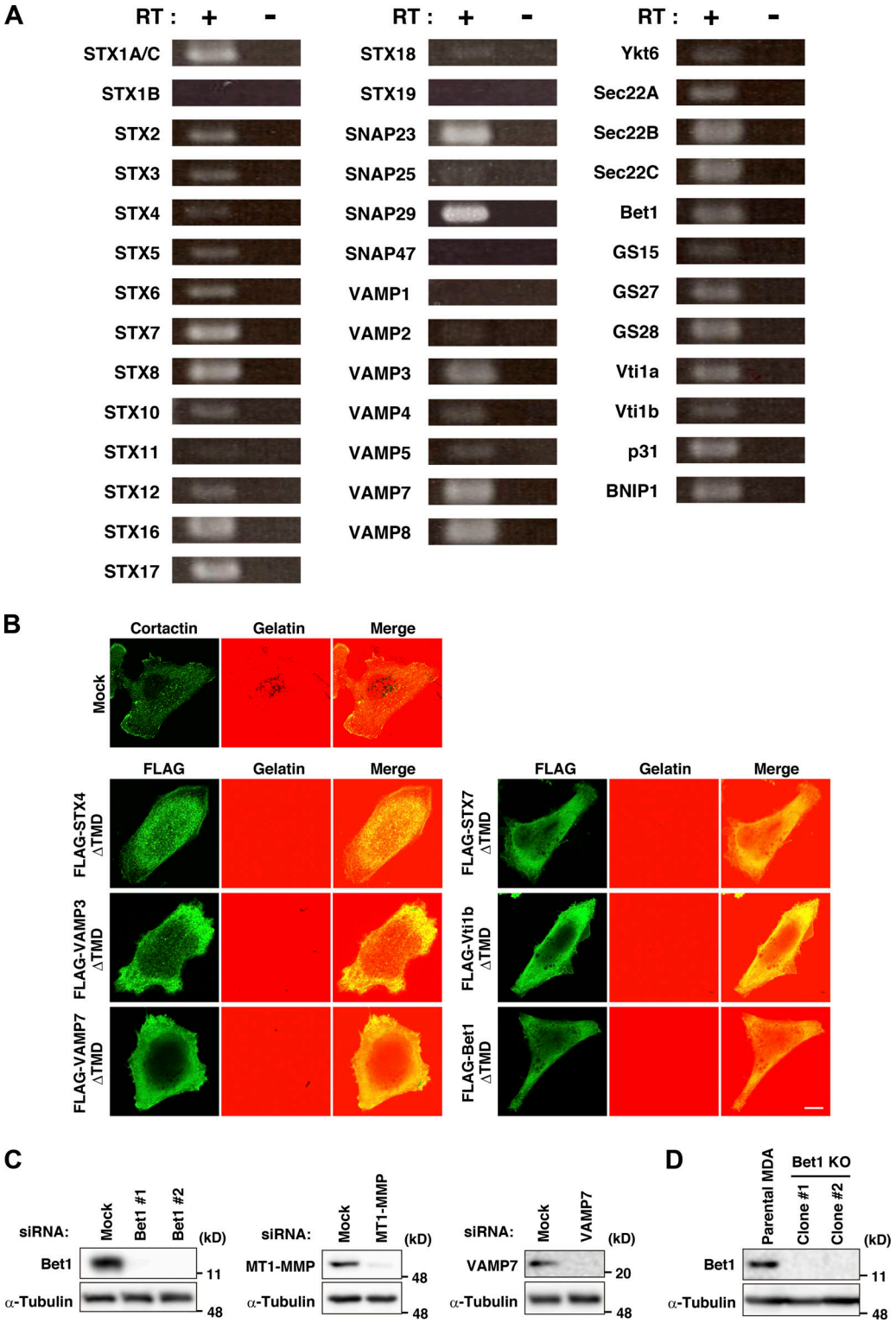


Figure S1. **Expression and screening of SNARE proteins that are involved in ECM degradation in MDA-MB-231 cells. (A)** 35 SNAREs are expressed in MDA-MB-231 cells. Total RNA was prepared from MDA-MB-231 cells, and RT-PCR was performed for cDNAs encoding 39 SNAREs, i.e., all SNARE genes identified in the human genome. **(B)** Representative pictures of gelatin degradation. MDA-MB-231 cells transiently expressing FLAG-tagged, TMD-deleted SNAREs were cultured on TRITC-gelatin for 7 h, fixed, and stained for FLAG or cortactin, and then the gelatin degradation was visualized by confocal microscopy. **(C)** siRNA-mediated knockdown of Bet1, MT1-MMP, and VAMP7. MDA-MB-231 cells transfected with the indicated siRNAs were lysed, and the indicated proteins were detected by immunoblotting with specific antibodies. α-tubulin was analyzed as a loading control. **(D)** CRISPR-mediated KO of Bet1. MDA-MB-231 cells in which the *Bet1* gene was disrupted were lysed, and Bet1 and α-tubulin were detected by immunoblotting. Scale bar: 10 µm.

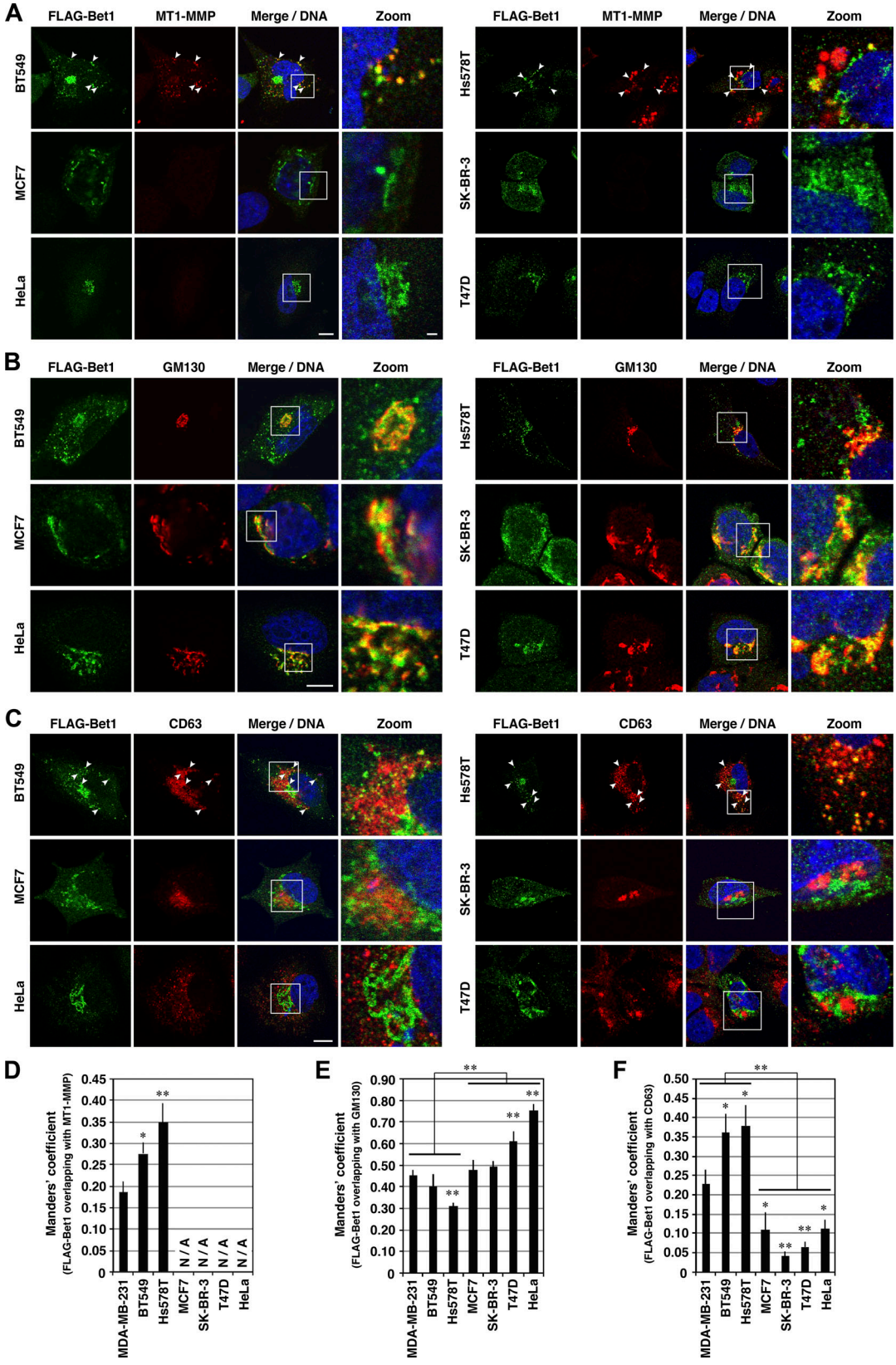


Figure S2. **Bet1 is localized in MT1-MMP–positive endosomes in invasive breast cancer cell lines BT549 and Hs578T, but not in noninvasive breast cancer cell lines MCF7, SK-BR-3, and T47D, or HeLa cells. (A–C)** The indicated cell lines transfected with FLAG-Bet1 were coimmunostained for FLAG and MT1-MMP (A), GM130 (B), or CD63 (C) and then visualized by confocal microscopy. Arrowheads indicate the colocalization. The areas enclosed by white squares are enlarged in the zoom panels. **(D–F)** Quantitation of colocalization between FLAG-Bet1 and MT1-MMP (D), GM130 (E), or CD63 (F) evaluated with Manders' overlap coefficient. Scale bar: 10 µm in a regular image; 2 µm in an inset. *, P < 0.05; **, P < 0.01; vs. MDA-MB-231.

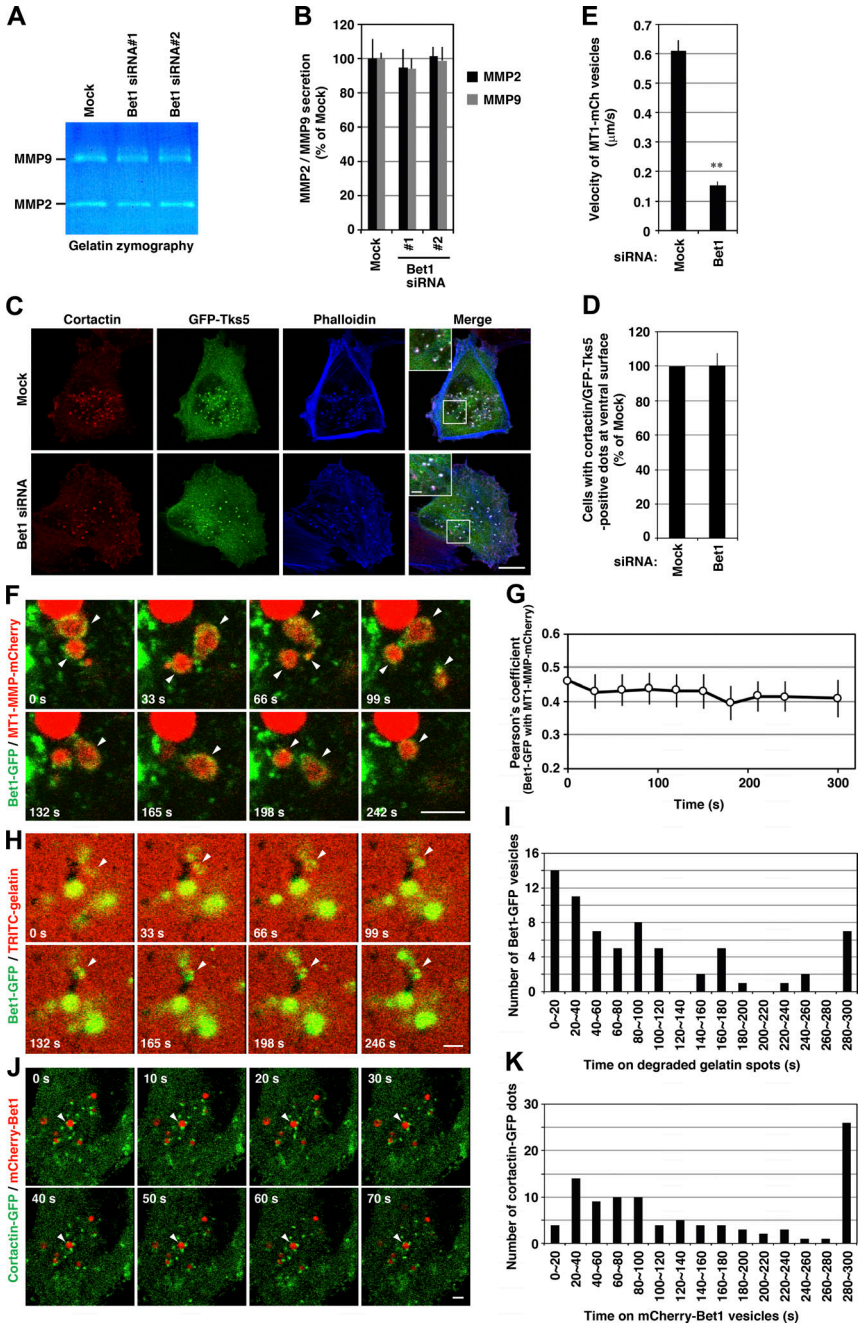


Figure S3. **Depletion of Bet1 does not affect the secretion of MMP2 and MMP9, but Bet1-positive endosomes are associated with gelatin-degraded areas and cortactin-positive dots in live cells. (A)** Gelatin zymography. MDA-MB-231 cells transfected with the indicated siRNAs were cultured for 48 h in serum-free OptiMEM. The conditioned media were separated by SDS-PAGE using 1 mg/ml gelatin-containing gel. The separated MMP2 and MMP9 in the conditioned media were renaturated and allowed to digest gelatin in the gel, and then the gel was stained with Coomassie Brilliant Blue. **(B)** Quantitation of gelatin digestion by MMP2 and MMP9. **(C)** Bet1 depletion does not affect Tks5-, F-actin-, and cortactin-positive invadopodial structures at the ventral cell surface. MDA-MB-231 cells stably expressing GFP-Tks5 were transfected with mock or Bet1 siRNA for 72 h, fixed, stained, and then visualized by confocal microscopy. **(D)** Quantitation of the ratio of the cells with GFP-Tks5– and cortactin-positive dots at the ventral cell surface as in C. **(E)** Quantitation of migration velocity of MT1-MMP-mCherry–positive vesicles as in Fig. 3, I and J. **(F)** Bet1 is localized in MT1-MMP–positive endosomes in live cells. Bet1-GFP was transiently expressed in MDA-MT1-mCh cells, and then time-lapse images of Bet1-GFP and MT1-MMP-mCherry were captured by confocal microscopy. Arrowheads indicate endosomes in which Bet1-GFP and MT1-MMP-mCherry were colocalized. **(G)** Evaluation of colocalization between Bet1-GFP and MT1-MMP-mCherry by using Pearson's correlation coefficient. **(H)** Bet1-positive endosomes are associated with gelatin degradation areas. MDA-MB-231 cells transiently expressing Bet1-GFP were cultured on TRITC-gelatin for 7 h, and then time-lapse images of them were captured. Arrowheads indicate Bet1-GFP–positive endosomes associated with gelatin degradation. **(I)** A histogram of the number and duration of Bet1-GFP–positive vesicles associated with gelatin degradation areas as in H. **(J)** mCherry-Bet1–positive endosomes are accompanied by cortactin-GFP–positive dots. mCherry-Bet1 and cortactin-GFP were transiently expressed in MDA-MB-231 cells, and then time-lapse images of them were captured. Arrowheads indicate mCherry-Bet1 endosomes associated with cortactin dots. **(K)** A histogram of the number and duration of cortactin-GFP–positive dots associated with mCherry-Bet1 endosomes as in J. Scale bar: 10 μm and 2 μm in the regular image and the inset, respectively, in C; 2 μm in F, H, and J. **, $P < 0.01$; vs. mock in E.

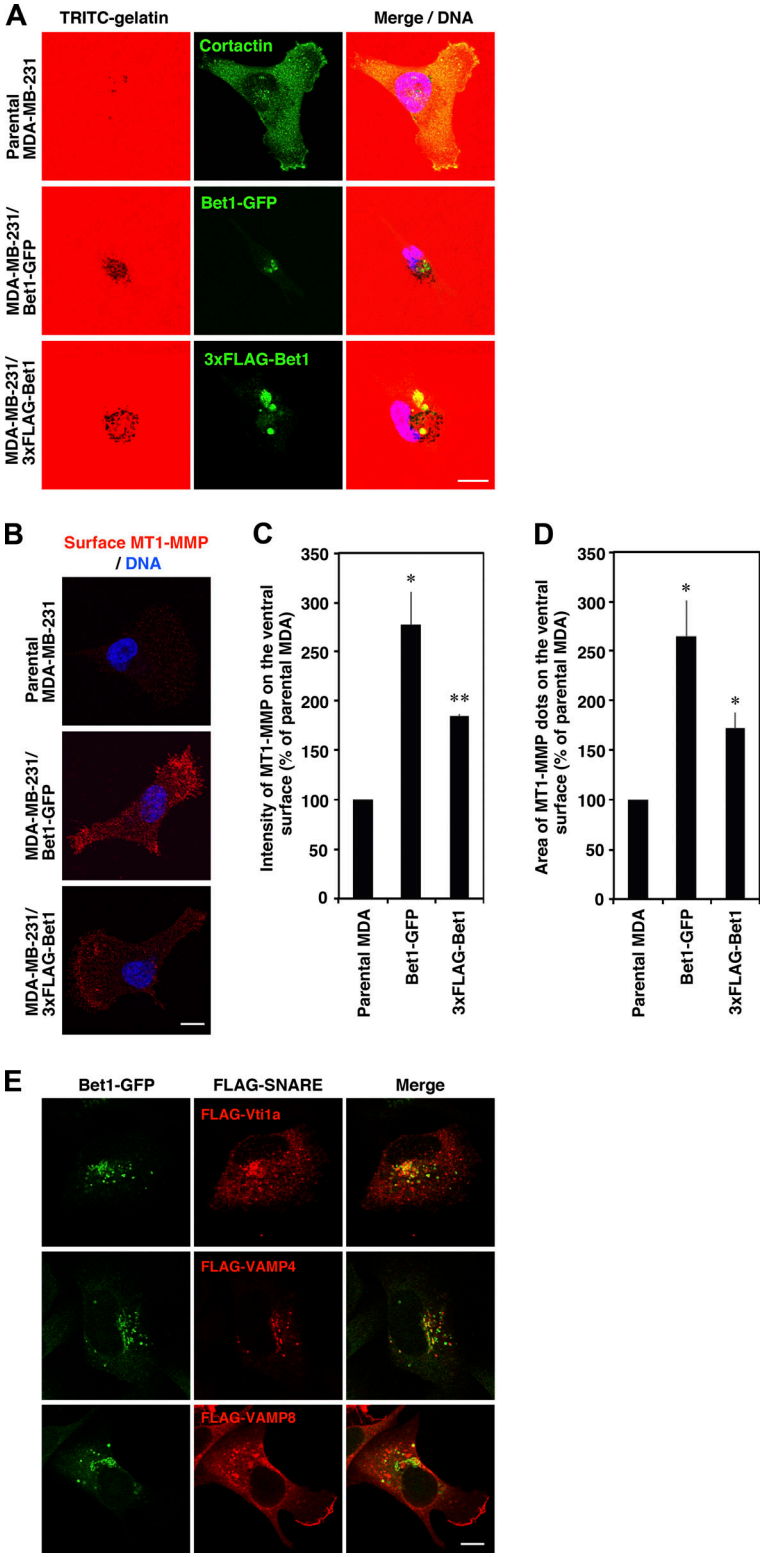


Figure S4. **Overexpression of Bet1 increases gelatin degradation and cell surface MT1-MMP, and Bet1 is colocalized with endosomal SNAREs. (A)** Representative pictures of gelatin degradation. Parental MDA-MB-231 cells and stable cells expressing Bet1-GFP or 3xFLAG-Bet1 (MDA/Bet1-GFP and MDA/3xFLAG-Bet1 cells) were cultured on TRITC-gelatin for 7 h, fixed, and stained for FLAG or cortactin, and then the gelatin degradation was visualized by confocal microscopy. **(B)** Representative pictures of MT1-MMP localized on the ventral cell surface. Cells spread on a gelatin-coated coverslip were incubated with an antibody to the extracellular domain of MT1-MMP on ice without permeabilization, fixed, and then analyzed by confocal microscopy. **(C and D)** Quantitation of surface MT1-MMP shown in B. MT1-MMP on the ventral cell surface was quantified as to intensity (C) and area (D). **(E)** Bet1-GFP is colocalized with FLAG-Vti1a, VAMP4, and VAMP8. MDA-MB-231 cells stably expressing Bet1-GFP were transiently transfected with the indicated FLAG-SNAREs, immunostained, and analyzed by confocal microscopy. Scale bar: 20 µm. *, $P < 0.05$; **, $P < 0.01$; vs. parental MDA.

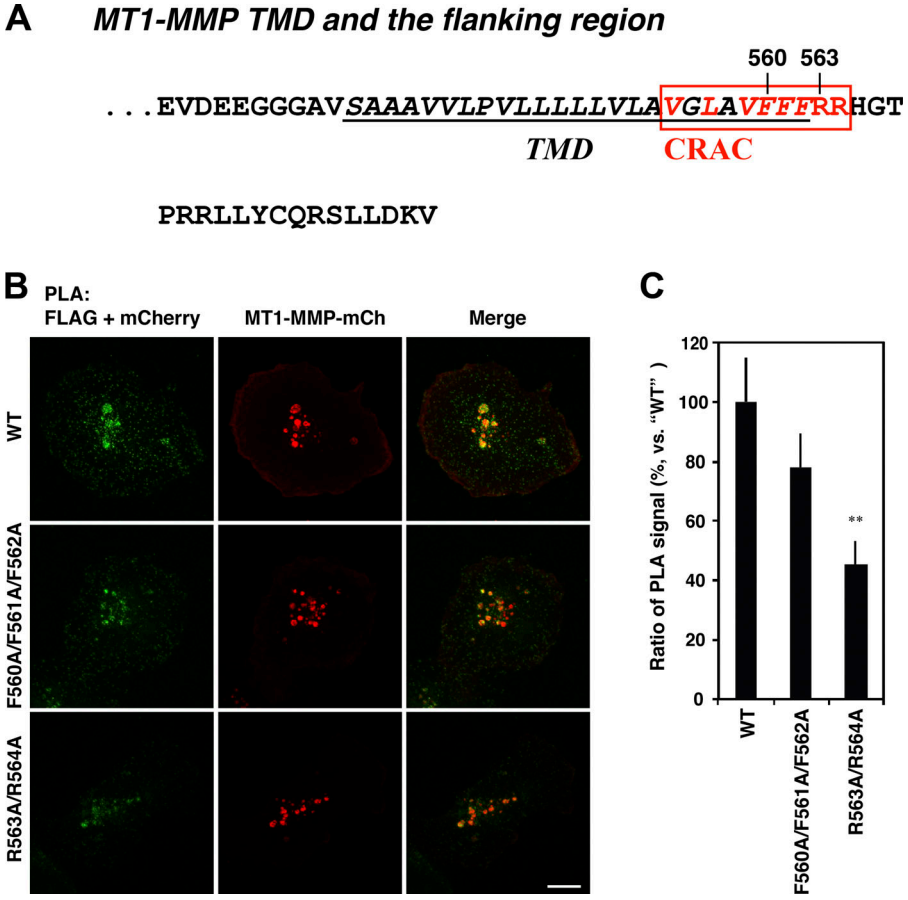
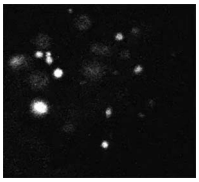
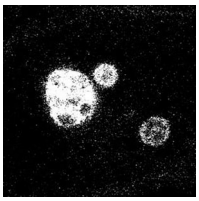


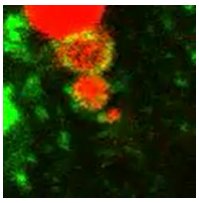
Figure S5. **Mutations in CRAC motif of MT1-MMP reduces the PLA signal between MT1-MMP and Bet1. (A)** Putative CRAC motif in MT1-MMP. **(B)** A mutant of MT1-MMP with R563A/R564A was in less proximity to 3xFLAG-Bet1. MDA-MB-231 cells stably expressing 3xFLAG-Bet1 were transfected with WT or mutant MT1-MMP-mCherry, stained, and analyzed by confocal microscopy. **(C)** Quantitation of PLA signals between MT1-MMP-mCherry and 3xFLAG-Bet1. Scale bar: 10 µm. **, P < 0.01; vs. WT..



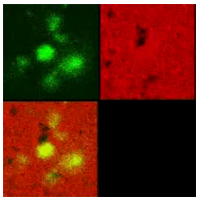
Video 1. **Time-lapse imaging of MT1-MMP-mCherry in mock-transfected MDA-MT1-mCh cells.** The images were captured every second and played back at 3 frames per second (fps).



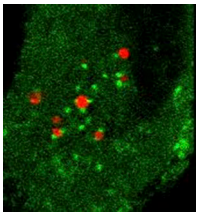
Video 2. **Time-lapse imaging of MT1-MMP-mCherry in Bet1 siRNA-transfected MDA-MT1-mCh cells.** The images were captured every second and played back at 3 fps.



Video 3. **Time-lapse imaging of Bet1-GFP and MT1-MMP-mCherry in MDA-MT1-mCh cells.** The images were captured every second and played back at 30 fps.



Video 4. **Time-lapse imaging of Bet1-GFP in MDA-MB-231 cells on TRITC-gelatin.** The images were captured every second and played back at 30 fps.



Video 5. **Time-lapse imaging of cortactin-GFP and mCherry-Bet1 in MDA-MB-231 cells.** The images were captured every second and played back at 10 fps.