Supplementary information, Data S1.

MATERIALS AND METHODS

Reagents and antibodies. Fibronectin (PHE0023) was from Life Technologies. Blebbistatin (ab120425) was from Abcam. Pravastatin sodium (T0672) was from TargetMol. Anti-Cpq (HPA023235) was from Sigma. Anti-Pigk (ab201693), anti-NDST1 (ab129248), anti-PCCA (ab187686), anti-Eogt (ab190693), anti-ITGAV (ab179475), anti-Tsg101 (ab125011), anti-CD63 (ab217345), anti-Alix (ab186728), and anti-Histone H3 (ab176882) were from Abcam. Anti-Syntenin-1 (505360) was from Zen bioscience. Anti-Calnexin (610523) and anti-Tim23 (611222) were from BD. For western blotting, the antibodies described above were used at 1:1000 except for anti-ITGAV which was used at 1:5000.

Cell culture. L929, U2OS, NRK, MGC803, MEF, HT1080 and NMuMG cells were cultured in DMEM (WISENT) supplemented with 10% FBS (BI). D2SC and BV2 cells were cultured in RPMI-1640 (WISENT) supplemented with 10% FBS and 0.1% 2-mercaptoethanol (M8211, Solarbio). All cells were cultured at 37°C with 5% CO_2.

Live-cell imaging. Cells were seeded in 35 mm glass-bottom dishes coated with (L929) or without (D2SC) 10 μg/ml fibronectin and cultured at 37 °C with 5% CO_2 overnight. Images were acquired with a Nikon A1 or Olympus F3000 confocal microscope.

Dye staining. L929 cells were cultured in 10 μg/ml fibronectin-coated dishes (D2SC cells were cultured without fibronectin) overnight and fixed by 4% paraformaldehyde for 15 min. Fixed cells were washed with PBS twice and incubated with 1 μg/ml WGA488 or 2.5 μM SYTO14 (S7576, Invitrogen) for 10 min, or 10 μg/ml Filipin III (70440, Cayman) for 1 h. Images were acquired with a Nikon A1 or Olympus Fv3000 confocal microscope.

Counting migrasomes or retractosomes in confocal images. Migrasomes and retractosomes are distinguished by size, morphology and location. Under a confocal microscope, migrasomes are large vesicular structure localized on the branch points or the ends of retraction fibers, while retractosomes are small dots which are always arranged in a string. Counting was carried out in a non-blinded manner.

Isolation of migrasomes from cultured cells. The isolation procedure was based on a modification of previous protocol.[5] Briefly, D2SC cells were grown in 150 mm dishes (NEST) coated with 1 μg/ml fibronectin in full RPMI (DMEM for L929 cells) medium for 16 h. The cells and migrasomes on plates were digested with trypsin and collected in 50 ml tubes, and conditioned medium was harvested for small EV isolation. All subsequent manipulations were performed at 4°C. Cells and large debris were removed by centrifugation at 1000× g for 10 min followed by 4000× g for 20 min. Crude migrasomes were then collected as the pellet by centrifugation at 18,000× g for 60 min. Migrasome fractionation was performed by density gradient

centrifugation, using Optiprep as the density medium (Sigma-Aldrich, D1556). The gradient was 2% (500 µl), 5% (500 µl), 8% (500 µl), 10% (500 µl), 12% (500 µl), 15% (500 µl), crude migrasomes(19% 800µl), 25% (500 µl) and 30% (500 µl). And the crude migrasome sample was prepared by resuspending the pellet with 137.5 µl dilution buffer and then mixing with 400 µl 1× extraction buffer and 252.5 µl 60% Optiprep. Second, the prepared gradient was centrifuged at 150,000× g for 4 h at 4°C in an MLS-50 rotor (Beckman). Third, samples were collected from top to bottom (530 µl per fraction). Each fraction was mixed with the same volume of PBS (530 µl) and centrifuged at 20,000× g for 45 min to collect the pellet. The samples were compatible with western blot analysis, negative staining EM and mass spectrometry.

Isolation of small EVs from cultured cells. The isolation procedure was based on a modification of previous protocol.[6] Briefly, D2SC cells were grown on 150 mm dishes (NEST) coated with 1 µg/ml fibronectin in RPMI medium (DMEM for L929 cells) with 10% FBS depleted of small EVs. After 16 h, conditioned medium was harvested and at the same time cells were harvested for migrasome or retractosome isolation. All subsequent manipulations were performed at 4°C. Cells and large debris were removed by centrifugation at 300×g for 10 min followed by 2000× g for 20 min and 10,000× g for 30 min in 50 ml tubes. The supernatant was passed through a 0.22 µm filter(16541-k, Minisart) and then centrifuged at 120,000× g for 70 min at 4°C in a Type 45 Ti rotor (Beckman). The crude small EV pellet was washed with PBS, followed by a second step of ultracentrifugation at 120,000× g for 70 min at 4°C in a

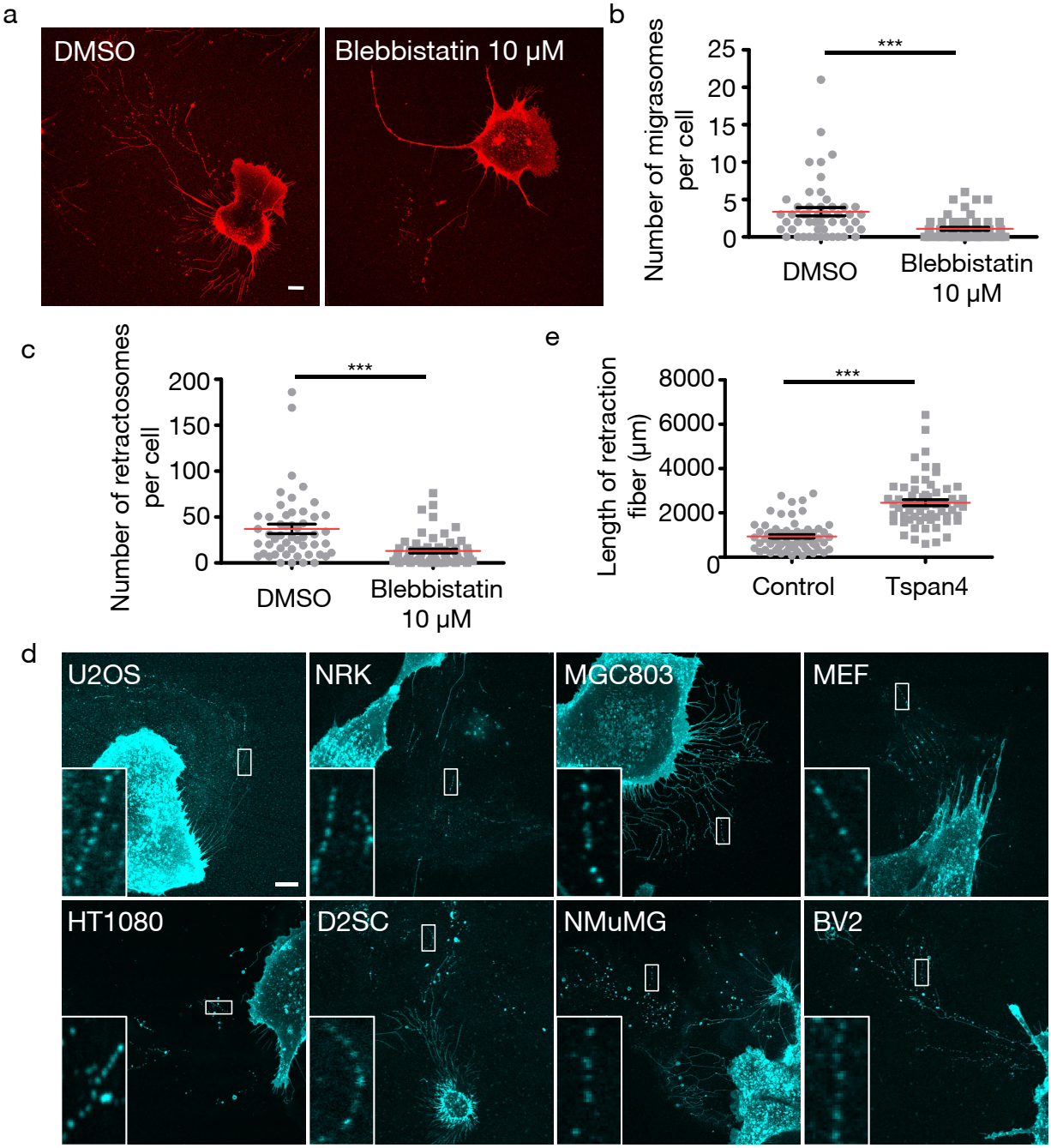
TLA-55 rotor (Beckman) to collect the crude small EVs in the pellet. Sucrose density gradient centrifugation was performed to further purify small EVs. Briefly, crude small EVs were resuspended in 500 µl of HEPES/sucrose stock solution (2.25 M sucrose, 20 mM HEPES/NaOH solution, pH 7.4). The gradient was 0.25 M, 0.50 M, 0.75 M, 1.00 M, 1.25 M, 1.50 M, 1.75 M, 2.00 M, crude small EVs(2.25 M) and 2.50 M (20 mM HEPES/NaOH, pH 7.4, 450 µl for each). The gradient was spun at 150,000× g at 4°C for 4 h. Gradient fractions of 450 µl were collected from top to bottom and each fraction was mixed with the same volume of PBS and centrifuged at 120,000× g for 70 min to collect the pellet. The samples were compatible with western blot analysis, negative staining EM and mass spectrometry.

Isolation of retractosomes from cultured cells. D2SC cells were grown in 150 mm dishes (NEST) coated with 1 µg/ml fibronectin in full RPMI medium (DMEM for L929 cells) for 16 h. The cells and retractosomes on plates were digested with trypsin and collected in 50 ml tubes, and conditioned medium was harvested for small EV isolation. All subsequent manipulations were performed at 4°C. Cells and large debris were removed by centrifugation at 1000× g for 10 min followed by twice at 4000× g for 10 min. The supernatant was passed through a 0.22 µm filter (16541-k, Minisart) and retractosomes were collected by centrifugation at 18,000× g for 60 min at 4°C. The crude retractosome pellet was washed with PBS, followed by a second centrifugation step at 18,000× g for 60 min at 4°C. The samples were compatible with western blot analysis, negative staining EM and mass spectrometry.

Negative staining and TEM imaging. Purified migrasomes, retractosomes or small EVs were resuspended in 50-100 μl PBS, then a 5 μl sample of each was mixed with the same volume of 2.5% glutaraldehyde (PB buffer, pH 7.4), and fixed for 30 min at room temperature. The sample was spread onto glow-discharged Formvar-coated copper mesh grids (Electron Microscopy Sciences, Hatfield) for about 5 min, then stained with 1% uranyl acetate for 30 s. Excess staining solution was blotted off with filter paper and the copper mesh grids were washed with water. After drying, grids were imaged at 10-100 kV using a transmission electron microscope H-7650.

Statistical analysis. Statistical analysis was performed in Graphpad Prism. Different experimental groups were compared with two-tailed *t*-tests (Fig. 1i, j, l, m, o; Supplementary information, Figs. S1b, c, e, and S3b, d). All data were obtained from independent experiments.

Figure S1


a
DMSO
Blebbistatin 10 μM
b
Number of migrasomes per cell
25
20
15
10
5
0
***
DMSO
Blebbistatin 10 μM
c
Number of retractosomes per cell
200
150
100
50
0
***
DMSO
Blebbistatin 10 μM
e
Length of retraction fiber (μm)
8000
6000
4000
2000
0
***
Control
Tspan4
d
U2OS
NRK
MGC803
MEF
HT1080
D2SC
NMuMG
BV2

Supplementary information, Fig. S1. Cytobiological characterization of retractosomes.

a Retractosome formation is impaired by blebbistatin. WT L929 cells were cultured for 12 h with DMSO or 10 μM blebbistatin, and images were acquired with an Olympus FV3000 confocal microscope.

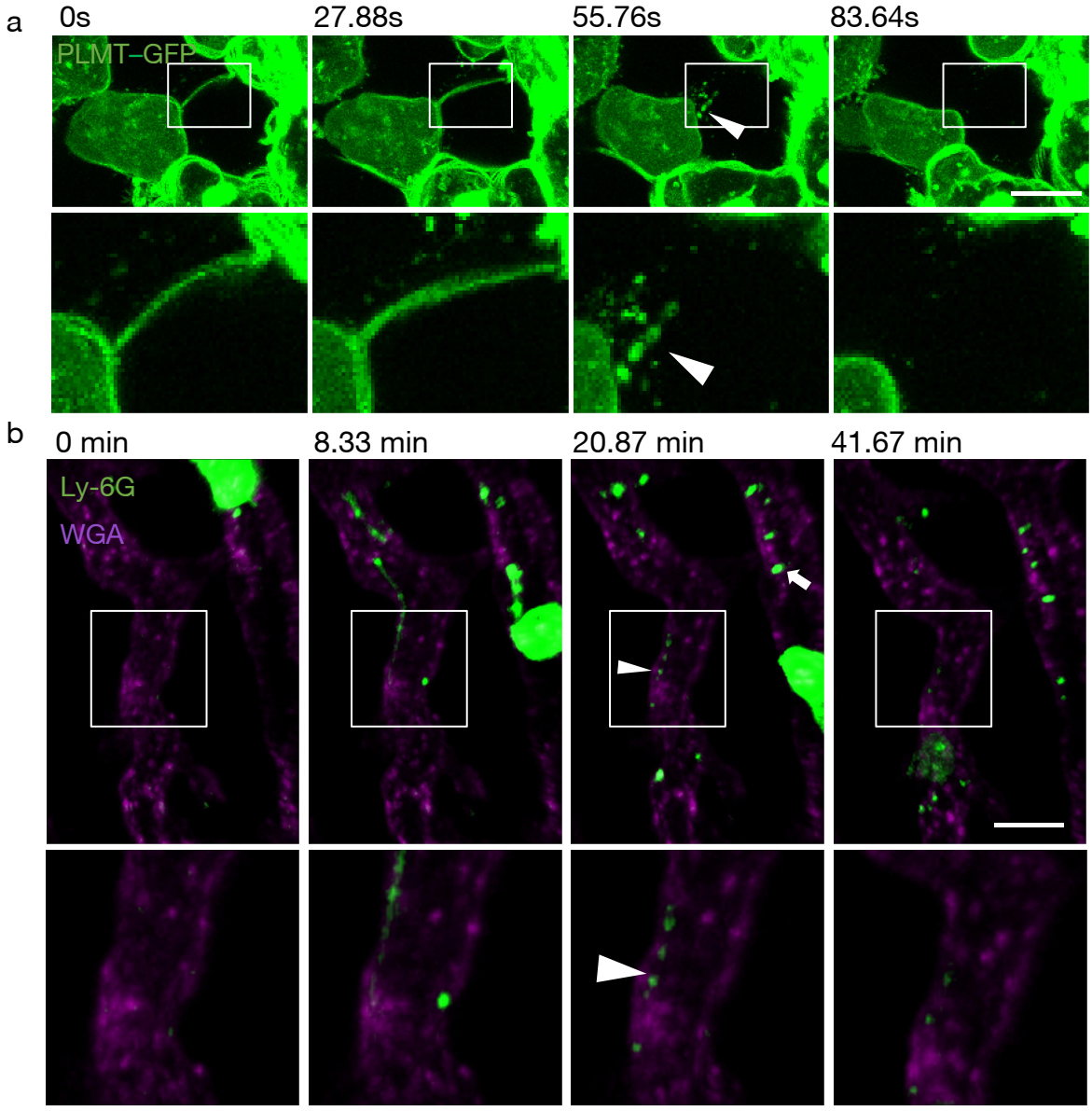
b Cells from assay shown in **a** were quantified for the number of migrasomes per cell. Data shown represent means ± SEM; n =60 cells for each treatment, each pooled from 3 independent experiments. Two-tailed unpaired t-test was used and ***$P < 0.001$.

c Cells from assay shown in **a** were quantified for the number of retractosomes per cell. Data shown represent means ± SEM; n = 60 cells for each genotype, each pooled from 3 independent experiments. Two-tailed unpaired t-test was used and ***$P < 0.001$.

d Retractosomes can be detected in different cell lines in the absence of Tspan4 overexpression. U2OS (human bone osteosarcoma epithelial cells), NRK (normal rat kidney epithelial cells), MGC803 (human gastric carcinoma cells), MEF (mouse embryonic fibroblasts), HT1080 (human fibrosarcoma cells), D2SC (mouse dendritic cells), NMuMG (mouse normal mammary gland cells) and BV2 (mouse microglial cells) were cultured for 12 h, dyed by WGA647 and observed by confocal microscopy. Insets show enlarged regions of interest. Scale bar, 10 μm.

e Cells from assay shown in **Fig. 1h** were quantified for the length of retraction fibers per cell. Data shown represent means ± SEM; n = 60 cells for each genotype, each pooled from 3 independent experiments. Two-tailed unpaired t-test was used and ***$P < 0.001$.

Figure S2

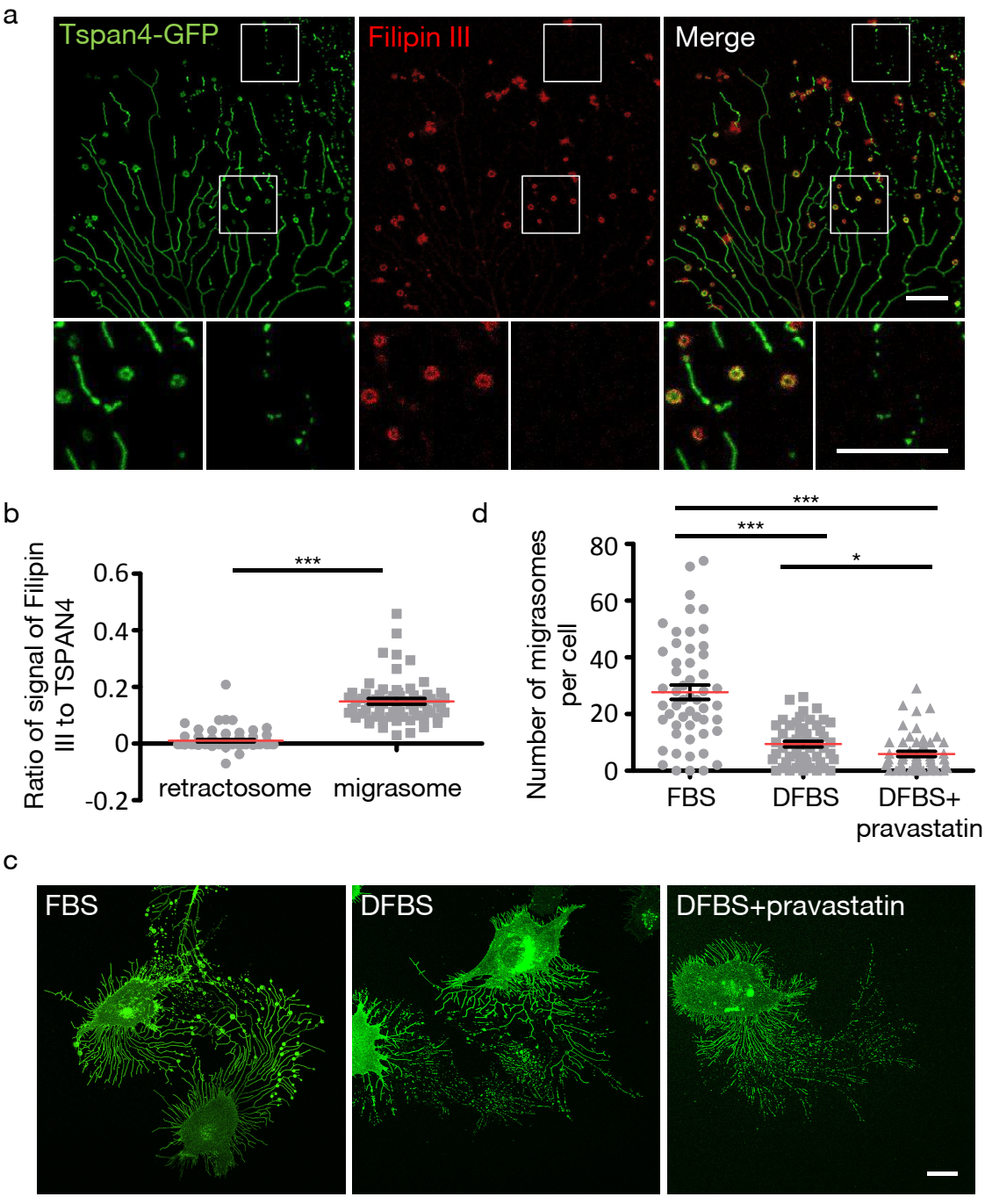

a
0s
27.88s
55.76s
83.64s
PLMT–GFP
b
0 min
8.33 min
20.87 min
41.67 min
Ly-6G
WGA

Supplementary information, Fig. S2. Retractosome generation *in vivo*.

a Retractosome generation in zebrafish embryos. A single blastomere of an embryo at 8-cell stage was injected with 100 pg PLMT–GFP mRNA to label plasma membranes. Time-lapse images were acquired at 27.88 s per frame with a Nikon A1 confocal microscope. 4.78 μm Z-stack images are shown here. Arrowhead indicates retractosomes. Scale bar, 10 μm.

b Retractosome generation by mouse neutrophils. Neutrophils were labeled with anti-mouse Ly-6G (Gr-1) PE, and blood vessels were labeled by AF647-WGA. Arrow indicates migrasomes and arrowhead indicates retractosomes. Scale bar, 10 μm.

Figure S3


a
Tspan4-GFP
Filipin III
Merge
b
Ratio of signal of Filipin III to TSPAN4
0.6
0.4
0.2
0
-0.2
***
retractosome
migrasome
d
Number of migrasomes per cell
80
60
40
20
0
***
***
*
FBS
DFBS
DFBS+ pravastatin
c
FBS
DFBS
DFBS+pravastatin

Supplementary information, Fig. S3. Cholesterol depletion promotes retractosome formation.

a Tspan4-GFP-expressing L929 cells were cultured for 12 h, fixed with paraformaldehyde, dyed with Filipin III to detect cholesterol, and imaged with a Nikon A1 confocal microscope. The panels underneath show migrasomes (left) and retractosomes (right). Scale bar, 10 μm.

b Retractosomes and migrasomes from assay shown in **a** were quantified for the ratio of Filipin III signal to Tspan4 signal. Data shown represent means ± SEM. n = 60 retractosomes or migrasomes from 3 independent experiments. Two-tailed unpaired t-test was used and ***$P < 0.001$.

c Tspan4-GFP-expressing L929 cells were cultured in normal medium (FBS) or cholesterol depletion medium (DFBS) with or without treatment of 30 μM pravastatin for 12 h, and images were acquired with an Olympus FV3000 confocal microscope. Scale bar, 10 μm.

d Cells from assay shown in **c** were quantified for the number of migrasomes per cell. Data shown represent means ± SEM. n = 60 cells from 3 independent experiments. Two-tailed unpaired t-tests were used and ***$P < 0.001$, *$P < 0.05$.

Figure S4

a

Remove culture medium

↓

Wash cells with PBS

↓

Harvest cells

↓ 1,000g 10 min

Supernatant

↓ 4,000g 10 min x2

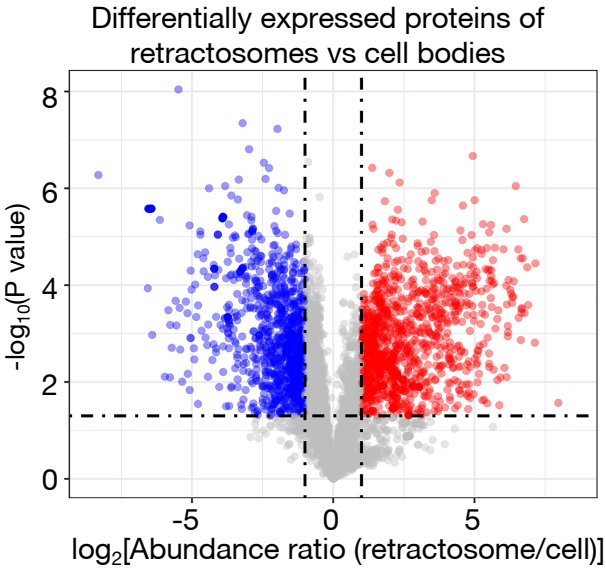
Supernatant

↓ 0.22 μm filter

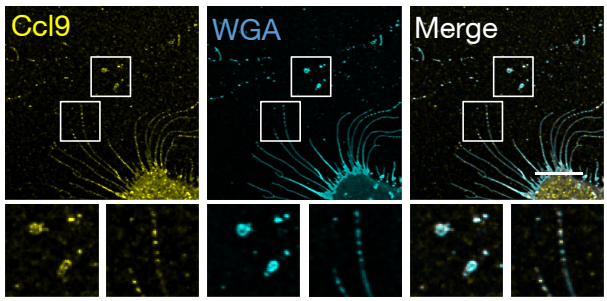
↓ 18,000g 60 min

Retractosomes

b



c



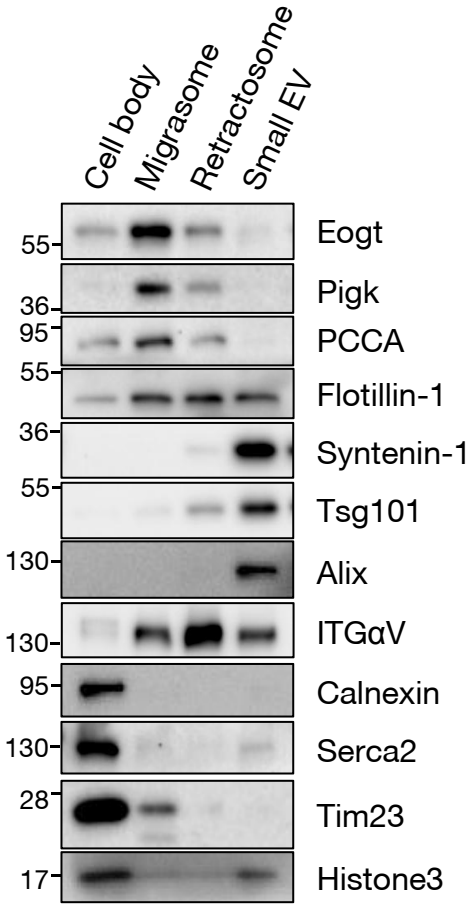
Supplementary information, Fig. S4. The protein composition of retractosomes is similar to migrasomes, but distinct from small EVs.

a Scheme for purification of retractosomes.

b Volcano blot showing the label-free mass spectrometry-based protein quantification of retractosomes vs cell bodies. The red dots represent retractosome:cell abundance ≥2, $P<0.05$; the blue dots represent retractosome:cell abundance ≤ 0.5, $P<0.05$. Data shown are from three biologically independent experiments. P values were calculated using a two-tailed, two-sample unequal variance t-test using Excel.

c Immunostaining of endogenous Ccl9 in D2SC cells. The bottom panels show migrasomes (left) and retractosomes (right). Scale bar, 10 μm.

Figure S5


Cell body
Migrasome
Retractosome
Small EV
55
Eogt
36
Pigk
95
PCCA
55
Flotillin-1
36
Syntenin-1
55
Tsg101
130
Alix
130
ITGαV
95
Calnexin
130
Serca2
28
Tim23
17
Histone3

Supplementary information, Fig. S5.

Samples from cell bodies, purified migrasomes, retractosomes and small EVs from D2SC cells were analyzed by western blotting using antibodies against the migrasome-specific markers Eogt, Pigk and PCCA; the small EV markers Flotillin-1, Syntenin-1, Tsg101 and Alix; the plasma membrane marker ITGαV; the ER markers Calnexin and Serca2; the mitochondrion marker Tim23; and the nuclear marker Histone3.

Supplementary information, Video S1. Breakage of retraction fibers.

Tspan4-mCherry-expressing L929 cells were cultured for 6 h, and time-lapse images were acquired at 7.5 min per frame with an Olympus FV3000 confocal microscope. Scale bar, 10 μm.