El Azzouzi et al., http://www.jcb.org/cgi/content/full/jcb.201510043/DC1

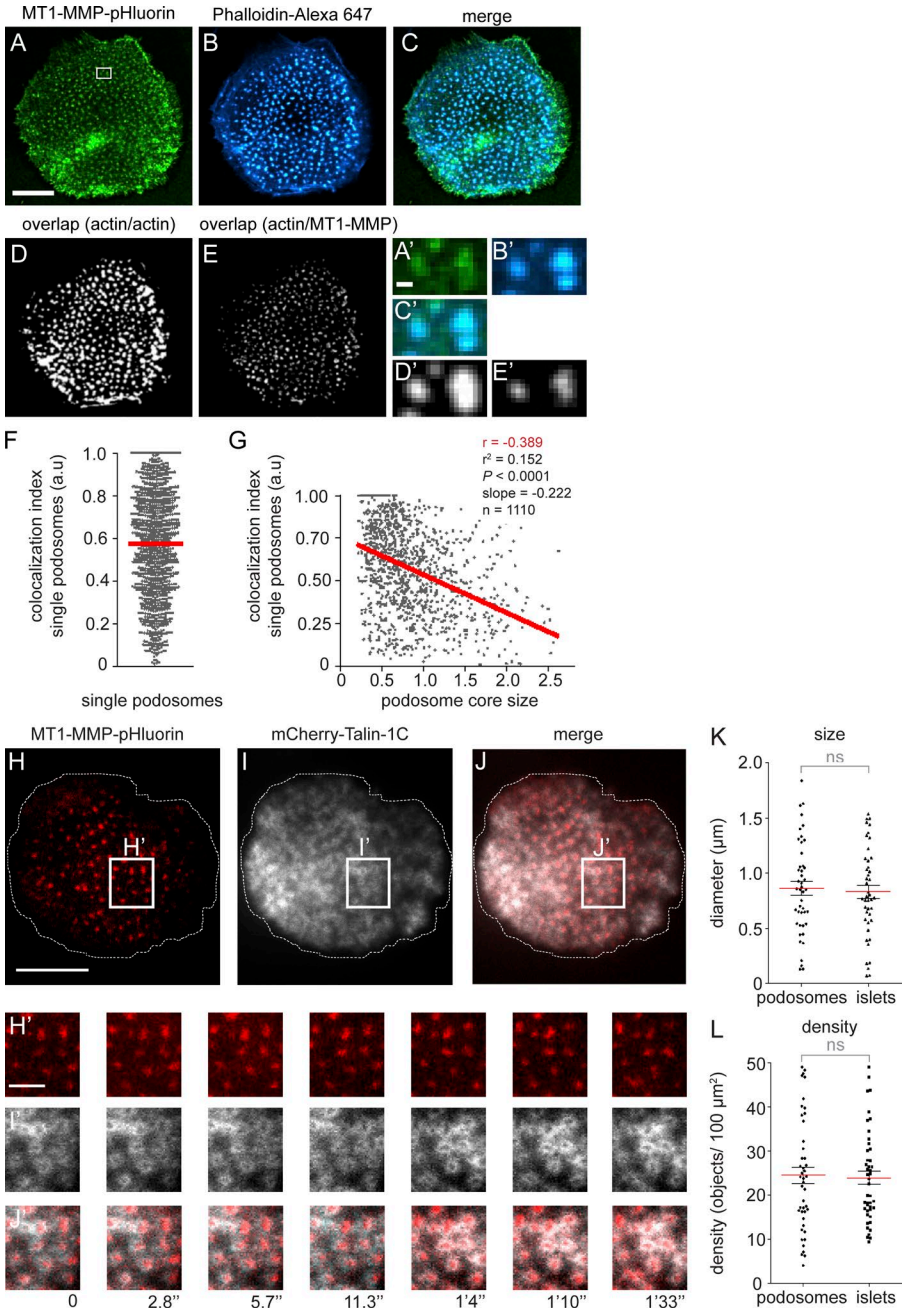


Figure S1. **Acquisition of MT1-MMP-pHluorin correlates negatively with podosome size, surface-exposed MT1-MMP codistributes dynamically with podosomes, and podosomes and MT1-MMP islets show similar sizes and densities.** (A–G) Correlation of MT1-MMP-pHluorin and F-actin at podosomes. (A–C) TIRF micrographs of macrophage expressing MT1-MMP-pHluorin (A, green) and stained for F-actin using phalloidin/Alexa Fluor 647 (B, blue), with merge (C). Bar, 10 µm. (D and E) Micrographs showing colocalizing pixels between actin/actin and actin/MT1-MMP-pHluorin, used for determination of the colocalization index. Yellow box in A indicates detail region shown in (A′–E′). Bars, 1 µm. (F) Colocalization index (0.57), as determined for 1,100 podosomes from four different cells; (G) correlation between colocalization index and podosome size. Correlation coefficient r: –0.389; r^2: 0.152, with a slope of 0.222, and P < 0.0001. (H–J) TIRF micrographs of macrophage expressing MT1-MMP-pHluorin (H, red) and mCherry-Talin-1C (I, white), to visualize podosome rings, with merge in J. Still images taken from Video 1. White boxes indicate detail regions shown in H′–J′. Time since start of the experiment is indicated in minutes and seconds. Bars: (H–J) 10 µm; (H′–J′) 2 µm. (K and L) Statistical evaluation of sizes of single podosomes and MT1-MMP islets (K) and densities (L) of podosomes and islets in macrophages. Each dot represents the respective value from a single cell. For each analysis, 3 × 15 cells from three different donors were evaluated. Red bar indicates mean ± SEM. ns, not significant.

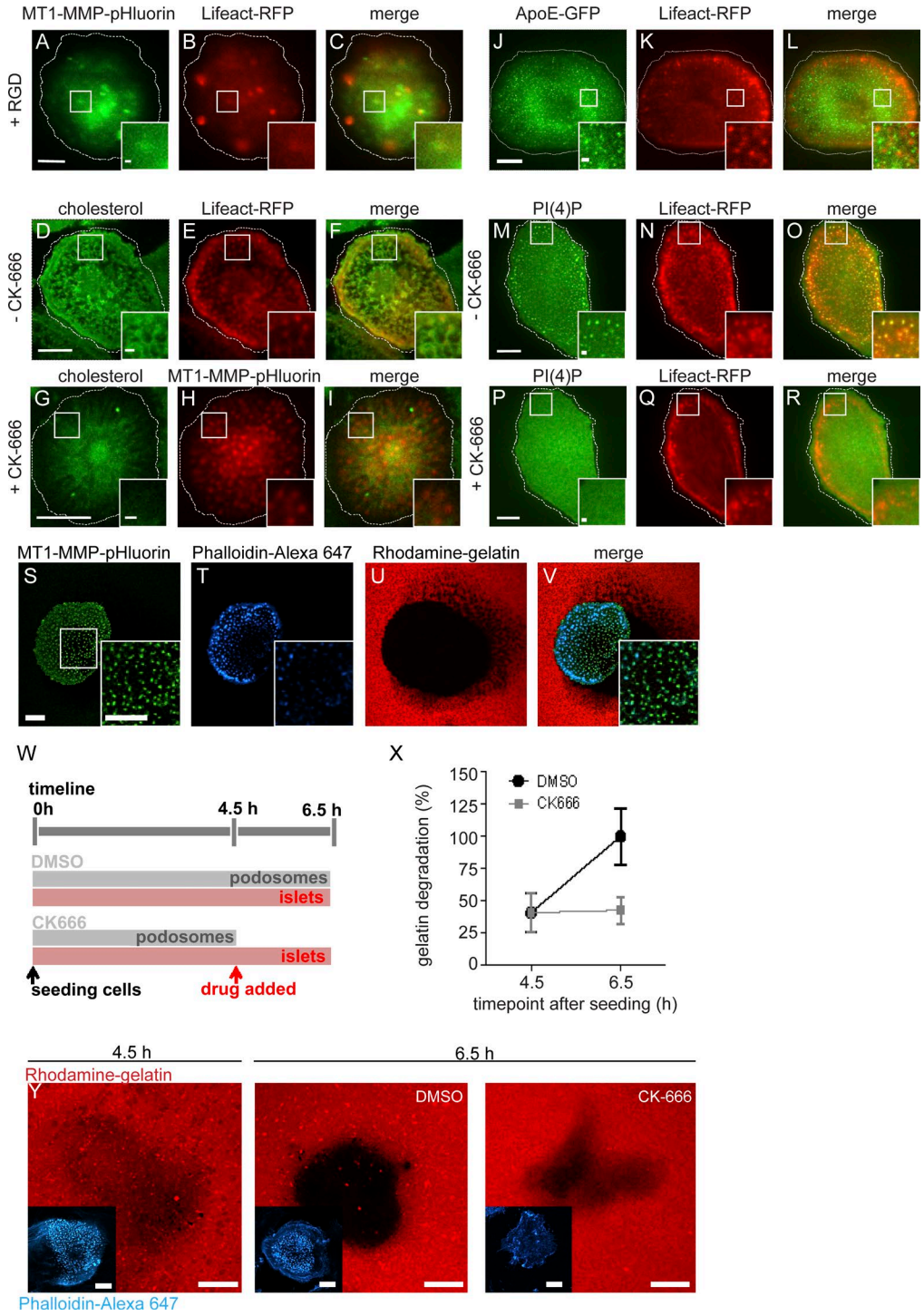


Figure S2. **The membrane lipids cholesterol and PI(4)P localize to podosomes, but not to MT1-MMP islets; ApoE-GFP does not localize to podosomes or islets; and MT1-MMP islets are not degradative on gelatin matrix.** (A–C) Immunofluorescence micrograph of macrophage expressing MT1-MMP-pHluorin (A, green) and Lifeact-RFP (B, red), seeded in the presence of integrin-binding RGD peptide (10 µM). (D–I) Immunofluorescence micrographs of macrophages expressing Lifeact-RFP (E, red) or MT1-MMP-pHluorin (H), and stained with filipin (405 nm excitation), to detect cholesterol (D and G, green). The macrophage in G–I was treated with CK-666. Note enrichment of cholesterol at F-actin–rich podosomes (F), but no enrichment at MT1-MMP islets (I). (J–L) Immunofluorescence micrographs of macrophage expressing ApoE-GFP (J, green) and Lifeact-RFP (K; red), with merge (L). Note the absence of ApoE-GFP from podosomes. (M–R) Immunofluorescence micrographs of macrophage expressing PI(4)P sensor OSH2-2xPH-GFP (M and P, green) and Lifeact-RFP (N and Q, red). Macrophage in P–R was treated with CK-666 to disrupt podosomes and induce MT1-MMP islet formation. Note enrichment of PI(4)P at podosomes, followed by dispersal after CK-666 treatment. Bars: 10 µm; (insets) 1 µm. (S–V) TIRF micrographs of macrophage expressing MT1-MMP-pHluorin (S, green), seeded on rhodamine-labeled gelatin (U, red) and stained for F-actin with phalloidin/Alexa Fluor 647 (T, blue) with merge (V). (W) Timeline of experiment for analysis of matrix degradation. Time points after seeding of cells on labeled matrix are indicated. Colored bars indicate presence of podosomes and/or islets in cell populations treated with indicated drugs. (X) Statistical evaluation of matrix degradation at 4.5- and 6.5-h time points after seeding of cells on gelatin matrix. Maximum of matrix degradation in control cells at 6.5 h was set to 100%. (Y) Representative images of matrix degradation at time points 4.5 h (left) and 6.5 h in cells treated for 2 h with DMSO as control (middle) or CK-666 (right). Insets show F-actin staining in blue. Bars: 10 µm; (insets) 1 µm.

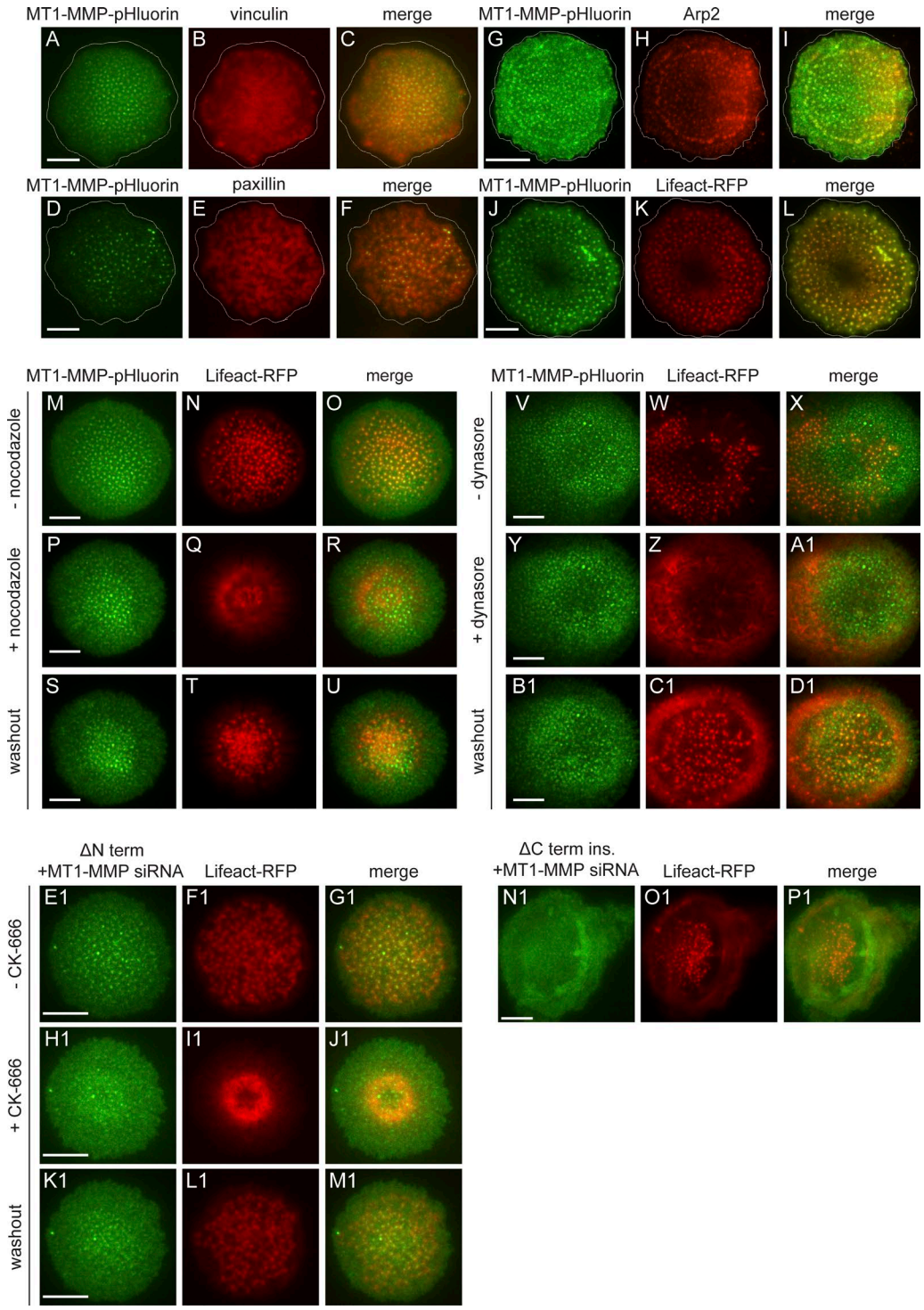


Figure S3. **Composition of reformed podosomes, impact of microtubule disruption or dynamin inhibition on islets, and localization of MT1-MMP mutants to islets.** (A–L) Composition of podosomes reformed after CK-666 washout. TIRF micrographs of macrophages expressing MT1-MMP-pHluorin (A, D, G, and J) and stained for vinculin (B), paxillin (E), and Arp2 (H) using respective antibodies or coexpressing Lifeact-RFP (K), with merges. (M–D1) Impact of microtubule disruption by nocodazole (M–U) or dynamin inhibition by dynasore (V–D1) on islet stability and podosome reformation. Stills from TIRF live-cell videos of macrophages expressing MT1-MMP-pHluorin (M, P, S, V, Y, and B1) and Lifeact-RFP (N, Q, T, WZ, and C1), with merges (O, R, U, X, A1, and D1) and treated with 1 µM nocodazole. (P–R) or 10 µM dynasore (Y-A1), with subsequent washout (S–U and B1–D1). Note disruption of podosomes in both cases (Q and Z) but reformation of podosomes mostly between islets in the nocodazole washout (U) and mostly at islets in the dynasore washout (D1). (E1–P1) Localization of MT1-MMPΔNterm and ΔCterm siRNA mutants in cells depleted of endogenous MT1-MMP by siRNA. Note that MT1-MMPΔNterm is naturally resistant to the used MT1-MMP siRNA, whereas the ΔCterm mutant was rendered insensitive by respective point mutations. Stills from TIRF live-cell videos of macrophages depleted for endogenous MT1-MMP and expressing indicated mutants. Note that MT1-MMPΔNterm localizes to islets (E1–G1), also upon disruption of podosomes by CK-666 (H1–J1), with reformation of podosomes at islets upon washout of CK-666 (K1–M1). In contrast, MT1-MMPΔCterm insensitive (insens.) does not localize to podosomes or islets (N1–P1). Bars, 10 µm.

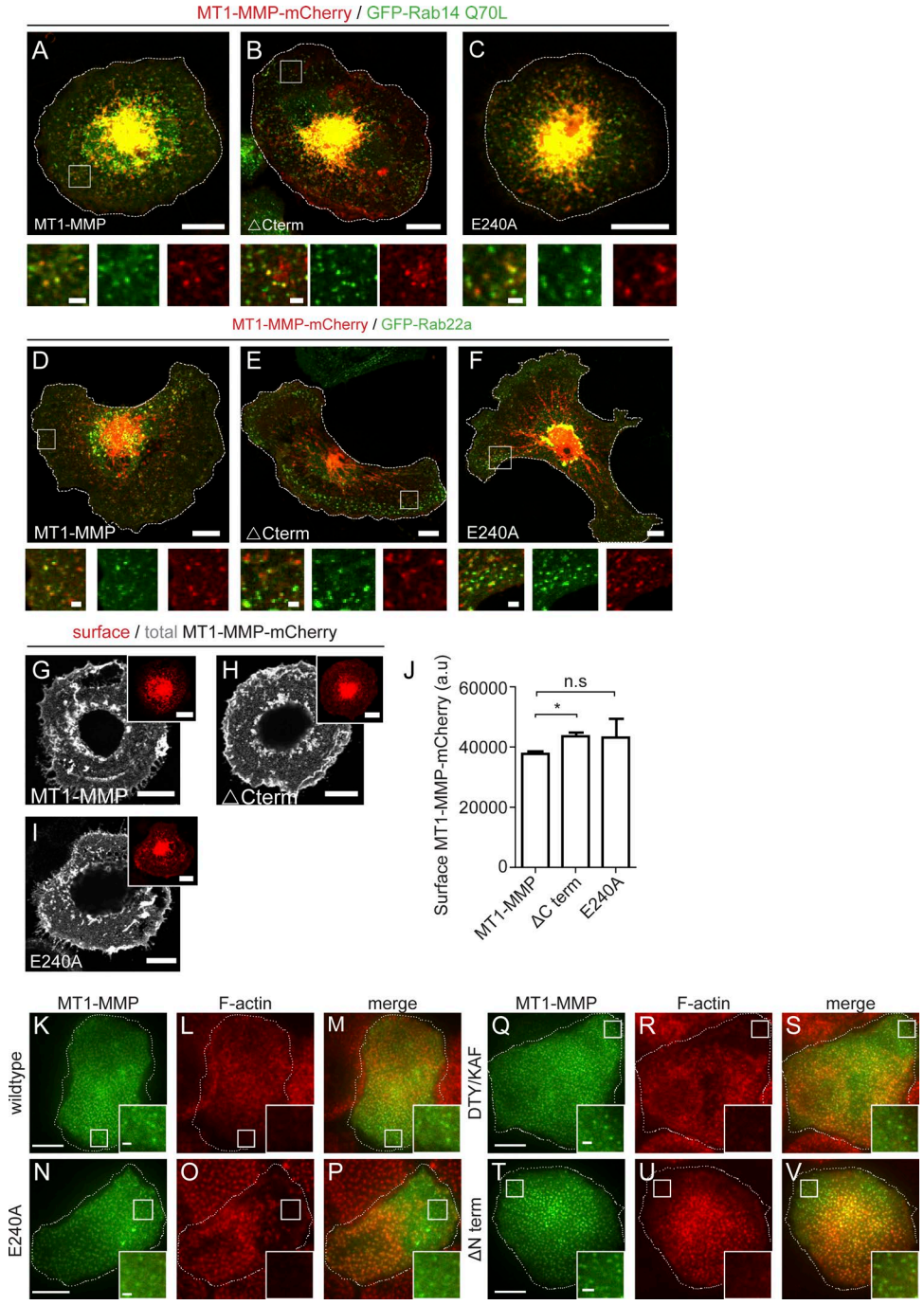


Figure S4. **Absence of the cytoplasmic region or catalytic inactivity does not influence MT1-MMP localization to vesicles or cell-surface exposure and ability of MT1-MMP mutants to form islets.** (A–F) Confocal micrographs of macrophages expressing mCherry-tagged (red) MT1-MMP WT (A and D), ΔCterm (B and E), or catalytically inactive E240A mutant (C and F), and coexpressing GFP-Rab14 Q70L (A–C) or GFP-Rab22a (D–F) to visualize vesicles. White boxes indicate areas of detail images below, with merge and each channel shown separately. Note pronounced colocalization of all three constructs to GFP-Rab14 and also to Rab22a vesicles. Dashed lines indicate cell circumference. Bars, 10 µm. (G–I). Confocal micrographs of macrophages expressing mCherry-tagged MT1-MMP WT (G), ΔCterm (H), and E240A (I) constructs. Cells were not permeabilized and stained for surface-localized MT1-MMP-mCherry using an anti-mCherry antibody (white). Insets show total cellular MT1-MMP-mCherry signals. Bars: 10 µm; (insets) 1 µm. (J). Statistical evaluation of surface-localized MT1-MMP-mCherry, based on Alexa Fluor 647 fluorescence intensity in cells expressing indicated constructs. Note that values of surface-localized ΔCterm and E240A constructs are at least as high as that of WT MT1-MMP. Bars represent mean ± SEM. *, $P < 0.05$. For specific values, see Table S2. (K–V) Localization of MT1-MMP-pHluorin constructs that were rendered siRNA insensitive and expressed in macrophages depleted for endogenous MT1-MMP and stained for F-actin. White boxes indicate detail regions shown as insets. Note localization of all constructs to F-actin/podosome-free islets.

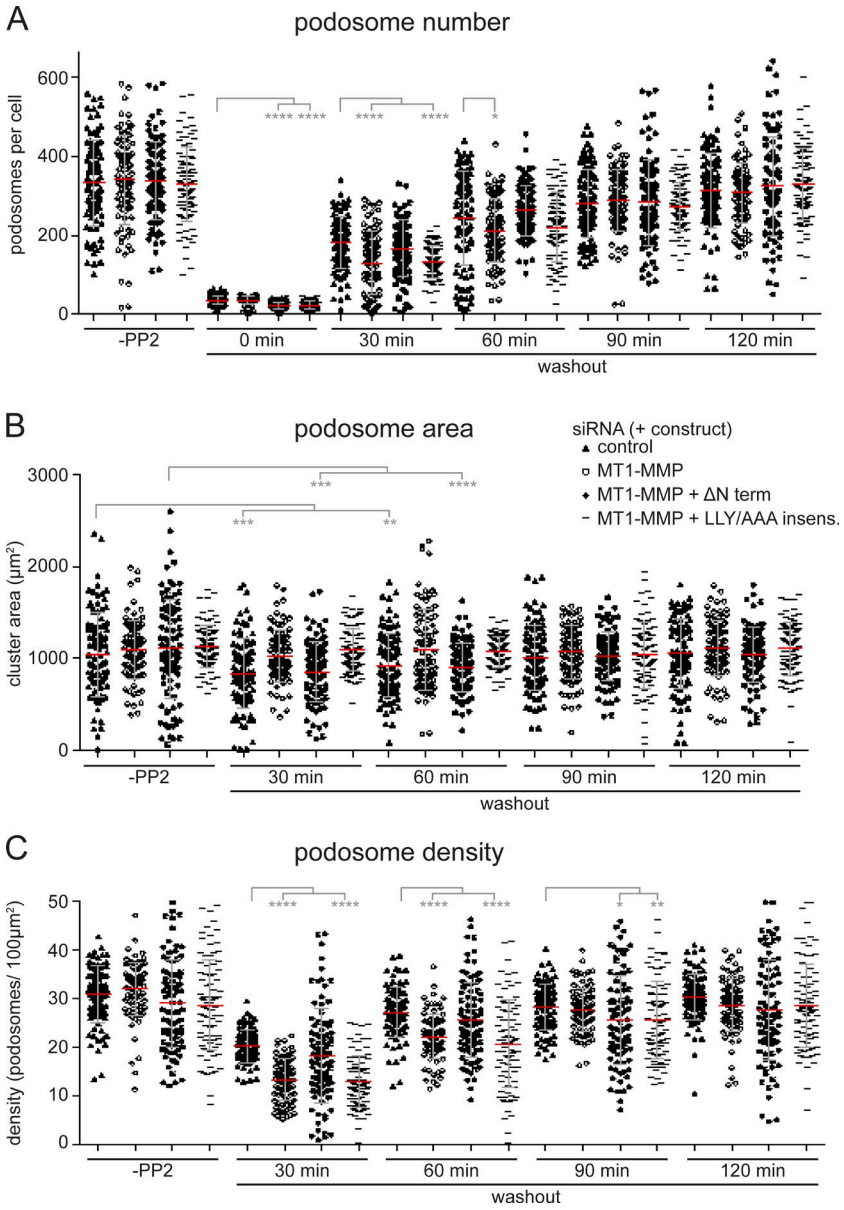
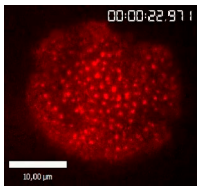
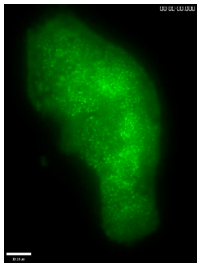


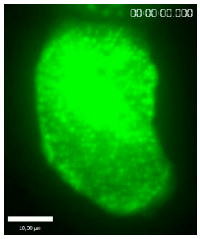
Figure S5. **The LLY motif in the MT1-MMP C terminus is crucial for the recovery of regular podosome number, area, and density during podosome reformation.** Statistical evaluations of podosome numbers (A), area covered by podosome groups ("clusters"; B), or podosome density (podosomes/100 µm²; C) during a podosome reformation assay. Parameters were evaluated in cells before disruption of podosomes ("-PP2") or after treatment with podosome-disrupting PP2 and washout of the drug for the indicated periods, in cells treated with control siRNA (black triangles) or with MT1-MMP-specific siRNA (open circles). In addition, subsets of MT1-MMP–depleted cells were also expressing a construct of the MT1-MMP C terminus that is not targeted by the siRNA (ΔNterm; filled circle) or of a full-length MT1-MMP construct mutated in the LLY motif, which was rendered siRNA insensitive (LLY/AAA insens.; line). Each dot in A–C represents a single cell, with $n = 3 \times 30$, for cells from three different donors. Note that podosomes are mostly absent at 0 min of the washout (A); podosome-covered area (B) and podosome density (C) were thus not evaluated for this time point. Values are given as means ± SD. *, $P < 0.05$; **, $P < 0.01$; ****, $P < 0.0001$. For specific values, see Table S2.



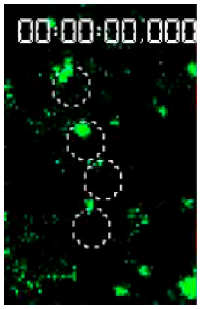
Video 1. **Dynamics of MT1-MMP-pHluorin and mCherry-talin-1C (from Fig. S1).** TIRF time-lapse video of a primary human macrophage expressing MT1-MMP-pHluorin (red; exposure time at 488 nm, 10 ms) and mCherry-Talin-1C (gray; exposure time at 568 nm, 50 ms). Acquisition rate: 20 time points/min; frame rate: 10 fps; sequence: 5 min and 21 s. Bars, 10 µm. Note that MT1-MMP-pHluorin signals are mostly surrounded by mCherry-Talin-1C, indicative of their podosomal localization.



Video 2. **MT1-MMP is present at podosomes and at podosome-free islets (from Fig. 2, A–C).** TIRF time-lapse video of a primary human macrophage expressing MT1-MMP-pHluorin (red; exposure time at 488 nm, 200 ms) and mCherry-Talin-1C (gray; exposure time at 568 nm, 200 ms). Acquisition rate: 2 time points/min; frame rate: 30 fps; sequence: 2 h. Bar, 10 µm. Note the presence of MT1-MMP at podosomes and at podosome-free regions termed "islets."



Video 3. **Podosomal MT1-MMP persists beyond disruption of the podosome structure itself (from Fig. 5, A–F).** TIRF time-lapse video of a primary human macrophage expressing MT1-MMP-pHluorin (green; exposure time at 488 nm, 600 ms) and Lifeact-RFP (red; exposure time at 568 nm, 50 ms). Acquisition rate: two time points/min; frame rate: 10 fps; sequence: 25 min and 6 s. Bar, 10 µm. Note the persistence of MT1-MMP signals even after CK-666–induced disruption of podosomes, indicated by absence of the F-actin reporter Lifeact-RFP.



Video 4. **MT1-MMP islets are sites of podosome reemergence (from Fig. 6, A′–C′).** TIRF time-lapse video of a primary human macrophage expressing MT1-MMP-pHluorin (green; exposure time at 488 nm, 300 ms) and Lifeact-RFP (red; exposure time at 568 nm, 300 ms). Acquisition rate: six time points/min; frame rate: 10 fps; sequence: 46 min and 40 s. Bar, 10 µm. Note the successive appearance of new podosomes, indicated by Lifeact-RFP, at MT1-MMP islets.

Video 5. **MT1-MMP islets can recruit material generated by podosome fission (from Fig. S4, A′–C′).** TIRF time-lapse video of a primary human macrophage expressing MT1-MMP-pHluorin (green; exposure time at 488 nm, 300 ms) and Lifeact-RFP (red; exposure time at 568 nm, 300 ms). Acquisition rate: six time points/min; frame rate: 10 fps; sequence: 1 h. Bar, 10 µm. Note fission of podosome, resulting in the generation of a new podosome core, which is subsequently recruited to a MT-MMP islet.

Table S1. **Tested potential islet components**

Component	Characteristic	Antibody, probe, or construct	Podosomes	Islets
F-actin[a]	Podosome core component	Phalloidin	+	–
		Lifeact-RFP	+	–
Arp2[a]	Arp2/3 complex subunit	Antibody	+	–
α-Actinin[a]	Actin-binding protein	Antibody	+	–
		GFP–α-actinin	+	–
Tks5[a]	Adaptor protein	Antibody	+/–	–
		Tks5-mCherry	+	–
Vinculin[a]	Adhesion plaque protein	Antibody	+	–
Talin[a]	Adhesion plaque protein	Antibody	+	–
		mCherry–talin-1C	+	–
Paxillin[a]	Adhesion plaque protein	Antibody	+	–
kindlin-3[a]	Integrin-interacting protein	Antibody	+	–
		EGFP–kindlin-3	+	–
c-Src	Tyrosine kinase	Antibody	–	–
	Podosome regulator	mTagRFP-T-C-Src-7	–	–
ILK[b]	integrin-interacting protein	ILK-GFP	+	–
β1 integrin[a]	Cell–matrix contact protein	Antibody	+	–
β2 integrin[b]	Cell–matrix contact protein	Antibody	+/–	–
β3 integrin[a]	Cell–matrix contact protein	Antibody	+	–
		β3 integrin–RFP	+	–
CD44[a]	Cell–matrix contact protein	Antibody	+/–	–
MT1-MMP[b]	Metalloproteinase	Antibody	+	+
		MT1-MMP-pHluorin	+	+
Flotillin-1[c]	Vesicle regulatory proteins	mCherry-flotillin1	–	–
Flotillin-2[c]		mCherry-flotillin2	+	–
CIP4[b]	Membrane curvature protein	EGFP-CIP4	–	–
FBP17[b]	Membrane curvature protein	Antibody	–	–
Apolipoprotein E[c]	Cholesterin vesicle protein	ApoE-GFP	–	–
Cholesterol[b]	Membrane lipid	Filipin	+	–
PtdIns(3,4,5)P3[b] and PtdIns(3,4)P2[b]	Membrane lipid	Akt-PH	–	–
PtdIns(4,5)P2[b]	Membrane lipid	PLCδ1-PH	–	–
PtdIns(3,4)P2[b]	Membrane lipid	TAPP1-PH	–	–
PtdIns(4)P[c]	Membrane lipid	OSH2-2xPH-GFP	+	–

Shown are tested proteins or lipid components, their characteristic function, the use of antibodies or probes to detect the endogenous form, or the use of overexpression constructs. + and – indicate respective presence at or absence from podosomes or MT1-MMP islets in primary human macrophages.
[a]Tested component was previously localized to macrophage podosomes.
[b]Tested component was previously localized to invadosomes of other cells.
[c]Tested component was not known to localize to invadosomes.

Table S2. **Values for podosome reformation assay and MT1-MMP constructs**

Podosome number

Time point	Number of values	(Mean ± SD)	
		Non-specific siRNA	MT1-MMP Kd
-PP2	90	323.3 ± 108.6	340.5 ± 113.9
0 min	90	35.57 ± 11.73	35.25 ± 9.33
30 min	90	180.2 ± 66.04	136.1 ± 79.04
60 min	90	243.3 ± 118.1	212.8 ± 80.86
90 min	90	281.0 ± 87.22	287.9 ± 78.62
120 min	90	314.1 ± 95.48	307.8 ± 76.43

Podosome area (µm^2)

Time point	Number of values	(Mean ± SD)	
		Non-specific siRNA	MT1-MMP Kd
-PP2	90	1054.23 ± 462.3	1101.54 ± 324.3
30 min	90	835.12 ± 381.1	1029.35 ± 280.2
60 min	90	922.14 ± 349.2	1100.94 ± 442.1
90 min	90	1010.64 ± 356.4	1073.82 ± 295.4
120 min	90	1051.78 ± 381.2	1107.36 ± 298.7

Density (podosome/100 µm^2)

Time point	Number of values	(Mean ± SD)	
		Non-specific siRNA	MT1-MMP Kd
-PP2	90	30.84 ± 5.76	31.87 ± 5.57
30 min	90	20.15 ± 3.13	13.24 ± 4.25
60 min	90	27.16 ± 5.02	22.12 ± 4.64
90 min	90	28.26 ± 4.74	27.67 ± 4.50
120 min	90	30.16 ± 4.74	28.58 ± 5.46

Podosomes per cell

Constructs	Number of values	Podosome number (mean ± SD)
pHluorin	15	296.80 ± 79.64
MT1-MMP	15	327.7 ± 146.1
E240A	15	334.9 ± 126.0
DTY/KAF	15	291.5 ± 118.6
ΔN-term	15	372.6 ± 161.1
ΔC-term	15	301.5 ± 111.8
LLY/AAA	15	331.6 ± 56.54

MT1-MMP surface exposure (a.u.)

Construct	Number of values	Surface MT1-MMP-mCherry (mean ± SEM)
MT1-MMP	90	37460 ± 1087
ΔC-term	90	43584 ± 1280
E240A	90	43231 ± 6199

Podosome number

Time point	Number of values	(Mean ± SD)			
		Non-specific siRNA	MT1-MMP Kd	MT1-MMP Kd + ΔN-term	MT1-MMP LLY/AAA insens.
-PP2	90	321.3 ± 106.5	338.3 ± 111.7	338.6 ± 100.4	328.2 ± 94.65
0 min	90	35.22 ± 10.12	34.15 ± 9.88	20.4 ± 8.51	22.21 ± 10.43
30 min	90	178.5 ± 64.76	131.5 ± 79.54	166.2 ± 71.6	134.4 ± 41.45
60 min	90	242.7 ± 121.9	211.6 ± 83.77	262.9 ± 64.64	219.9 ± 88.25
90 min	90	282 ± 84.84	288.4 ± 80.21	283.6 ± 108.2	273.6 ± 62.22
120 min	90	312.5 ± 91.62	305.1 ± 82.31	325.7 ± 125.5	330.4 ± 91.49

Podosome area (μm^2)

Time point	Number of values	(Mean ± SD)			
		Non-specific siRNA	MT1-MMP Kd	MT1-MMP Kd + ΔN term	MT1-MMP LLY/AAA insens.
-PP2	90	1045 ± 450.7	1105 ± 315.2	1104 ± 544.2	1132 ± 230.7
30 min	90	836.4 ± 387.6	1028 ± 284.8	854.6 ± 331.1	1093 ± 230.8
60 min	90	927.5 ± 344.2	1096 ± 440.7	897.5 ± 268.8	1098 ± 166.9
90 min	90	1011 ± 354.6	1065 ± 290.9	1027 ± 262.4	1033 ± 354.3
120 min	90	1055 ± 384.6	1110 ± 312.1	1043 ± 294.2	1106 ± 293.8

Density (podosome/100 μm^2)

Time point	Number of values	(Mean ± SD)			
		Non-specific siRNA	MT1-MMP Kd	MT1-MMP Kd + ΔN-term	MT1-MMP LLY/AAA insens.
-PP2	90	30.74 ± 5.66	30.61 ± 5.49	28.95 ± 8.91	28.62 ± 9.32
30 min	90	19.24 ± 3.24	12.55 ± 4.35	18.21 ± 9.61	12.9 ± 5.29
60 min	90	26.89 ± 5.07	21.97 ± 4.25	25.58 ± 7.71	20.61 ± 8.96
90 min	90	27.89 ± 4.66	27.07 ± 4.54	25.61 ± 9.12	25.45 ± 7.95
120 min	90	29.63 ± 4.78	28.18 ± 5.29	27.53 ± 10.31	28.13 ± 8.55

Rhodamine-gelatin degradation				
Time point	Number of values	(Mean ± SEM)		
		Before drug addition	DMSO	CK-666
4.5 h	3 × 30	40.71% ± 15.48%	/	/
6.5h	3 × 30	/	100.0% ± 21.80%	42.65% ± 10.27%

Percentage of cells with islets		
Constructs	Number of values	Podosome number (mean ± SD)
pHluorin	90	2.22 ± 1.92
MT1-MMP	90	54.44 ± 6.94
E240A	90	50.00 ± 8.82
DTY/KAF	90	47.78 ± 13.88
ΔN-term	90	35.56 ± 6.94
ΔC-term	90	16.67 ± 6.67
LLY/AAA	90	23.33 ± 3.33