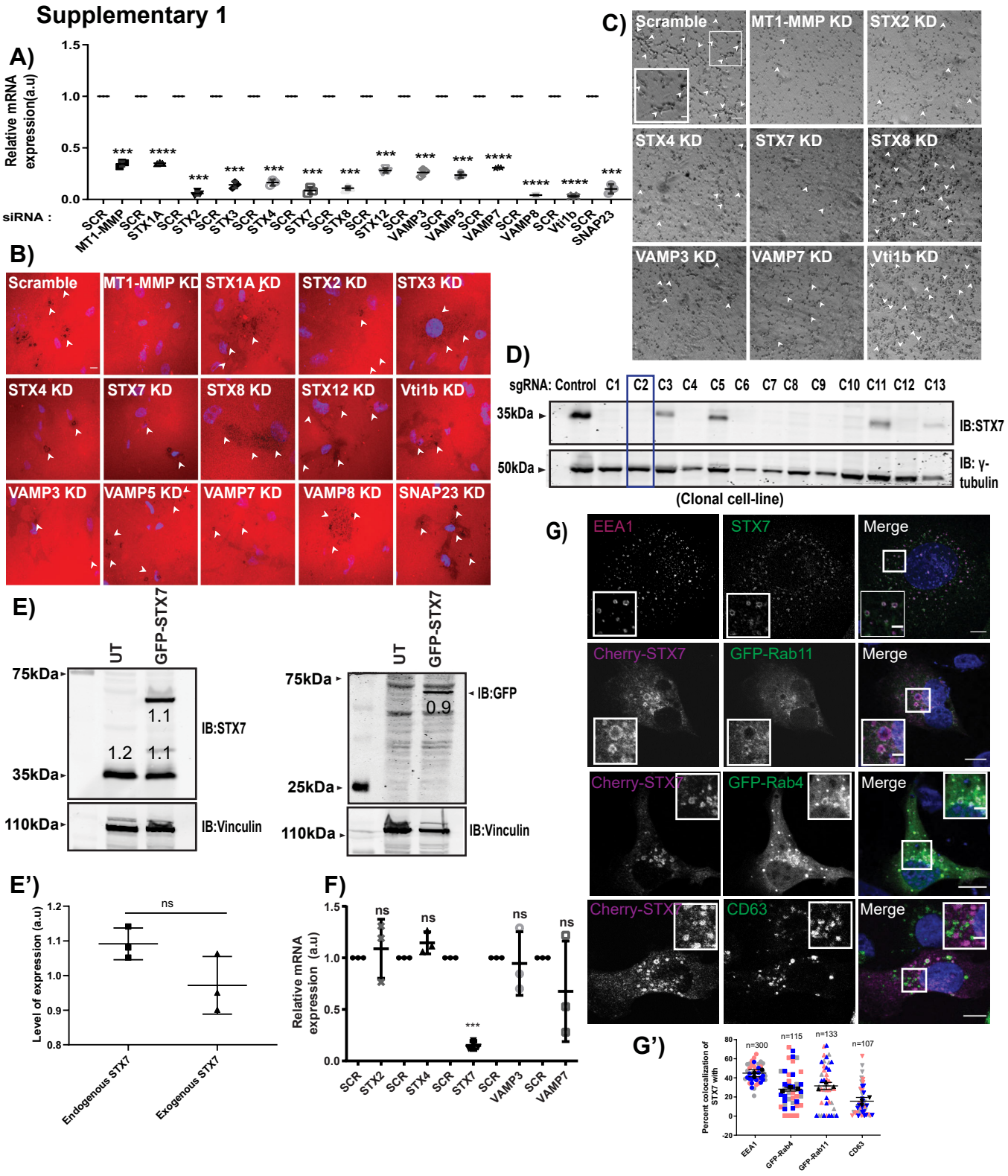


Fig. S1. Screening of SNAREs of endocytic and recycling pathway. A) Indicated SNAREs were depleted using siRNA in MDA-MB-231. The efficacy of gene suppression was quantified using qPCR. (N=3). Two-tailed Student's t test, *** P < 0.001, **** P < 0.0001. B) Indicated molecules were depleted using siRNA in MDA-MB-231. Post-transfection cells were seeded on Alexa Fluor 568 labeled gelatin-coated coverslip for 12h. Arrowhead indicates the degradation spot. (N=3, n=300 cells, Scale bar 10 μm). C) Indicated molecules were depleted using siRNA in MDA-MB-231. Post-transfection, cells were seeded on Matrigel-coated cell inserts for 20h. Arrowhead indicates the cells that invaded through the Matrigel. (N=3, n=50,000 cells, Scale bar 10 μm). D) Different clones of MDA-MB-231 STX7KO clonal cells (STX7KO) were obtained after cells were transfected with Cas9 protein complexes with either control sgRNA or sgRNA-targeting STX7. The knockout efficiency was determined by immunoblotting using anti- STX7 and anti-γ-Tubulin (loading control).The boxed region indicates the clone used in the study. E and E') UT and GFP-STX7 transfected MDA-MB-231 cells were lysed and proceed for immunoblotting using anti-STX7, anti-vinculin and anti-GFP. Anti-Vinculin was used as a loading control. The level of expression of GFP-STX7 (exogenous) compared to endogenous STX7 was quantified and plotted. Two-tailed Student's t-test, P >0.05-ns (non-significant). F) STX7 was depleted using siRNA in MDA-MB-231, and the relative expression of indicated SNARE molecules were checked via qPCR (N=3). Two-tailed Student's t test, *** P < 0.001, ns-non significant. G and G') MDA-MB-231 cells were immunostained with EEA1/STX7, co-expressed with Cherry-STX7 and GFP-Rab11 or GFP-Rab4 and Cherry-STX7/CD63. Inset represents the zoomed-in view of the boxed region. Percentage co-localization of STX7 with respective compartments was quantified and plotted (N=3, Scale bar 10 μm, inset 3 μm). The data are displayed using SuperPlots, each biological replicate is distinctly color-coded and each dot represents identified percentage colocalization in a field of view (frame), described in the Material and methods. All the graphs represent means ± SD. N- number of experimental repeats, n- number of cells analyzed.

Supplementary 2

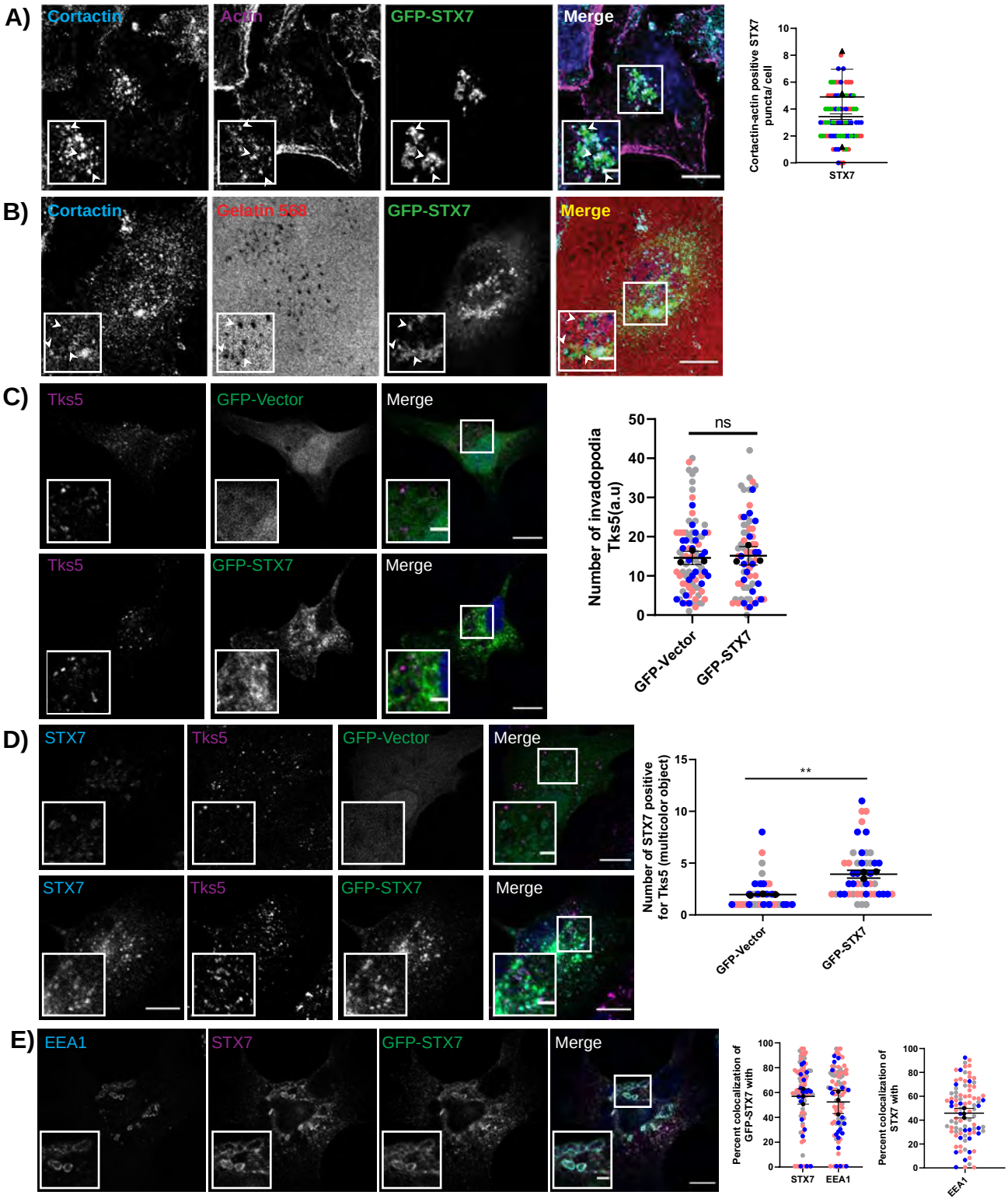


Fig. S2. STX7 is invadopodia associated. A) GFP-STX7 transfected MDA-MB-231 cells were plated over gelatin-coated coverslip and immunostained with cortactin followed by labeling with Phalloidin-568 (F-actin) and DAPI. Inset represents zoomed in view of the boxed region. Arrowhead indicates STX7-Cortactin-actin positive punctae. Graph shows STX7 punctae positive for Cortactin and actin per cell. (N=3, n=77, Scale bar 10µm, Inset 3 µm). B) GFP-STX7 transfected MDA-MB-231 cells were plated over Alexa fluor 568 labeled gelatin-coated coverslip for 12h, followed by immuno-staining with cortactin. DAPI was used to label the nucleus. Inset represents zoomed-in view of the boxed region. Arrowhead indicates STX7 localization near degradation spot (N=2, n=40. Scale bar 10µm, Inset 3 µm). C) GFP-Vector and GFP-STX7 transfected MDA-MB-231 cells were seeded on the unlabelled gelatin-coated coverslip, immunostained with Tks5 followed by labelling with DAPI. Inset represents zoomed in view of the boxed region. Number of Tks5 punctae were counted and plotted. {N=3, n=84(GFP-Vector), n=70(GFP-STX7), Scale bar 10µm, Inset 3 µm}. Two-tailed Student's t-test, P >0.05- ns (non-significant). D) GFP-Vector and GFP-STX7 transfected MDA-MB-231 cells were seeded on the unlabelled gelatin-coated coverslip, immunostained with Tks5, STX7 followed by DAPI staining. Inset represents zoomed in view of the boxed region. Number of STX7 and Tks5 punctae were counted and plotted. {N=3, n=42(GFP-Vector), n=47(GFP-STX7), Scale bar 10µm, Inset 3 µm}. Two-tailed Student's t-test, ** P < 0.01. E) GFP-STX7 transfected MDA-MB-231 cells were immunostained using STX7 and EEA1. Inset represents zoomed in view of the boxed region. Graph represents the percent colocalisation of GFP-STX7 with STX7 or EEA1. (N=3, n=94, Scale bar 10µm, Inset 3 µm). The data that are displayed using SuperPlots, each biological replicate is distinctly color-coded and each dot represents identified multicolor objects/percentage colocalization in a field of view (frame), described in the Material and methods. All the graphs represent means ± SD. N- number of experimental repeats, n- number of cells analyzed.

Supplementary 3

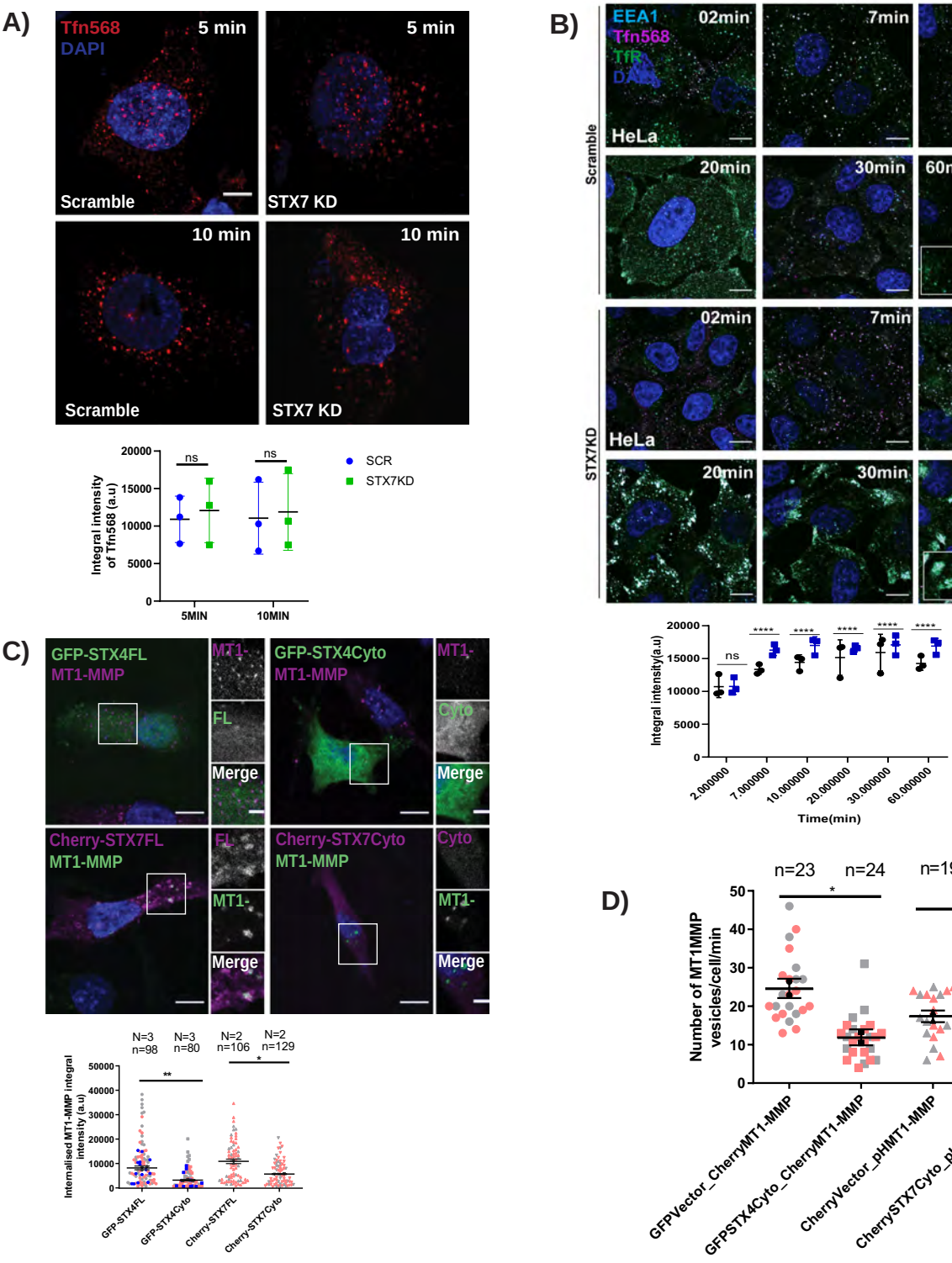


Fig. S3. STX7 is involved in recycling but not endocytosis. A) Control siRNA and STX7 depleted MDA-MB-231 cells were serum-starved and incubated with Alexa Fluor 568 labeled transferrin for 1h at 4˚C. Cells were shifted to 37 ˚C to chase the bound cargo for 5 and 10min. Cells were fixed and stained with DAPI to label the nucleus. Integral intensity of the endocytosed transferrin was measured and plotted. (N=3, n≥300, Scale bar 10 μm). Two-tailed Student's t test, P>0.05, ns-non significant. B) HeLa cells were treated as mentioned above, and the bound cargo was chased for 2-60min. Cells were immunostained with EEA1 and Transferrin receptor (TfR). Inset represents the zoomed-in view of the boxed region. Integral intensity of the labeled Tfn-568 with due course of time was calculated and plotted. (N=3, n≥300 cells, Scale bar 10 μm, inset 3 μm), One way ANOVA, **** P < 0.0001, P>0.05-ns-non significant. C) MDA-MB-231 cells were transfected with GFP-STX4FL/Cyto and Cherry-STX7FL/Cyto. Post- transfection cells were incubated with antibodies against MT1-MMP at 4°C for 1h, then shifted to 37°C for 15 min. Cells were fixed, stained with DAPI to label the nucleus, and imaged with a confocal microscope. The insets represent the zoomed-in view of the boxed region showing the MT1-MMP antibody uptake by the cells. The observed signal corresponds to internalized anti-MT1-MMP antibody (Scale bars = 10 μm, inset = 3 μm). Two-tailed Student's t-test, *< 0.05, ** P < 0.01. D) MDA-MB-231 cells were co-expressed with GFP-Vector/ChMT1-MMP, GFP-STX4Cyto/Cherry-MT1-MMP, Cherry-Vector/pHMT1-MMP and Cherry-STX7Cyto/pHMT1-MMP. Cells were allowed to adhere to the gelatin-coated imaging dishes for 6h and then subjected to TIRF microscopy. The number of MT1-MMP vesicles that are available in the TIRF plane and could form a track spanning minimum 4 consecutive frames captured over 1 minute duration was quantified and plotted {N = 2, n=23(GFP-Vector_ChMT1-MMP), n=24(GFP-STX4Cyto_ChMT1-MMP), n=20(Cherry-Vector_pHMT1-MMP), n=20(Cherry-STX7Cyto_pHMT1-MMP). Two-tailed Student's t test, *< 0.05. See Materials and methods for details. The data that are displayed using SuperPlots, each biological replicate is distinctly color-coded and each dot represents identified objects/integral intensity in a field of view (frame), described in the Material and methods. All the graphs represent means ± SD. N- number of experimental repeats, n- number of cells analyzed.

Supplementary 4

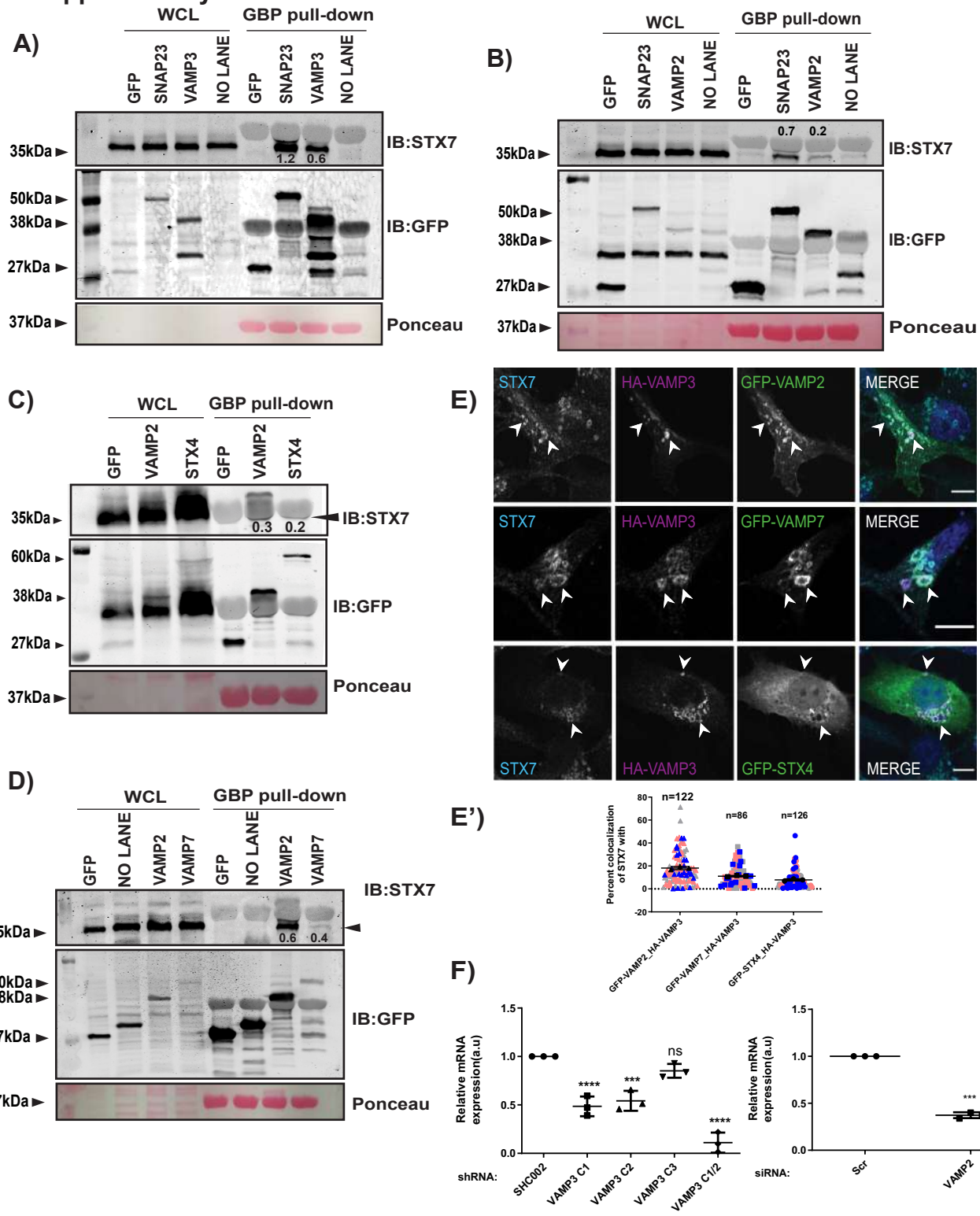
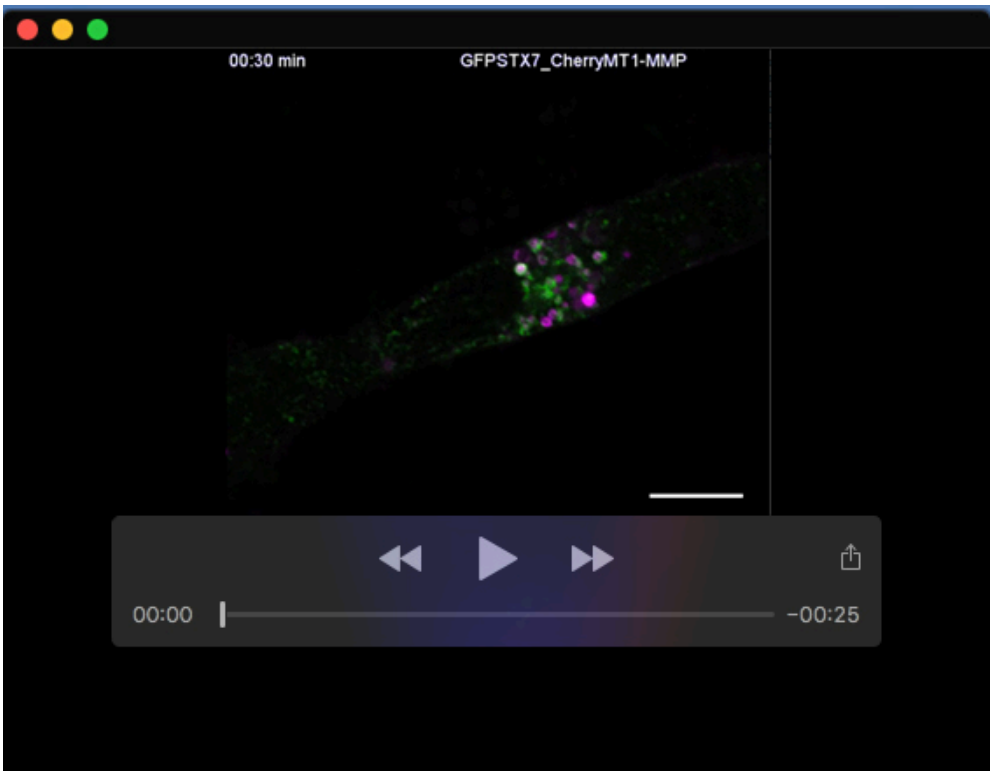
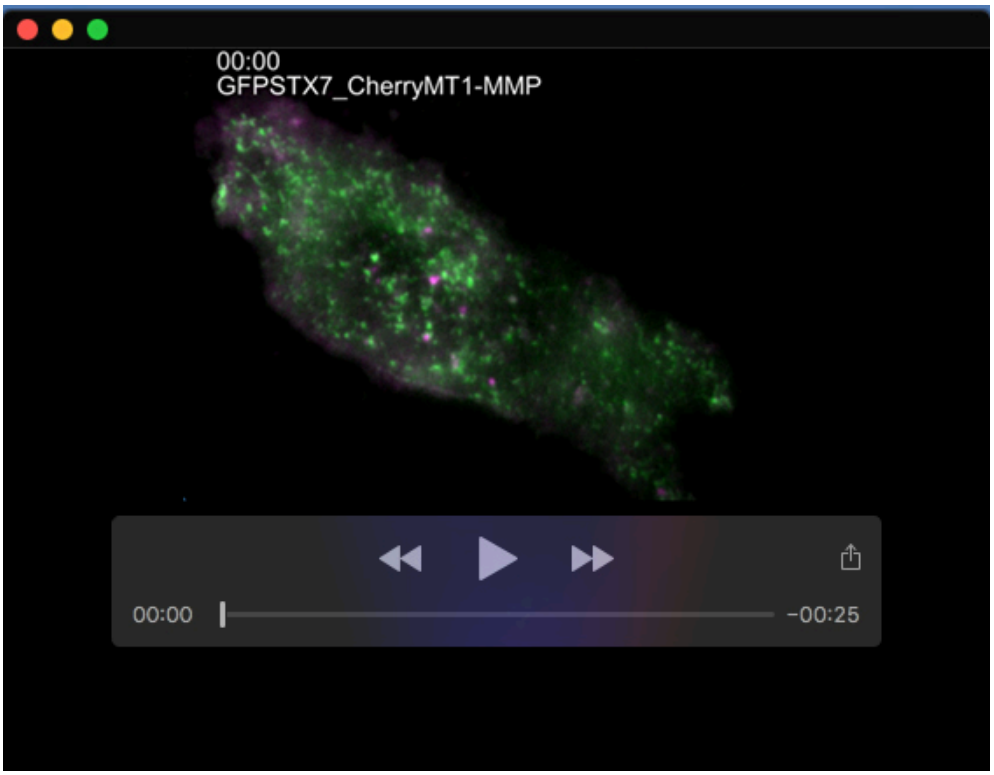


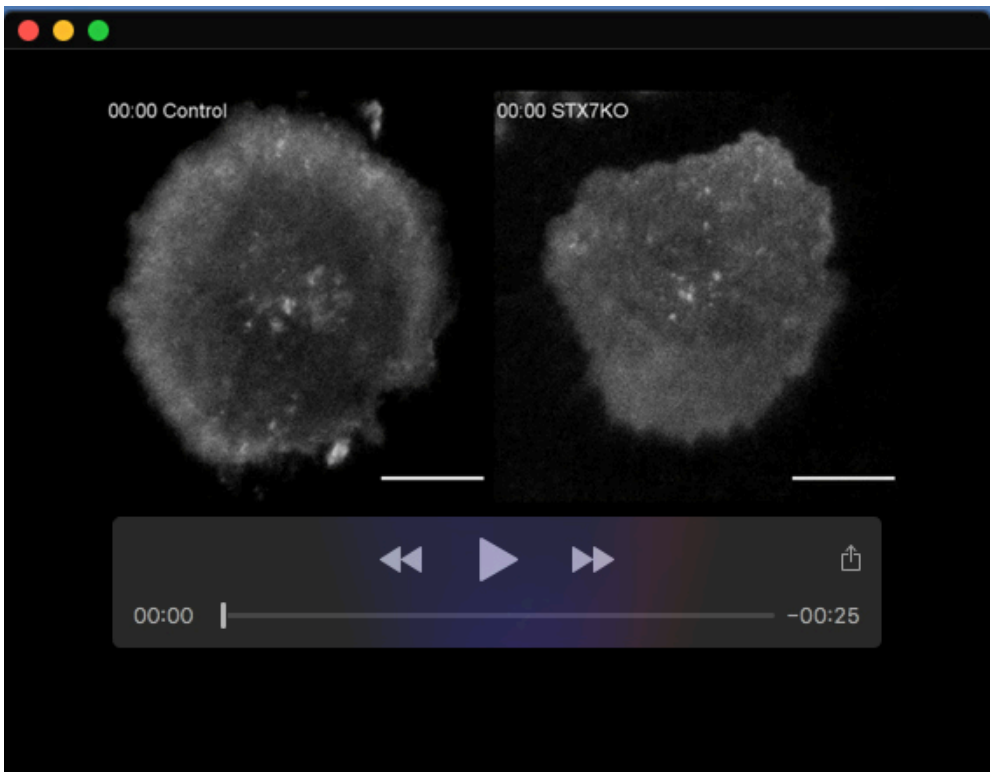
Fig. S4. A and B) GBP pull-down was performed with lysates from MDA-MB-231 cells expressing GFP- vector, GFP- tagged SNAP23, VAMP3 and VAMP2, pre-treated with 1mM NEM. Purified GST–GBP (25µg) was incubated with glutathione sepharose beads and allowed to bind with the respective lysates (300µg) for 5h. Beads were washed, boiled, and advanced for immunoblotting sequentially using anti-STX7, anti-GFP antibodies. The number represents the normalized value of prey protein (STX7) with respect to the precipitated protein (GFP-tagged protein). C and D) GBP pull-down was performed with lysates from HEK293 cells expressing GFP-Vector, GFP-tagged VAMP2, STX4 and VAMP7, pre-treated with 1mM NEM. Purified GST–GBP (25µg) was incubated with glutathione sepharose beads and allowed to bind with the respective lysates GFP-Vector (300µg), GFP- VAMP2 (300µg), GFP- STX4 (900µg) and GFP- VAMP7 (900µg) for 5h. Beads were washed, boiled, and advanced for immunoblotting sequentially using anti-STX7, anti-GFP antibodies. The number represents the normalized value of prey protein (STX7) with respect to the precipitated protein (GFP-tagged protein). Black arrowhead indicates the band of interest. E and E') MDA-MB-231 cells were independently co-expressed with HA-VAMP3 and GFP-VAMP2/GFP-VAMP7/GFP-STX4, fixed using 4% PFA, immunostained with STX7, and labeled with DAPI to label the nucleus. The percentage of co-localization was quantified and plotted. Arrowhead indicates the colocalized objects. {N=3, n=122 (GFP-VAMP2_HA-VAMP3), n=86 (GFP-VAMP7_HA-VAMP3), n=126(GFP-STX4_HA-VAMP3); Scale bar 10 µm}. The data is displayed using SuperPlots, each biological replicate is distinctly color-coded and each dot represents identified multicolor objects/percentage colocalization in a field of view (frame), described in the Material and methods. F) Gene silencing efficiency of VAMP3 and VAMP2 in MDA-MB-231 was detected by qPCR (N=3), One way ANOVA, *** P< 0.001, ****P < 0.0001, ns-non-significant. N- number of experimental repeats, n- number of cells analyzed. The graph represents means±SD.



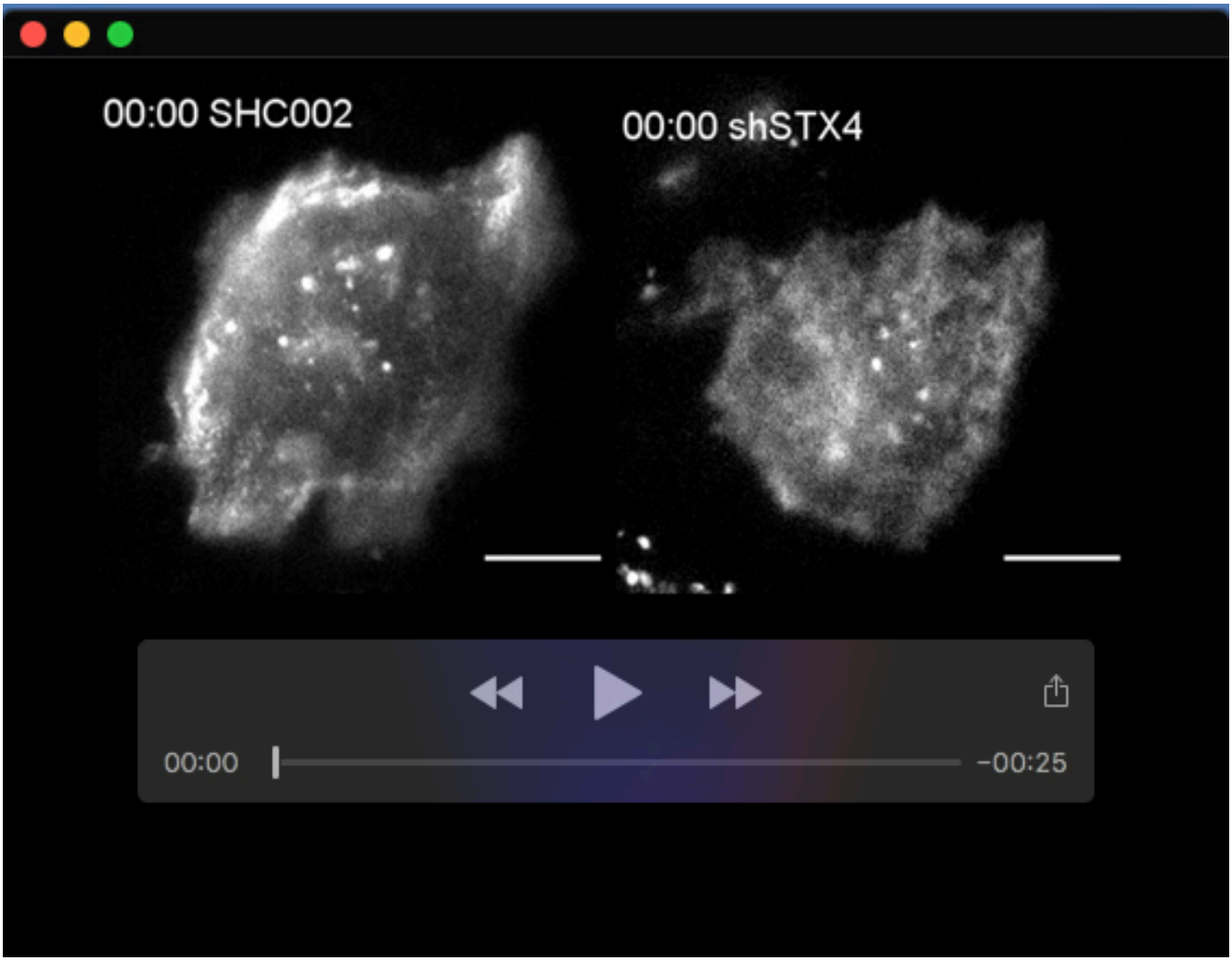
Movie 1. MDA-MB-231 cells cotransfected with Cherry-MT1-MMP and GFP-STX7. Cells were analyzed using a live-cell confocal microscope (Fig. 2E). Scale bar 10 µm, Inset 3 µm. Exposure time: 4.0 µs/pixel.



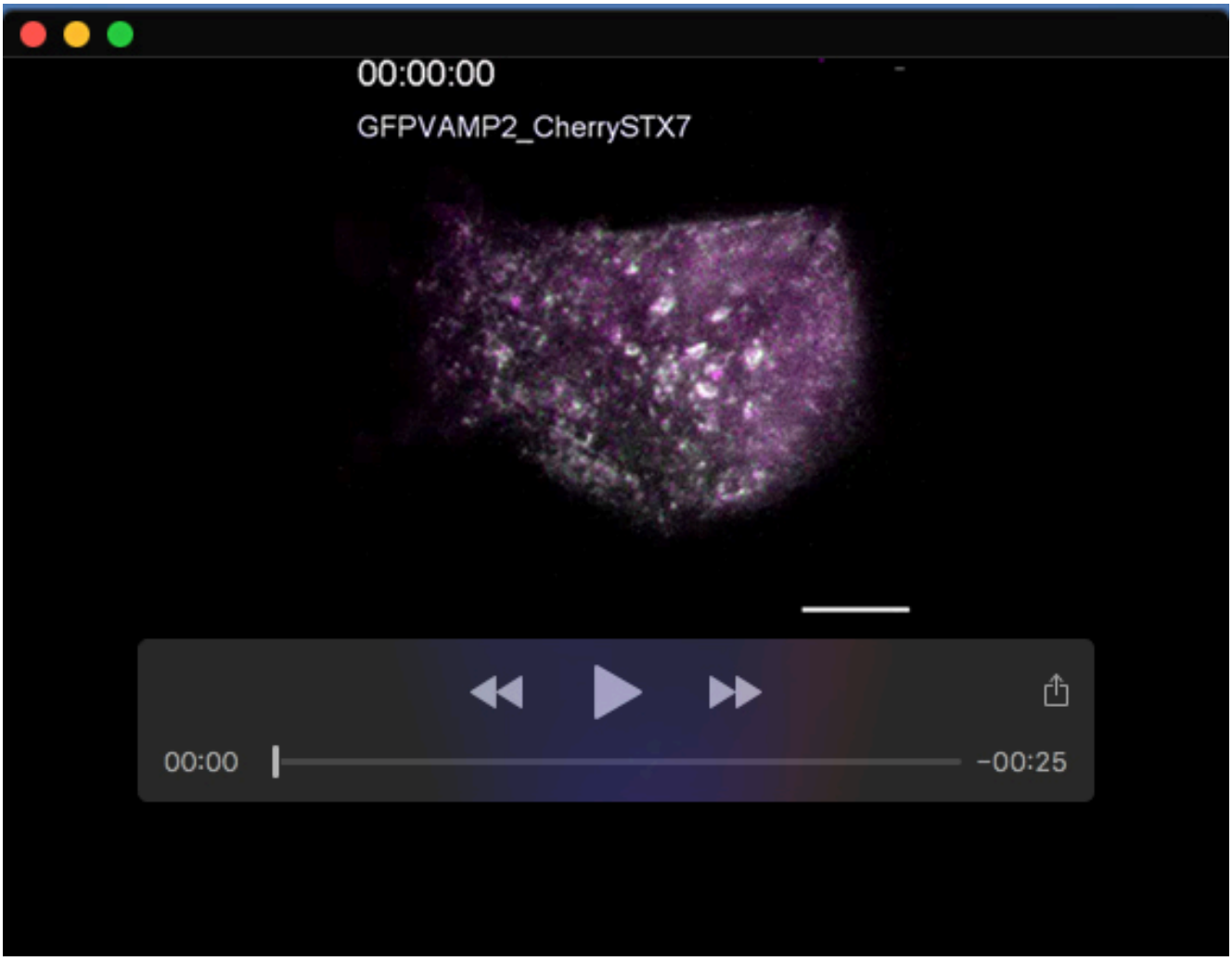
Movie 2. MD A-MB-231 cells cotransfected with Cherry-MT1-MMP and GFP-STX7. Cells were plated on a gelatin-coated imaging dish, and videos were captured using a TIRF microscope (Fig. 3A). Scale bar 10 µm. Exposure time, TIRF Red: 800ms, TIRF Green: 500ms.



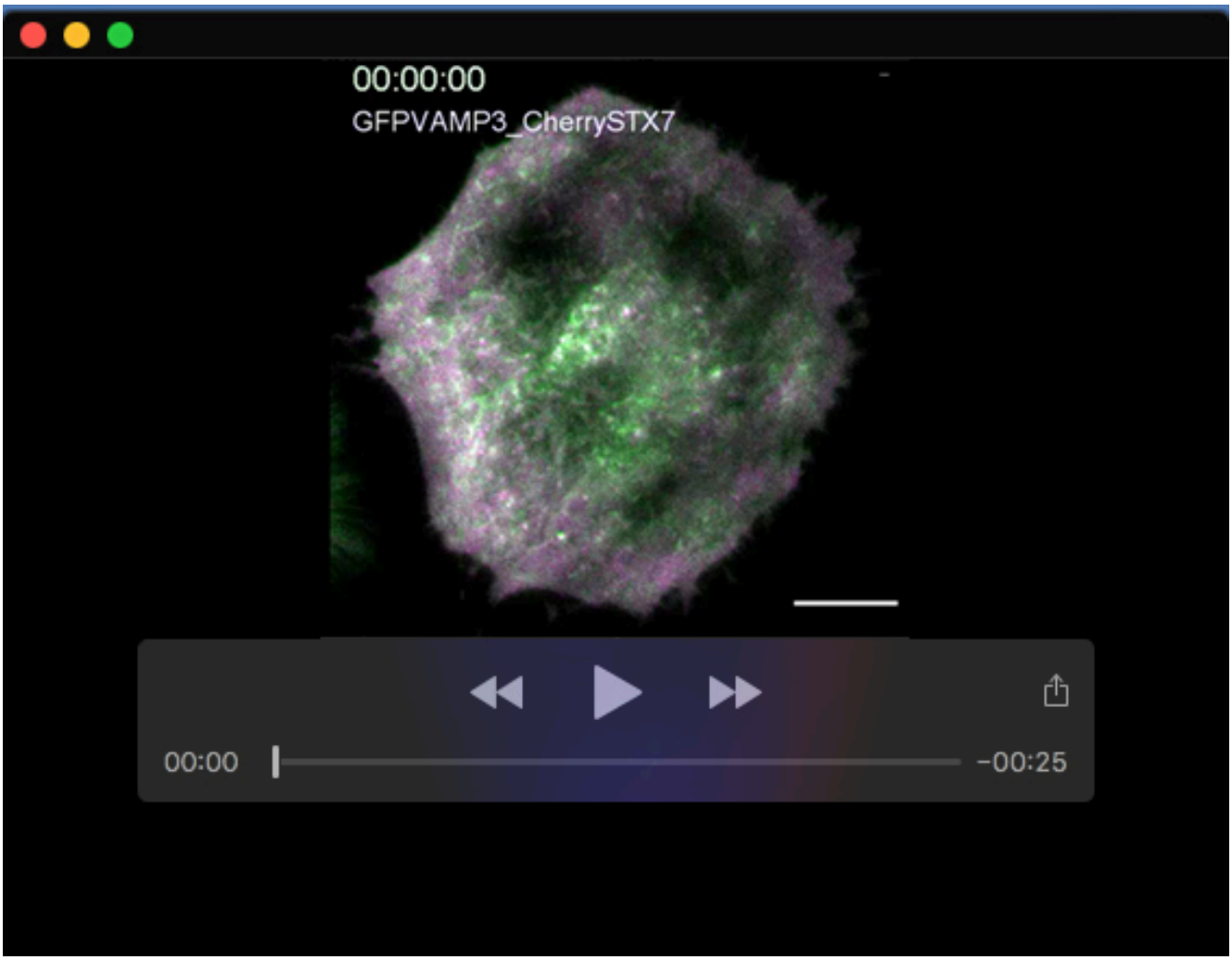
Movie 3. MDA-MB-231 control and STX7KO clonal cells (STX7KO) were transfected with pHluorinMT1-MMP. Cells were plated on a gelatin-coated imaging dish, and videos were captured using a TIRF microscope (Fig. 3A). Scale bar 10 µm. Exposure time, TIRF Green: 600ms (Control); 800ms (STX7KO).



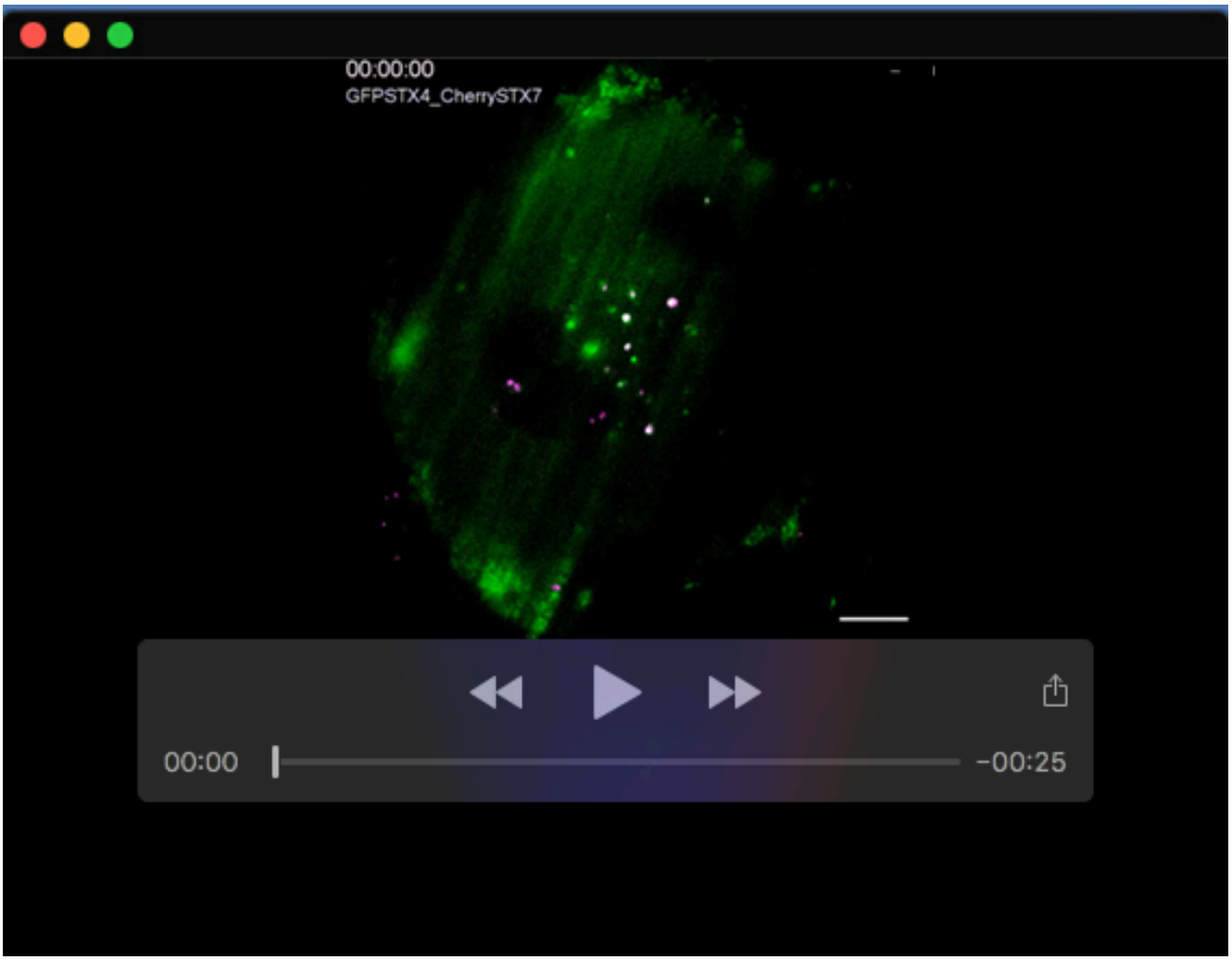
Movie 4. MDA-MB-231 cells were transfected with SHC002 and shSTX4. Depleted cells were transfected with pHluorinMT1-MMP. Cells were plated on a gelatin-coated imaging dish, and videos were captured using a TIRF microscope (Fig. 3A). Scale bar 10 µm. Exposure time, TIRF Green: 400ms (SHC002); 500ms (shSTX4).



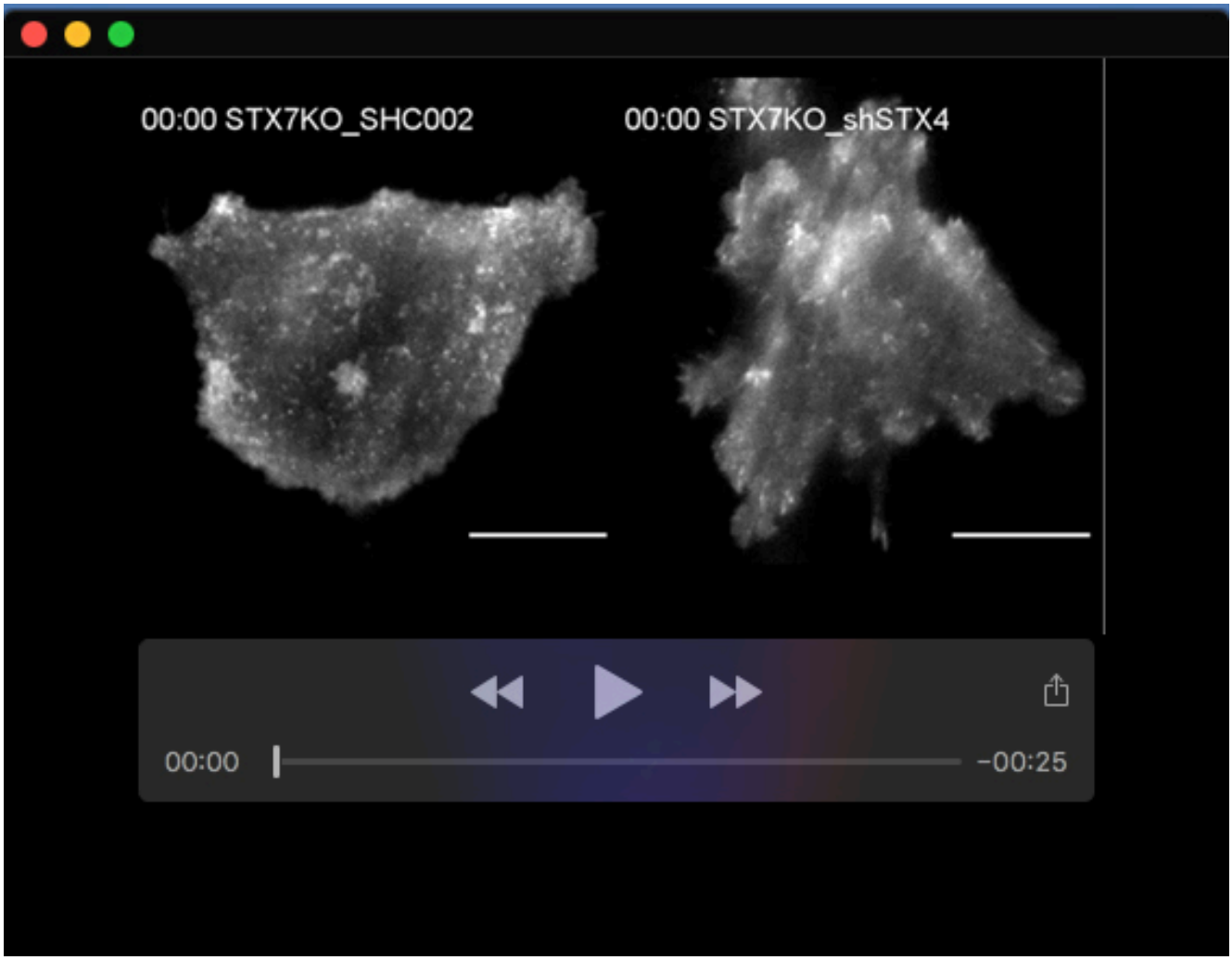
Movie 5. MDA-MB-231 cells cotransfected with Cherry-STX7 and GFP-VAMP2. Cells were plated on a gelatin-coated imaging dish, and videos were captured using a TIRF microscope (Fig. 4D). Scale bar 10 µm. Exposure time, TIRF Red: 200ms, TIRF Green: 50ms.



Movie 6. MDA-MB-231 cells cotransfected with Cherry-STX7 and GFP-VAMP3. Cells were plated on a gelatin-coated imaging dish and videos were captured using a TIRF microscope (Fig. 4D). Scale bar 10 µm. Exposure time, TIRF Red: 100ms, TIRF Green: 100ms.



Movie 7. MDA-MB-231 cells cotransfected with Cherry-STX7 and GFP-STX4. Cells were plated on a gelatin-coated imaging dish, and videos were captured using a TIRF microscope (Fig. 4D). Scale bar 10 µm. Exposure time, TIRF Red: 600ms, TIRF Green: 300ms.



Movie 8. MDA-MB-231 STX7KO clonal cells (STX7KO) were transfected with SHC002 and shSTX4. After 48h of depletion, cells were transfected with pHluorinMT1-MMP. Cells were plated on a gelatin-coated imaging dish, and, videos were captured using a TIRF microscope (Fig. 4I). Scale bar 10 µm. Exposure time, TIRF Green: 400ms.

Table S1. Oligonucleotide sequences for the siRNA SMARTpool Gene SMARTpool siRNA sequence (5'–3')

Scramble	GGCCAGACGCCCAACCAUA
Scramble	GCGAGCAGCCACCAAAUUG
Scramble	GGGAGUACCUGGCGUUUCC
Scramble	CGAACGGCCUGUACGAUGA
MT1MMP	GGAUGGACACGGAGAAUUU
MT1MMP	GGAAACAAGUACUACCGUU
MT1MMP	GGUCUCAAAUGGCAACAUA
MT1MMP	GAUCAAGGCCAAUGUUCGA
STX1A	GGAACACGCGGUAGACUAU
STX1A	AAACAAAGUUCGUUCCAAG
STX1A	GGAGGAGAUUCGAGGCUUC
STX1A	GAGGUGAAGCGGAAGCACA
STX2	UAGACAAGCUCUCAAUGAA
STX2	CUUGUGAUCCUUGGAAUUA
STX2	UAGCAACCAUAUCCCAAGA
STX2	GUUAAAGGCUAUUGAACAA
STX3	GAUCAUUGACUCACAGAUU
STX3	AAGAAACUCUACAGUAUCA
STX3	AGGGUGAGAUGUUAGAUAA
STX3	AAACUCGGCUUAACAUUGA
STX4	GGACAAUUCGGCAGACUAU
STX4	GCGUCACAGUGGUUGGAUA
STX4	GCGAGGUGUUUGUGUCCAA
STX4	CGUCAACACAAGAAUGAGA
SNAP23	UAACAGAACUCAACAAAUG
SNAP23	CAACUAAACCGCAUAGAAG
SNAP23	GAAACUCAUUGACAGCUAA
SNAP23	ACAGAGAUCGUAUUGAUAU
VAMP5	UGACGGAAAUUAUGCGUAA

VAMP5	GAUAUGAGCUCAACCUUCA
VAMP5	GAAUAGAGUUGGAGCGGUG
VAMP5	GCAGCAGCGUUCAGACCAA
STX12	CCACAAAUCAGCUCGCCAA
STX12	GAGGAUCAGUAUAUCGGUA
STX12	ACACUACAGUCUCGUAAUA
STX12	GCUCAGAGGUGCACGUCGA
STX7_siRNA#1	GAGUUUGUUGCUCGAGUAA
STX7_siRNA#1	CAGCAGAUUAUCAGCGCAA
STX7_siRNA#1	UGUCAUUGGAGUUGCGAUU
STX7_siRNA#1	GGAGCACACUGUCGCACUA
STX7_siRNA#2	CAAAGAAACAGAUAAGUAC
STX7_siRNA#2	GCGAUUAUCAGUCUCAUCA
STX7_siRNA#2	GUCAAGGGCAGCAGAUUAU
STX7_siRNA#2	GAGUUUGUUGCUCGAGUAA
STX8	AAUGAAACCAGGCGGGUAA
STX8	GAAUGAGGGUGCCGAACCA
STX8	GAUCUUGUAACUCGAGAGA
STX8	GAGUGAAGAGGCUAAGCGA
VAMP8	CCACUGGUGCCUUCUCUUA
VAMP8	GUCCUUAUCUGCGUGAUUG
VAMP8	GAAAUGAUCGUGUGCGGAA
VAMP8	GGGAAAACUUGGAACAUCU
VTI1B	CCAAAGUAUUGAACGUUCU
VTI1B	GAGCAUAUGAAUCGGCUAC
VTI1B	UGGAGGAGGAGCUACGUUA
VTI1B	UUGCUAAACUCCAUCGGGA
VAMP3	GGAUUACUGUUCUGGUUAU
VAMP3	GAGUUAACGUGGACAAGGU
VAMP3	GGCAGGCGCUUCUCAAUUU
VAMP3	UCAAGUAGAUGAGGUGGUG
VAMP7	GUACUCACAUGGCAAUUAU

VAMP7	GAACGUUCCCGAGCCUUUA
VAMP7	CGAGUUCUCAAGUGUCUUA
VAMP7	GCCAAGACAGGAUUGUAUA
VAMP2	GCGCAAAUACUGGUGGAAA
VAMP2	CAUCAUAGUUUACUUCAGC
VAMP2	GGGAGUGAUUUGCGCCAUC
VAMP2	UCAUGAGGGUGAACGUGGA

Table S2. shRNA Information- MISSION shRNA Plasmid DNA Control Vectors

S.No	Name	Sequence
1	SHC002 MISSION pLKO.1-puro Non-Mammalian shRNA Control (TRC1/1.5)_ Non human or mouse shRNA	CCGGCAACAAGATGAAGAGCA CCAACTCGAGTTGGTGCTCTTC ATCTTGTTGTTTTT
2	TRC ID: TRCN0000065023 STX4_AAI44_D10 (Clone1)	CCGGCCGTCAACACAAGAATG AGAACTCGAGTTCTCATTCTTG TGTTGACGGTTTTTG
3	TRC ID: TRCN0000065027 STX4_AAI44_E2 (Clone2)	CCGGGCTGCACGACATATTCA CTTTCTCGAGAAAGTGAATATG TCGTGCAGCTTTTTG
4	TRC ID: TRCN0000380073 STX4_ LAC36_F12 (Clone3)	GTACCGGGTGACTCGACAGGC CTTAAATCTCGAGATTTAAGGC CTGTCGAGTCACTTTTTTG
5	TRC ID: TRCN0000029814 VAMP3_AAE51_F11 (Clone1)	CCGGCGGGATTACTGTTCTGG TTATCTCGAGATAACCAGAACA GTAATCCCGTTTTT
6	TRC ID: TRCN0000029815 VAMP3_AAE51_F12 (Clone2)	CCGGGCAGCCAAGTTGAAGAG GAAACTCGAGTTTCCTCTTCAA CTTGGCTGCTTTTT
7	TRC ID: TRCN0000029817 VAMP3_AAE51_G2 (Clone3)	CCGGCAGGCGCTTCTCAATTT GAAACTCGAGTTTCAAATTGAG AAGCGCCTGTTTTT

Supplementary 5 : BLOT TRANSPARENCY

Figure 1C' (1)

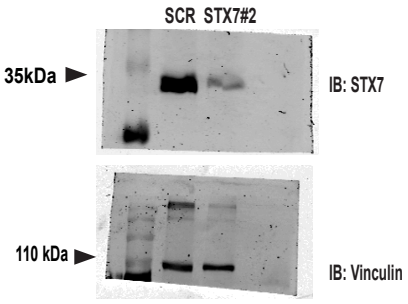
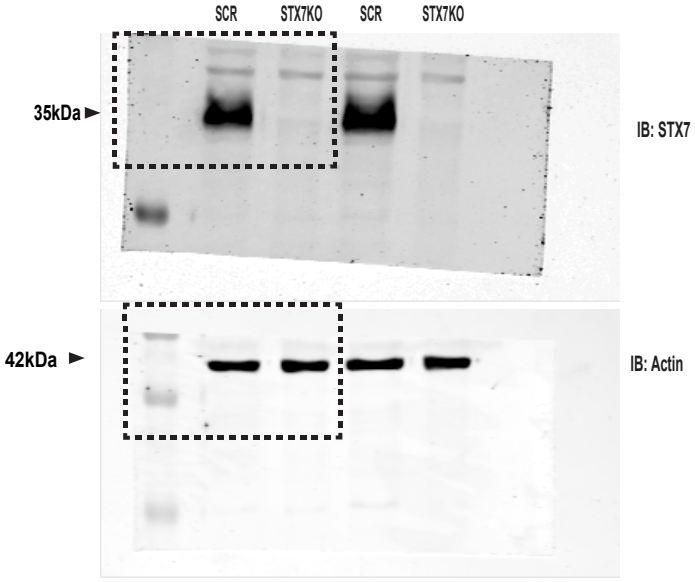
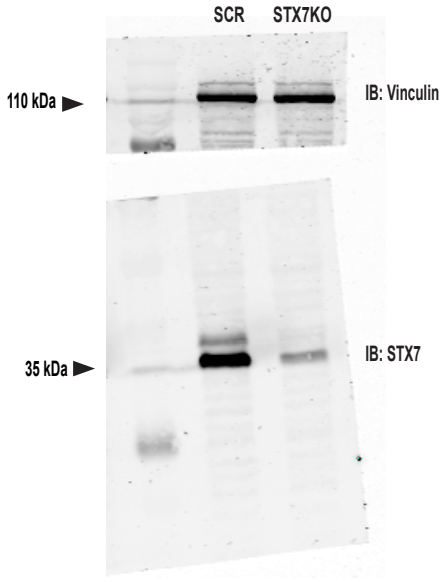


Figure 1C' (2)



STX7KO Clonal cell line

Figure 1C' (3)



STX7KO non-clonal cell line

Figure 1E

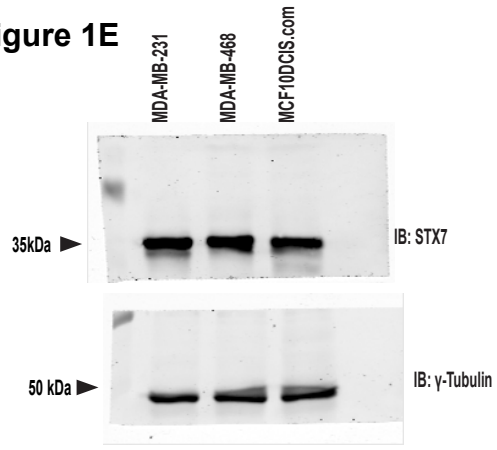
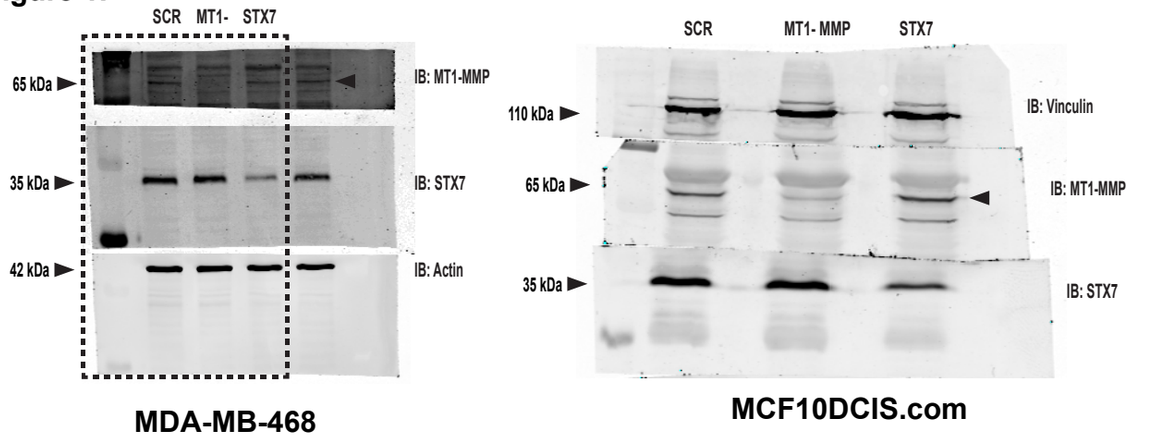
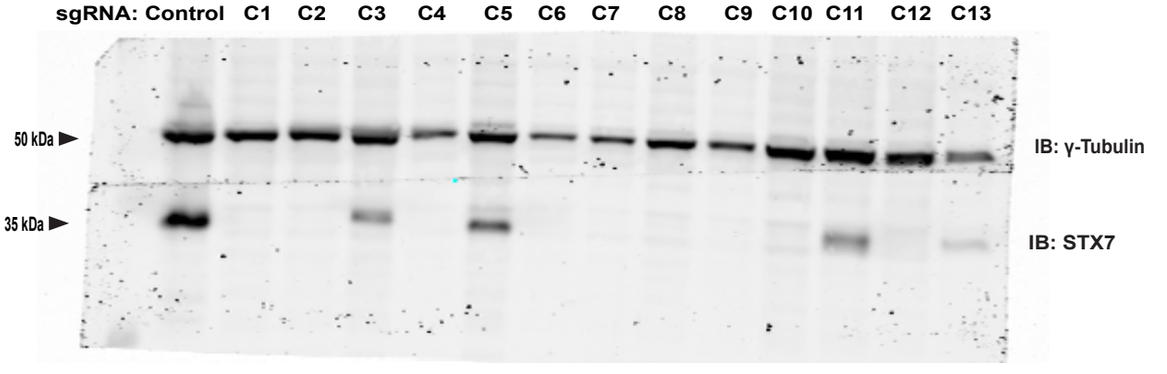


Figure 1F



Supplementary 1D



Supplementary 1E

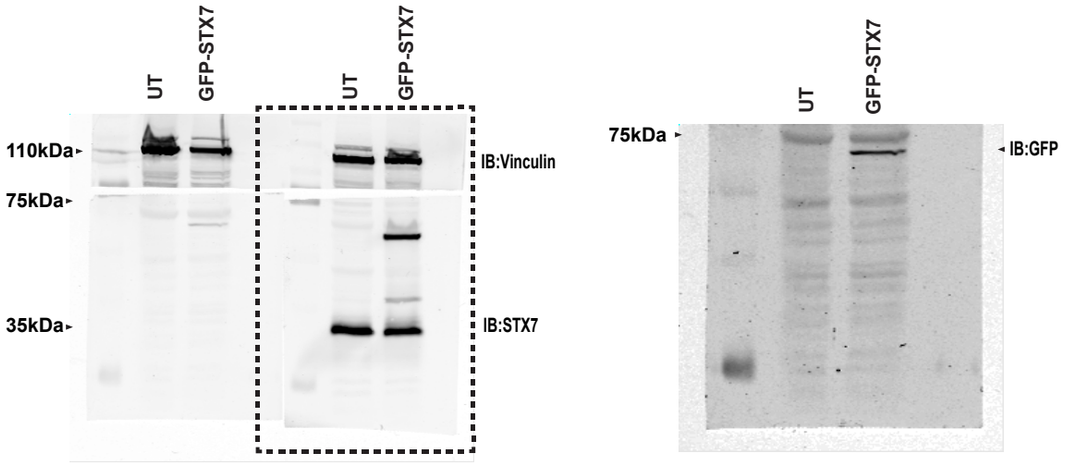


Figure 3B

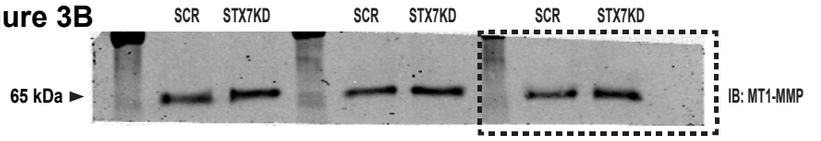


Figure 3B

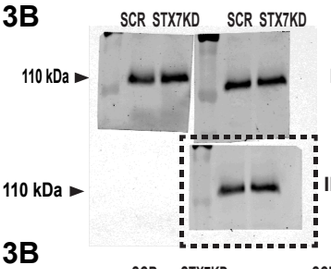


Figure 3B

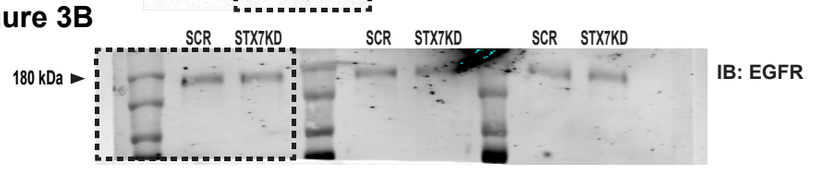


Figure 3B

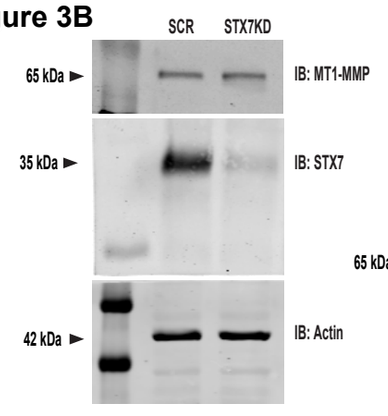


Figure 3D

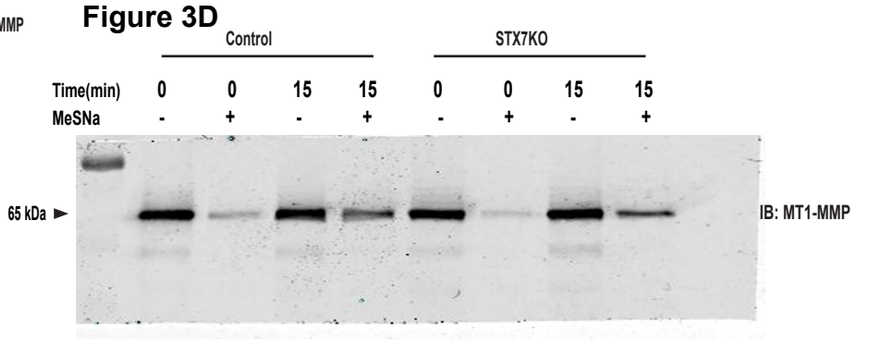


Figure 4E

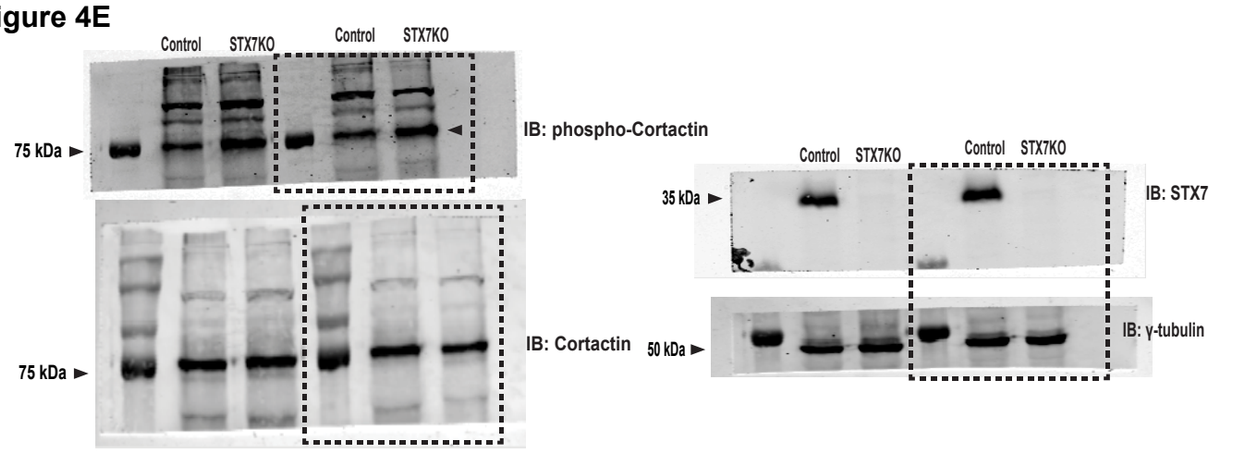


Figure 5A

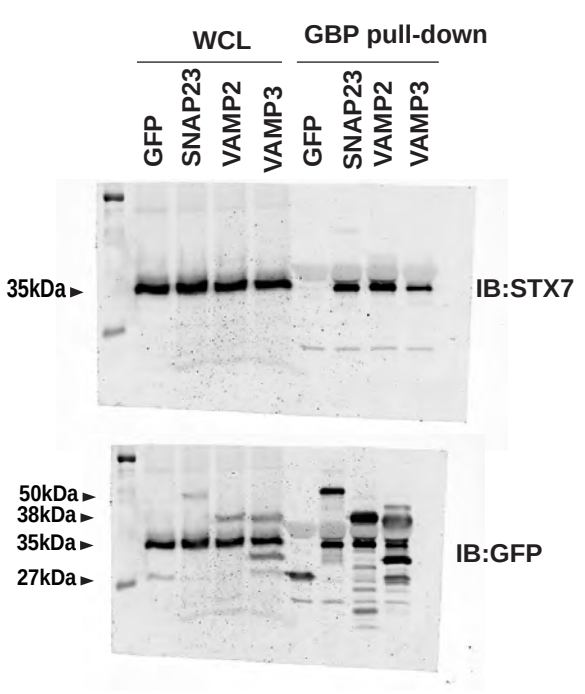


Figure 5B

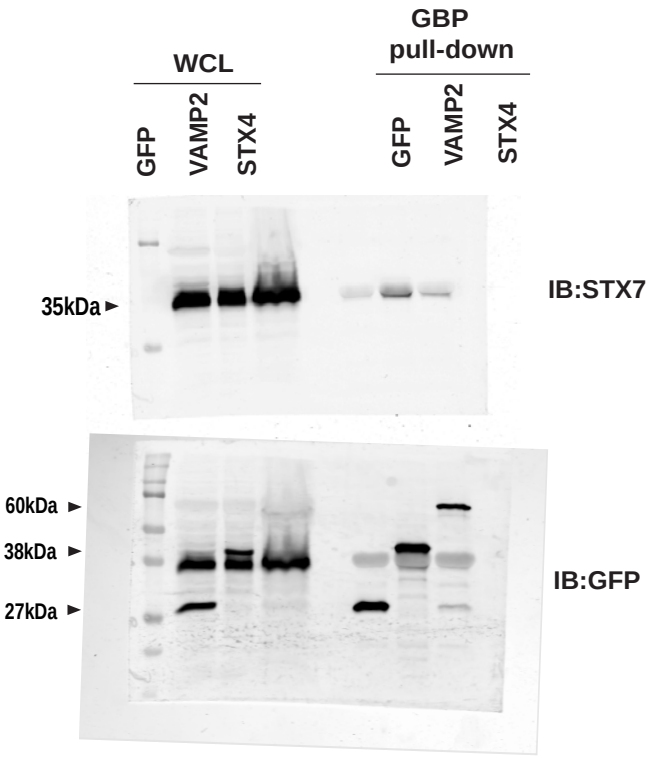
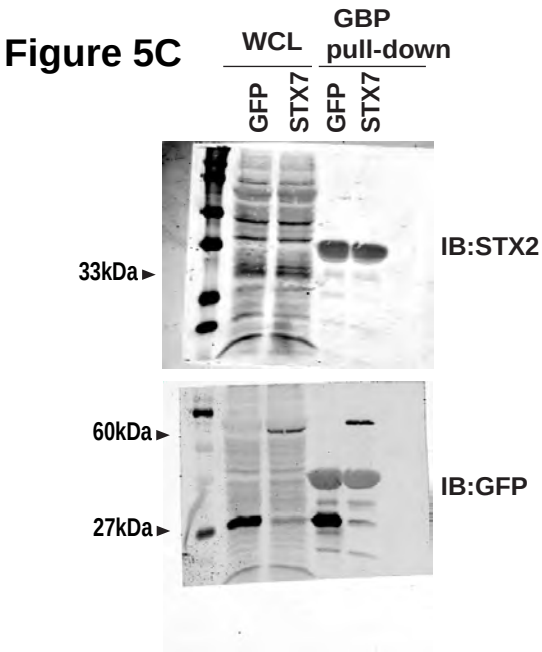
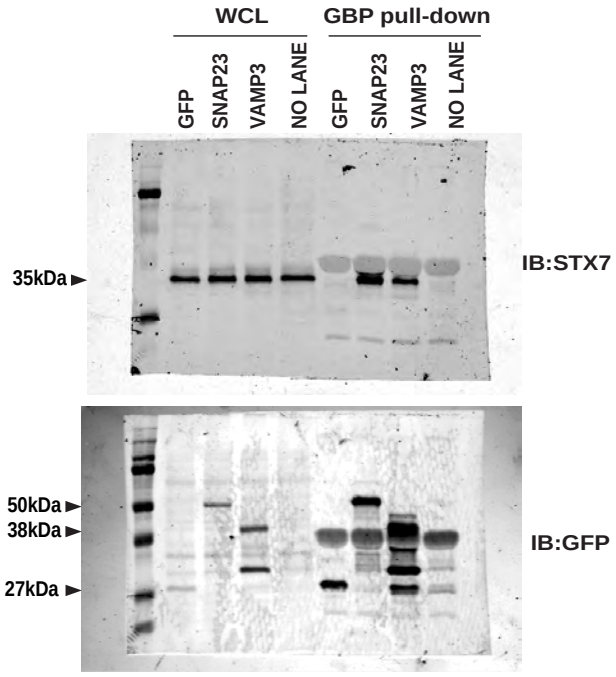


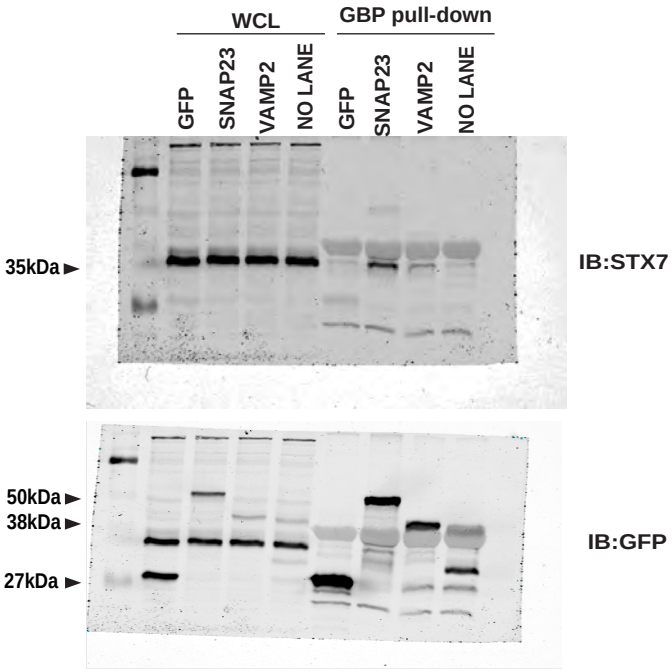
Figure 5C



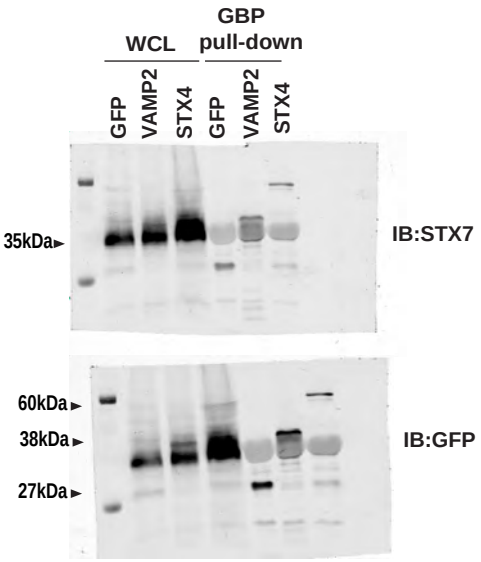
Supplementary 4A



Supplementary 4B



Supplementary 4C



Supplementary 4D