Related to figure 1 and 2.

A
DMSO
anti-PMEL(NKI)
anti-LAMP1
anti-PMEL
anti-LAMP1
merge
YM201636
anti-PMEL(NKI)
anti-LAMP1
anti-PMEL
anti-LAMP1
merge

B
Correlation coefficient (LAMP1/PMEL)
0,8
0,75
0,7
0,65
0,6
***
DMSO
YM201636

C
anti-LAMP1
anti-TYRP1
anti-LAMP1
anti-TYRP1
merge
anti-LAMP1
anti-TYRP1
anti-LAMP1
anti-TYRP1
merge

D
Correlation coefficient (LAMP1/TYRP1)
0,7
0,65
0,6
0,55
0,5
**
DMSO
YM201636

E
DMSO
anti-PSEN2
anti-PMEL(NKI)
anti-PSEN2
anti-PMEL
merge
YM201636
anti-PSEN2
anti-PMEL(NKI)
anti-PSEN2
anti-PMEL
merge

F
Correlation coefficient (PSEN2/PMEL)
0,6
0,55
0,5
0,45
***
DMSO
YM201636

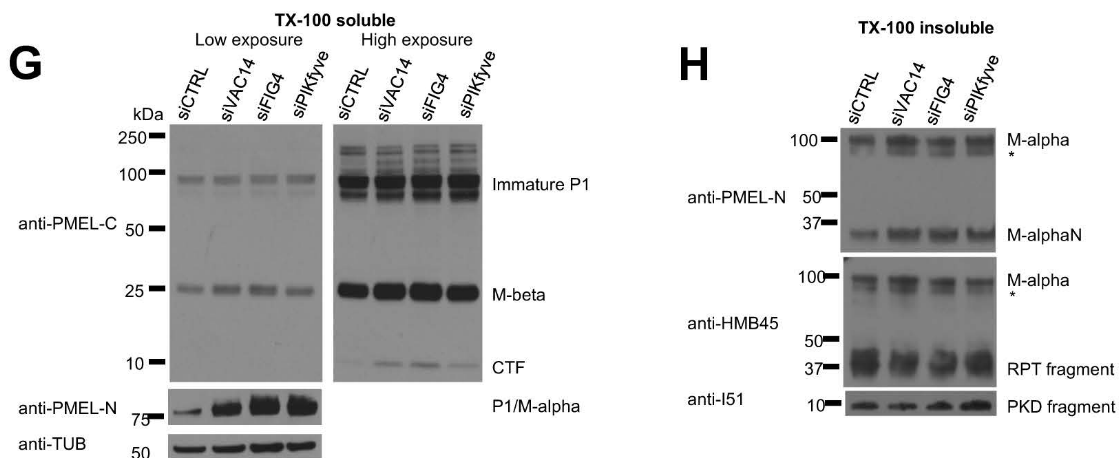

Figure S1 related to figure 1.

A, C, E) MNT-1 cells treated for 2 h with DMSO or 1.6 µM YM201636 were fixed, permeabilized and immuno-labeled using anti-PMEL-NKI (red) (recognizing mainly processed PMEL in stage II melanosomes) and anti-LAMP1 (green) antibodies (A) or anti-TYRP1 (green) (a marker for pigmented melanosomes) and anti-LAMP1 (red) antibodies (C) or anti-PSEN2 (green) and anti-PMEL-NKI (red) antibodies (E). DAPI was used to stain nuclei. Panels on the right show magnifications of the boxed regions. (Scale bars: 10µm). B, D, F) Quantification of colocalization between LAMP1 and PMEL fluorescence (B), LAMP1 and TYRP1 fluorescence (D) and PSEN2 and PMEL fluorescence (F). G-H) Triton X-100-soluble (G) and Triton X-100-insoluble (H) lysates of MNT-1 cells treated with control siRNAs or siRNAs against VAC14, FIG4 and PIKfyve were analyzed by immunoblotting using antibodies against the PMEL C-terminus (anti-PMEL-C), the PMEL N-terminus (anti-PMEL-N), the PMEL RPT domain (anti-PMEL-HMB45), the PMEL PKD domain (anti-PMEL-I51) and Tubulin (anti-TUB) as equal loading marker. The different PMEL fragments are annotated on the right. Stars indicate M-alpha fragments derived from another isoform generated by alternative splicing. Right panels show higher exposures. Data are represented as mean ± SEM.

Related to figure 3

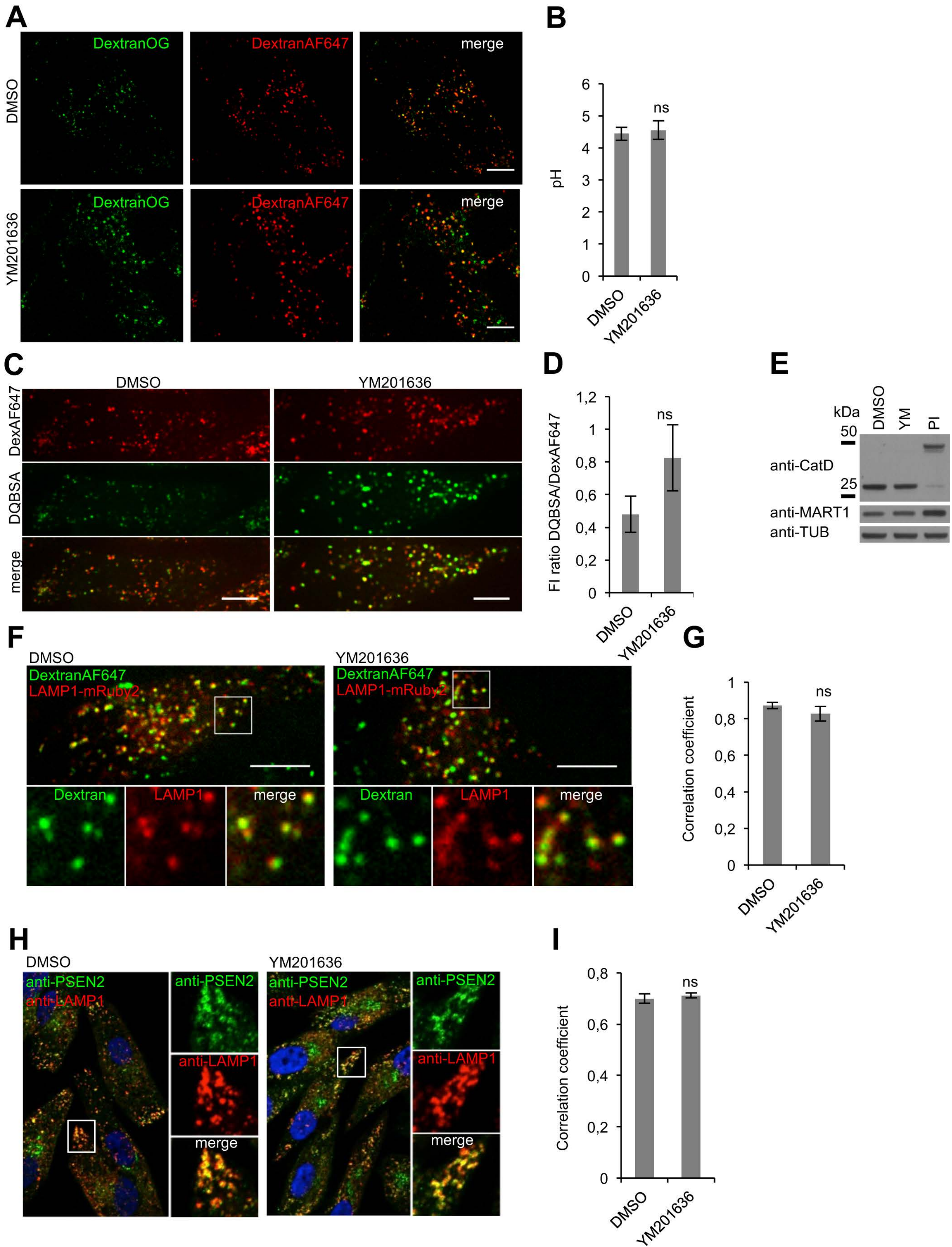

Figure S2 related to figure 3.

A) Endolysosomal pH of mock and 1.6 µM YM201636 treated cells measured by ratiometric fluorescence imaging using internalized pH-sensitive DextranOregan green (Dextran OG) and pH-insensitive DextranAF647. B) Quantification of the pH, measured by ratiometric fluorescence of Dextran OG and DextranAF647. C) MNT-1 cells were pre-treated with DMSO or 1.6 µM YM201636 for 2 h and then Dextran-AF647 (DexAF647) and DQ-BSA green were co-internalized by 2 h pulse and 1 h chase. Snapshots of live cells were taken at a spinning disc microscope. Green fluorescence indicates proteolytic cleavage of DQ-BSA, while the red fluorescence of DexAF647 serves as a fluid phase marker. (Scale bars: 10µm). D) Quantification of the fluorescence ratio between DQBSA and DexAF647 in C). E) Immuno-blot analysis of MNT-1 cells treated for 24 h with DMSO, 1.6 µM YM201636 or a protease inhibitor mixture (100 µM leupeptin, 10 µM pepstatin A and 10 µM E-64d) using antibodies against CathepsinD (CatD), MART1 and Tubulin. F) LAMP1-mRuby2 expressing MNT-1 cells were treated for 2 h with 1.6 µM YM201636 or DMSO and then DextranAF647 was internalized by 2 h pulse and 1 h chase in the presence of YM201636 or DMSO. Lower panels show magnifications of the boxed regions. G) Quantification of colocalization between DextranAF647 and LAMP1-mRuby2 fluorescence. (Scale bars: 10µm). H) MNT-1 cells treated for 2 h with DMSO or 1.6 µM YM201636 were fixed, permeabilized and immuno-labeled using anti-LAMP1 (green) antibodies and anti-PSEN2 antibodies (red). I) Quantification of colocalization between LAMP1 and PSEN2 fluorescence. Data are represented as mean ± SEM.

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Related to figure 3

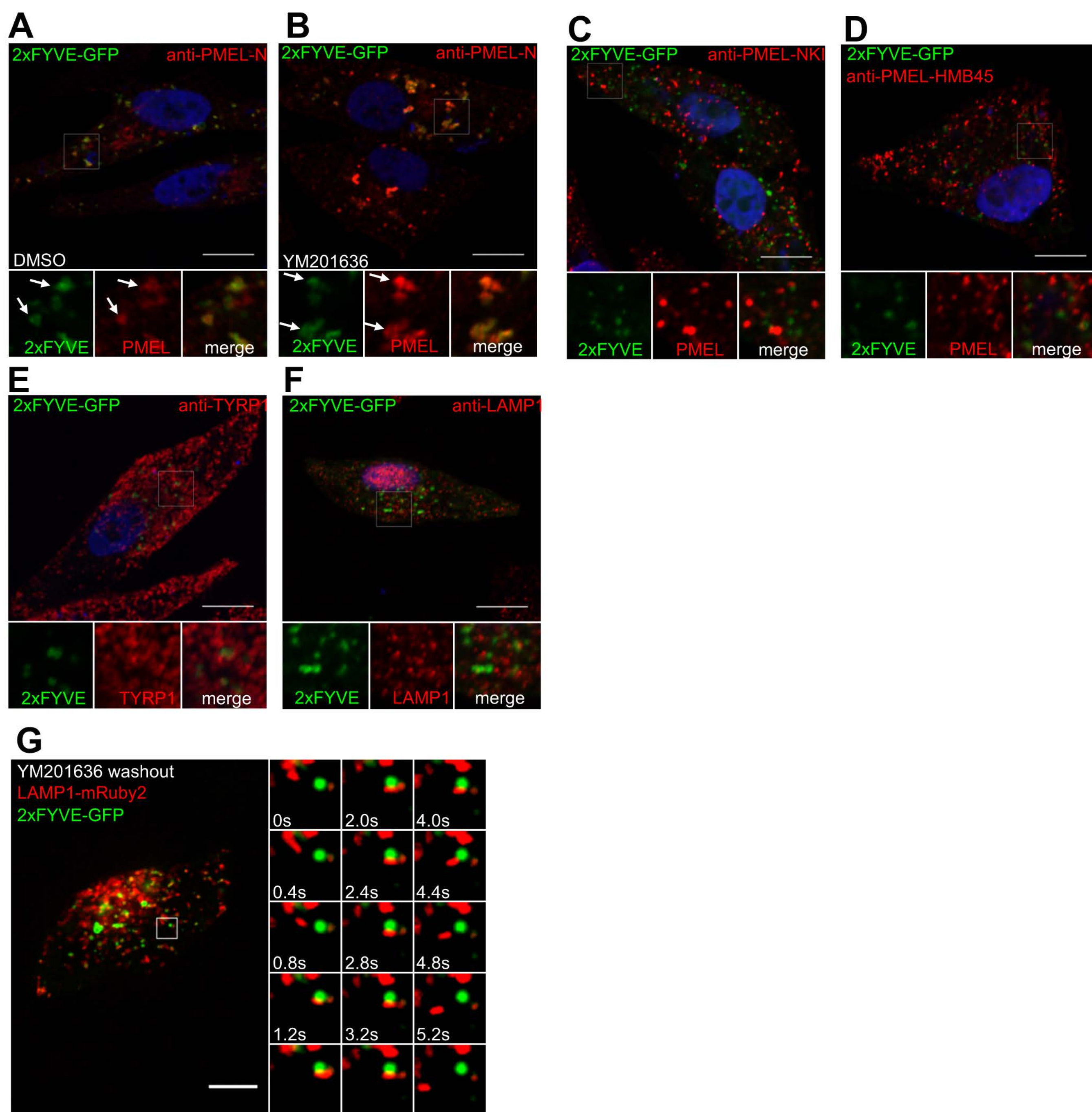

Figure S3 related to figure 3.

A-B) MNT-1 cells expressing 2xFYVE-GFP (green) were treated for 2 h with DMSO A) or YM201636 B), fixed, permeabilized and immuno-labeled using anti-PMEL-N antibody (red) and DAPI (blue) to stain nuclei. C-F) MNT-1 cells expressing 2xFYVE-GFP (green) were fixed, permeabilized and immuno-labeled using anti-PMEL-NKI C), anti-PMEL-HMB45 D), anti-TYRP1 E) and anti-LAMP1 F) antibodies (red) and DAPI (blue) to stain nuclei. Lower panels show magnifications of the boxed regions. (Scale bars: 10µm). G) MNT-1 cells were co-transfected with LAMP1-mRuby2 (red) and 2xFYVE-GFP (green), to mark endolysosomes and stage I melanosomes, respectively. After cells were treated for 2 h with 1.6 µM YM201636 and subsequently YM201636 was washed out for 1 h. Movies were taken at a frame rate of 0.4 s by spinning disc microscopy. The left panels show the first frame of the movies. The right panels show stills of the magnified regions. (Scale bars: 10 µm).

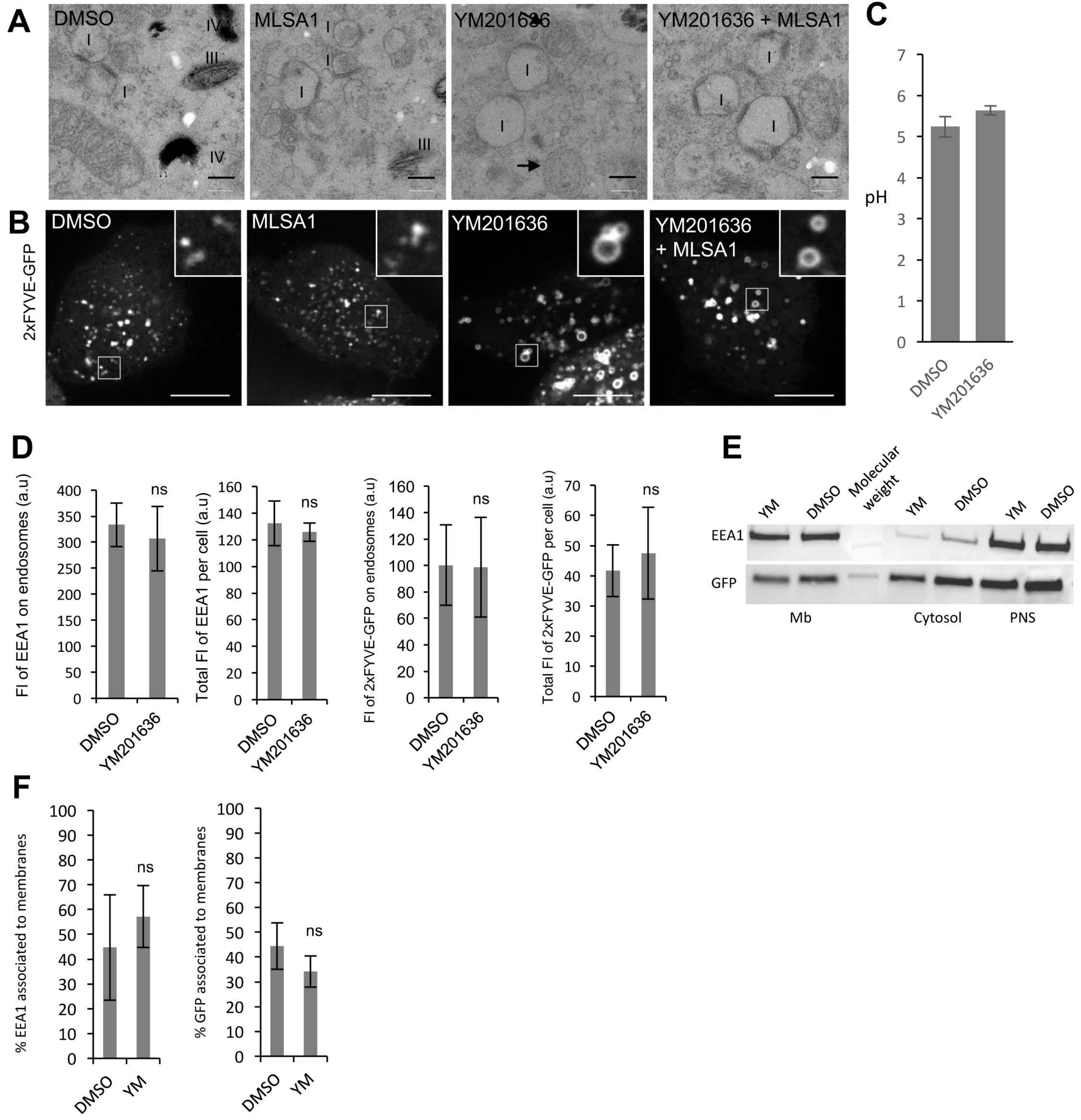
A
DMSO
MLSA1
YM201636
YM201636 + MLSA1
I
III
IV
B
2xFYVE-GFP
DMSO
MLSA1
YM201636
YM201636 + MLSA1
C
pH
DMSO
YM201636
D
FI of EEA1 on endosomes (a.u)
Total FI of EEA1 per cell (a.u)
FI of 2xFYVE-GFP on endosomes (a.u)
Total FI of 2xFYVE-GFP per cell (a.u)
ns
E
YM
DMSO
Molecular weight
EEA1
GFP
Mb
Cytosol
PNS
F
% EEA1 associated to membranes
% GFP associated to membranes
YM

Figure S4 related to figure 6.

A) EM analysis of MNT-1 cells treated for 24 h with DMSO or 1.6 µM YM201636, 100 µM MLSA-1 or 1.6 µM YM201636 + 100 µM MLSA-1. “I” marks stage I melanosomes and Arrows highlight aberrant unpigmented melanosomes containing unstructured aggregates. (Scale bars: 200 nm). B) Snapshots live 2xFYVE-GFP transfected MNT-1 cells treated for 2 h with DMSO, 1.6 µM YM201636, 100 µM MLSA-1 or 1.6 µM YM201636 + 100 µM MLSA-1. Magnifications of the boxed regions illustrate morphology and size of 2xFYVE-GFP compartments (Scale bars: 10 µm). C) MNT1 cells were treated for 2 h with 1.6 µM YM201636 or DMSO. Dextran-OregonGreen (DextranOG) (green) and DextranAF647 (red) were co-internalized by 5 minutes pulse and 8 minutes chase into stage I melanosomes. The pH of mock and 1.6 µM YM201636 treated cells is measured by ratiometric fluorescence imaging using internalized pH-sensitive DextranOregan green and pH-insensitive DextranAF647. D) Quantification of mean fluorescence intensity (FI) of EEA1 (Figure 3E) and 2xFYVE-GFP (Figure 5A) on endosomes and in total cell after 2h of DMSO or YM201636 treatment. E-F) Cytosol-membrane fractions of MNT-1 cells treated with DMSO or 1.6 µM YM201636. The membrane fraction (Mb), cytosol and Post Nuclear Supernantant (PNS) containing both membranes and cytosol, were immunoblotted using antibodies against EEA1 or GFP to detect 2XFYVE-GFP. F) Quantification of the mean intensity of EEA1 or GFP on membranes.

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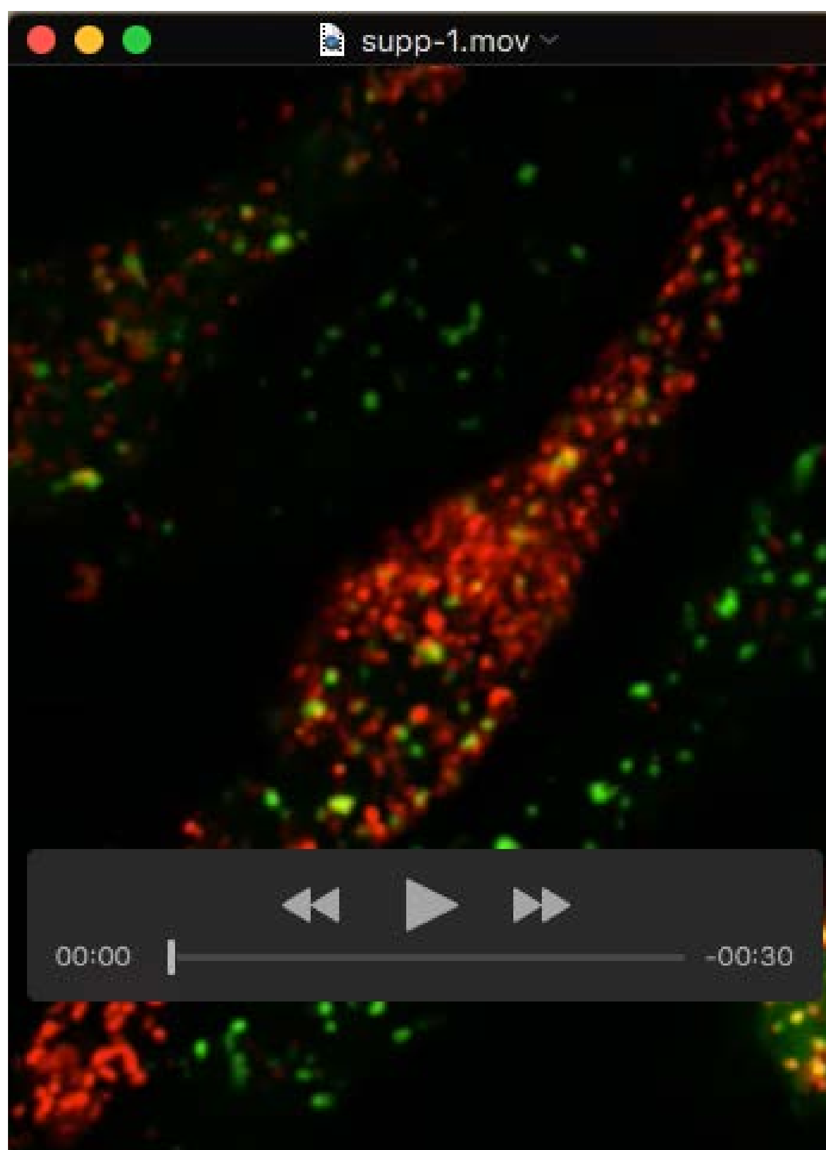

Movie 1 (related to Figure 3I).
MNT-1 cells were co-transfected with LAMP1-mRuby2 and 2xFYVE-GFP and treated for 2 h with DMSO. The movie was taken at 0.4 sec frame rate and 300 frames are shown.

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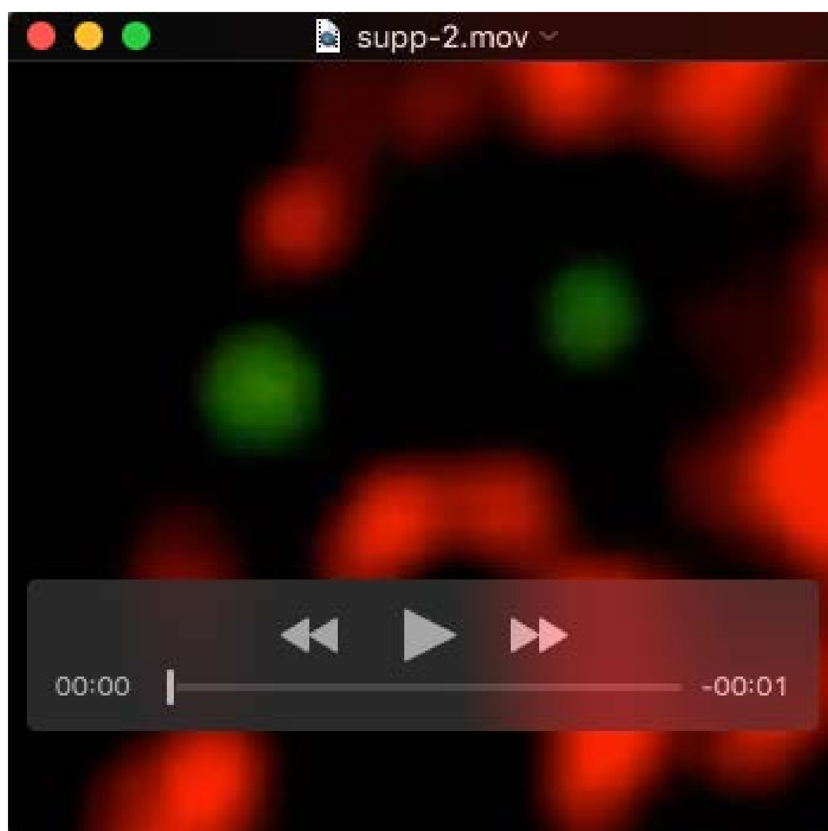
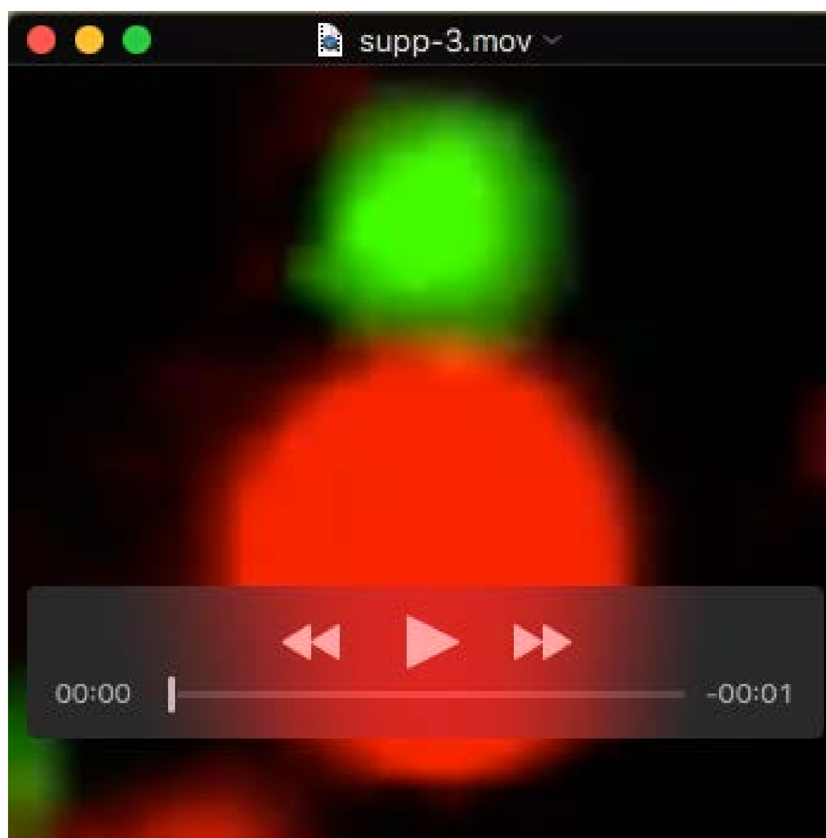


Movie 2 (related to Figure 3I).
MNT-1 cells were co-transfected with LAMP1-mRuby2 and 2xFYVE-GFP and treated for 2 h with DMSO. Movie shows magnification of boxed region in Figure 3I. The movie was taken at 0.4 sec frame rate and 10 frames are shown.



Movie 3 (related to Figure 3L).
Dextran-AF555 (Dex555) (red) was internalized by 4 h pulse and 20 h chase into lysosomes of MNT-1 cells overexpressing 2xFYVE-GFP (localized on stage I melanosomes). Then cells were treated for 2 h with DMSO. Movies were taken at a frame rate of 1.4 s and 10 frames are shown.

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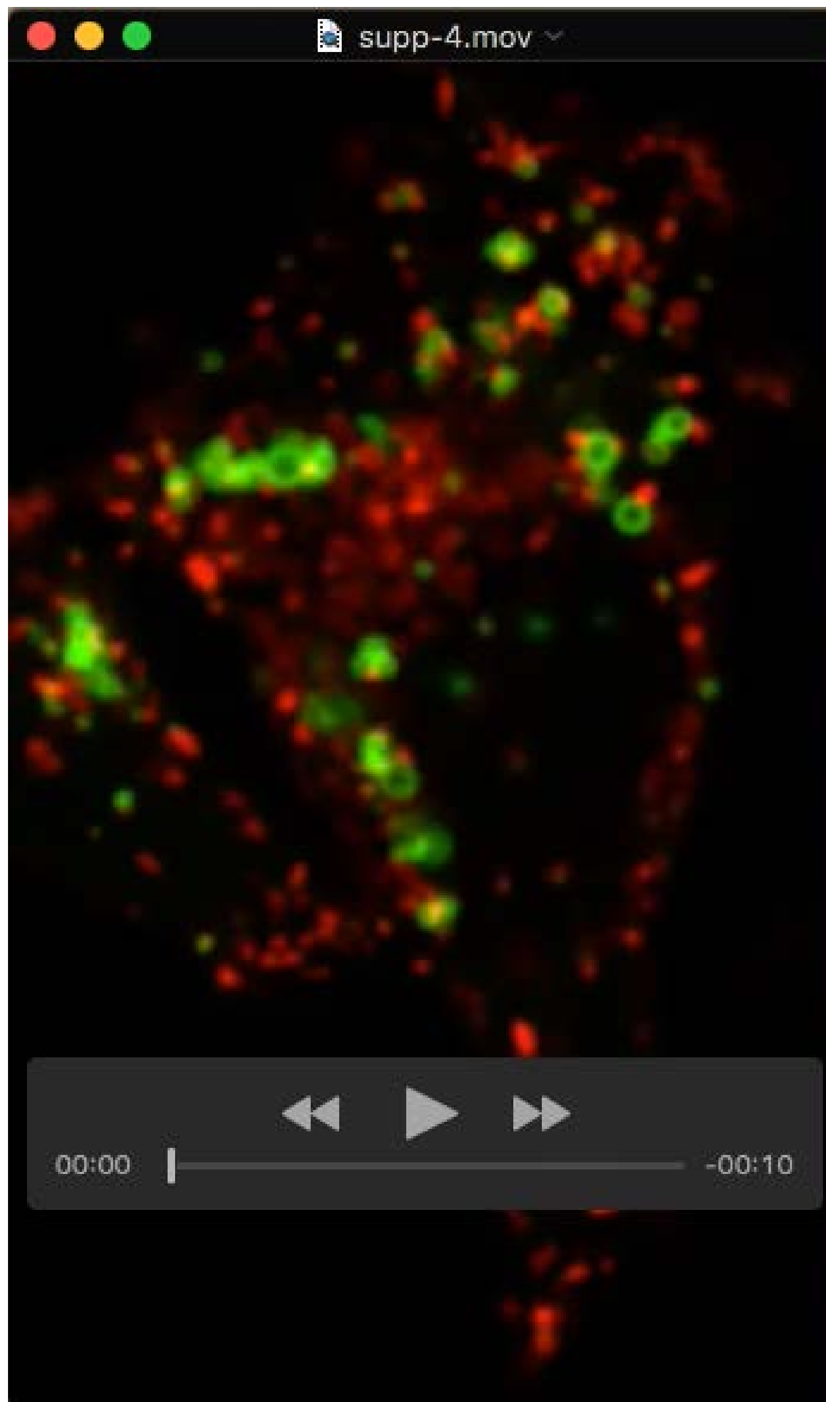
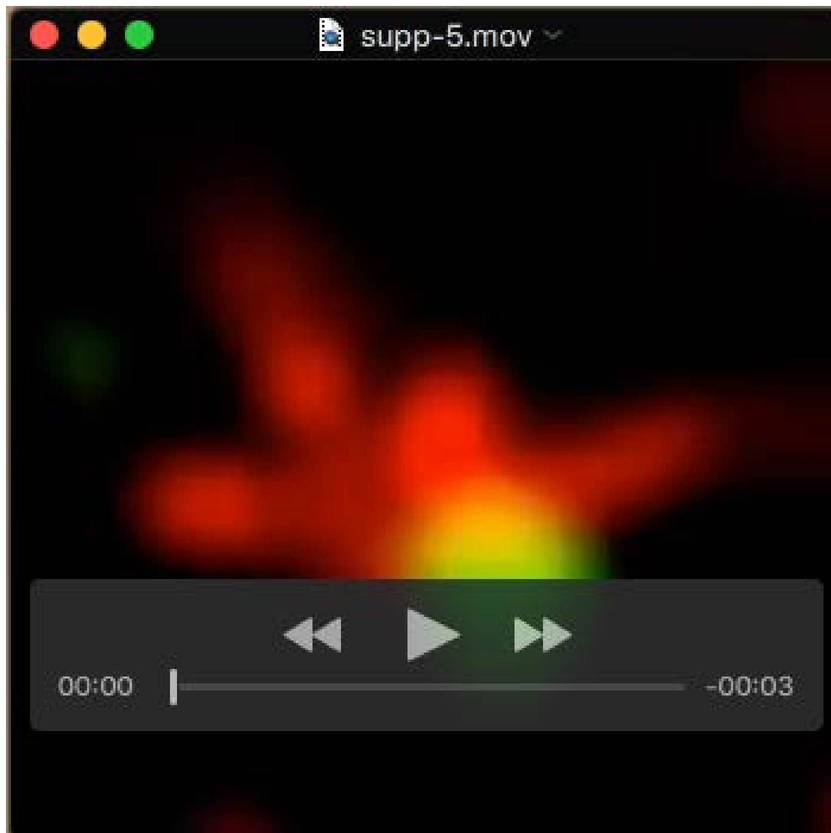
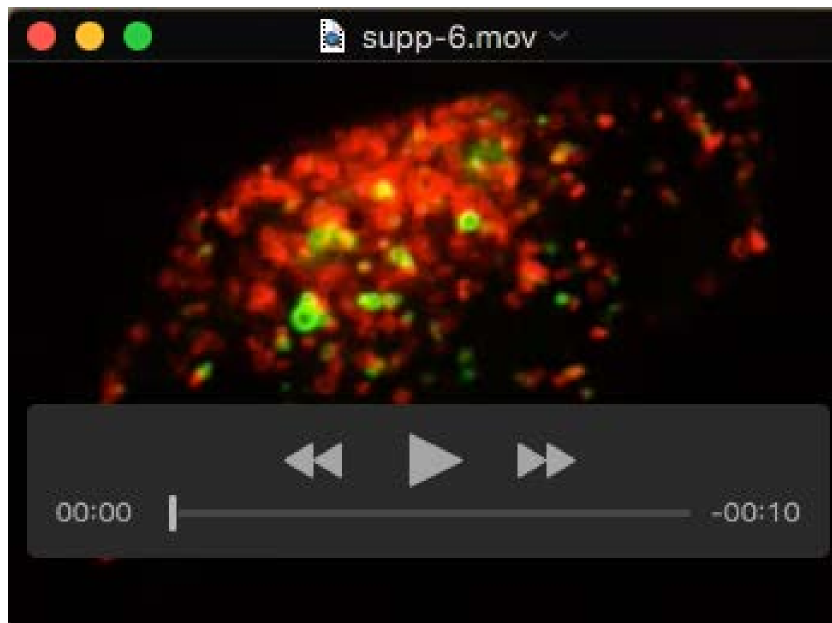


Movie 4 (related to Figure 3J).
MNT-1 cells were co-transfected with LAMP1-mRuby2 and 2xFYVE-GFP and treated for 2 h with 1.6 µM YM201636. The movie was taken at 0.4 sec frame rate and 100 frames are shown.



Movie 5 (related to Figure 3J).
MNT-1 cells were co-transfected with LAMP1-mRuby2 and 2xFYVE-GFP and treated for 2 h with 1.6 µM YM201636. Movie shows magnification of boxed region in Figure 5B. The movie was taken at 0.4 sec frame rate and 30 frames are shown.



Movie 6 (related to Figure S3G).
MNT-1 cells were co-transfected with LAMP1-mRuby2 and 2xFYVE-GFP and treated for 2 h with 1.6 µM YM201636 before YM201635 was washed out for 1 h. The movie was taken at 0.4 sec frame rate and 100 frames are shown.

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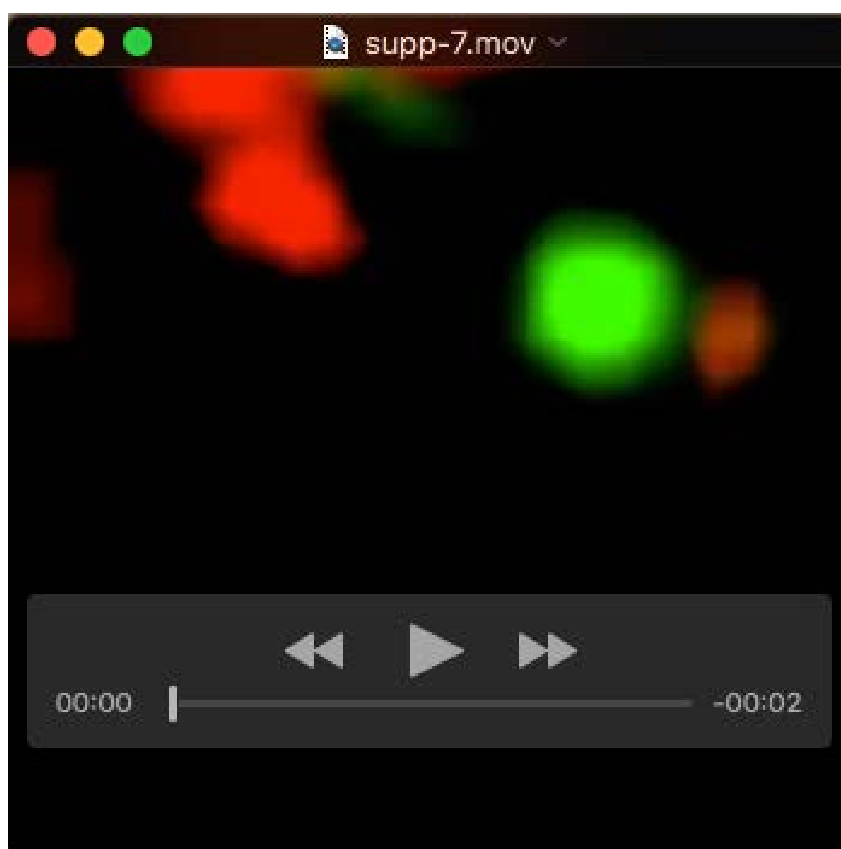
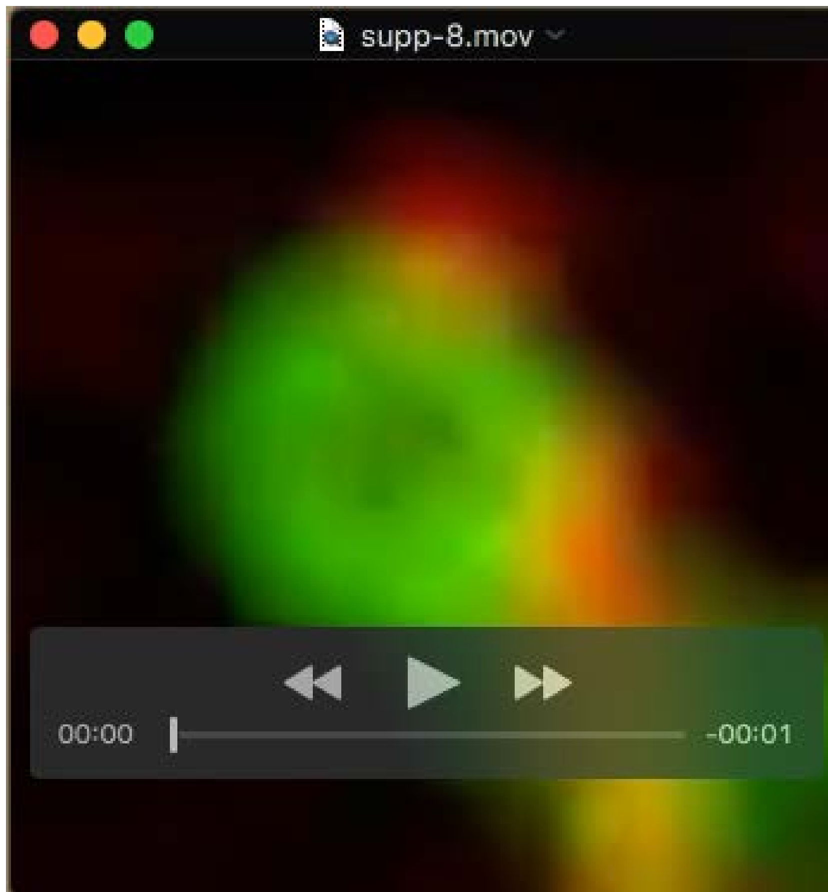


Movie 7 (related to Figure S3G).
MNT-1 cells were co-transfected with LAMP1-mRuby2 and 2xFYVE-GFP and treated for 2 h with 1.6 µM YM201636 before YM201635 was washed out for 1 h. Movie shows magnification of boxed region in Figure 5C. The movie was taken at 0.4 sec frame rate and 15 frames are shown.



Movie 8 (related to Figure 3N).
Dextran-AF555 (Dex555) (red) was internalized by 4 h pulse and 20 h chase into lysosomes of MNT-1 cells overexpressing 2xFYVE-GFP (localized on stage I melanosomes). Then cells were treated for 2 h with 1.6 µM YM201636. Movies were taken at a frame rate of 1.4 s and 10 frames are shown.

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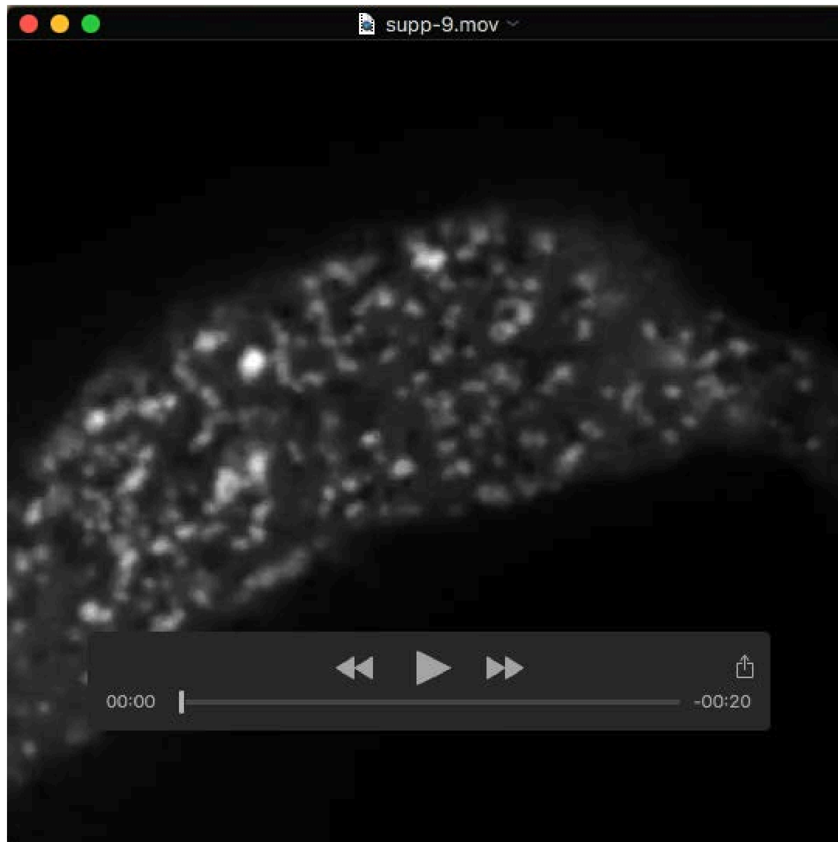

Movie 9 (related to Figure 5A).
MNT-1 cells were transfected with 2xFYVE-GFP and treated for 2 h with DMSO. The movie was taken at 0.2 sec frame rate and 200 frames are shown.

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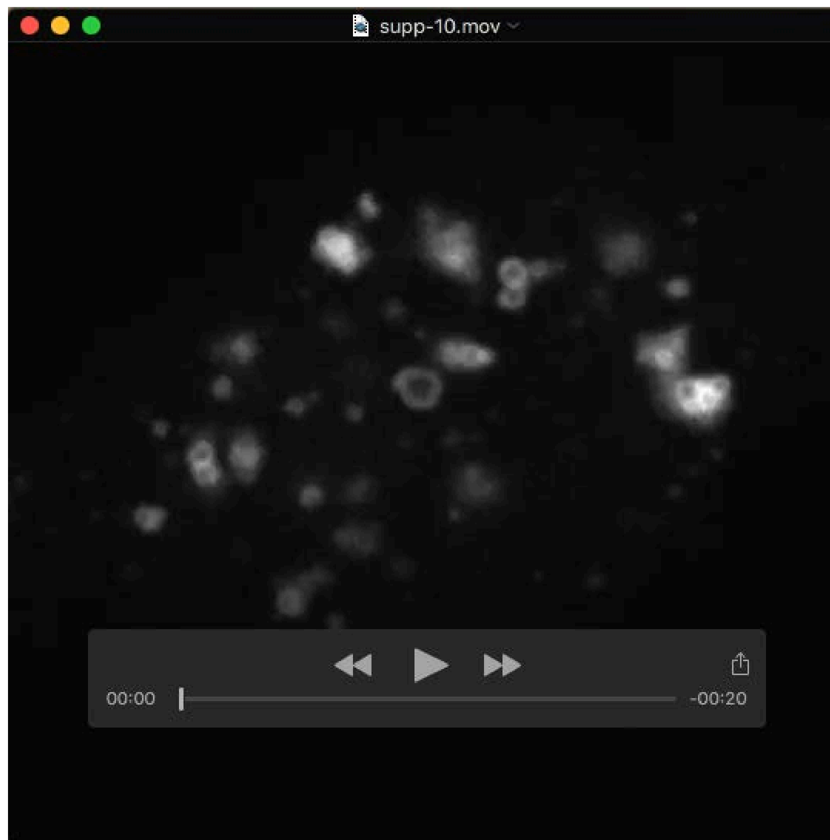
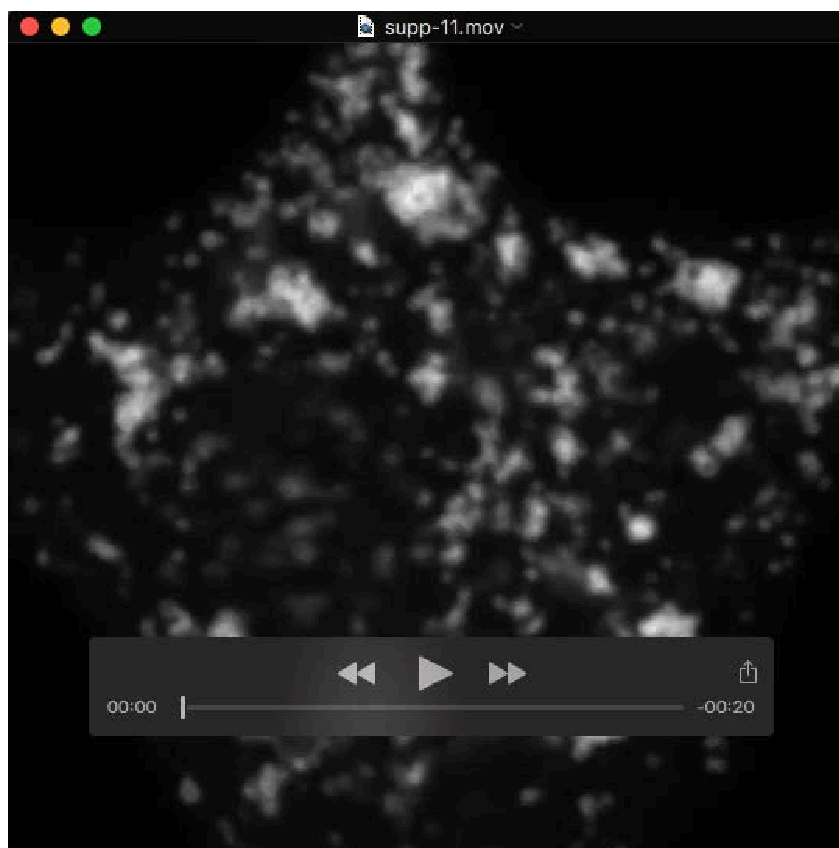
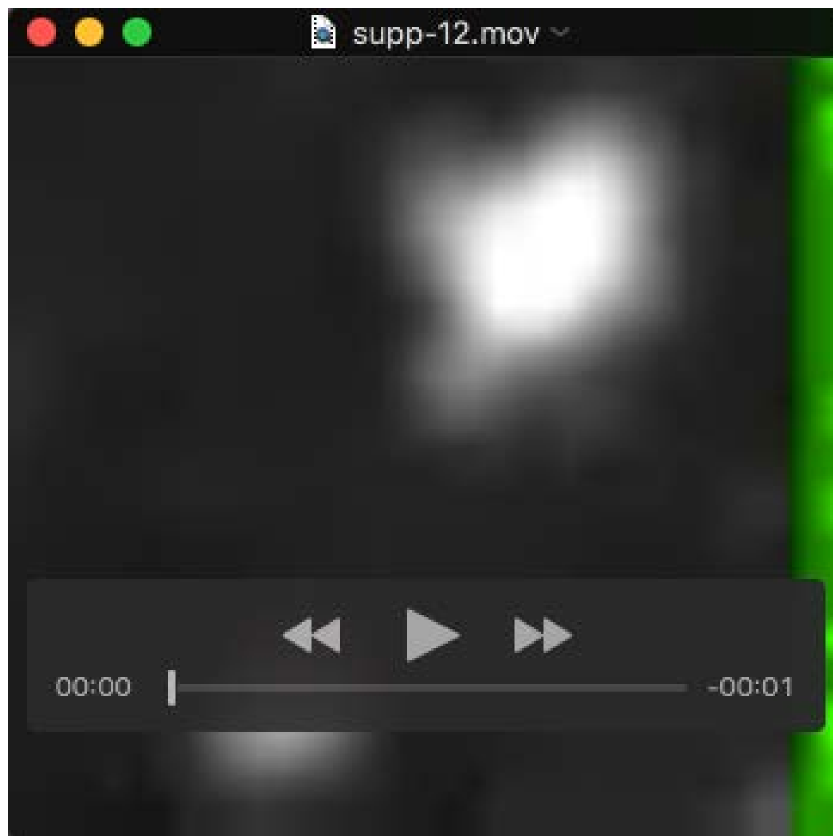


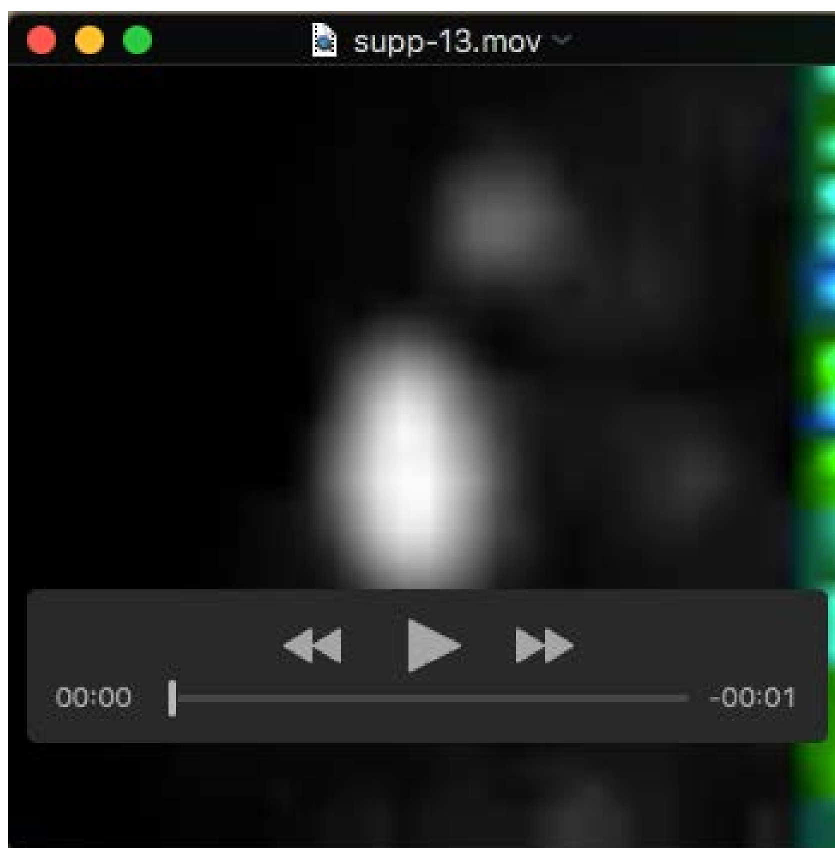
Movie 10 (related to Figure 5A).
MNT-1 cells were transfected with 2xFYVE-GFP and treated for 2 h with 1.6 µM YM201636. The movie was taken at 0.2 sec frame rate and 200 frames are shown.



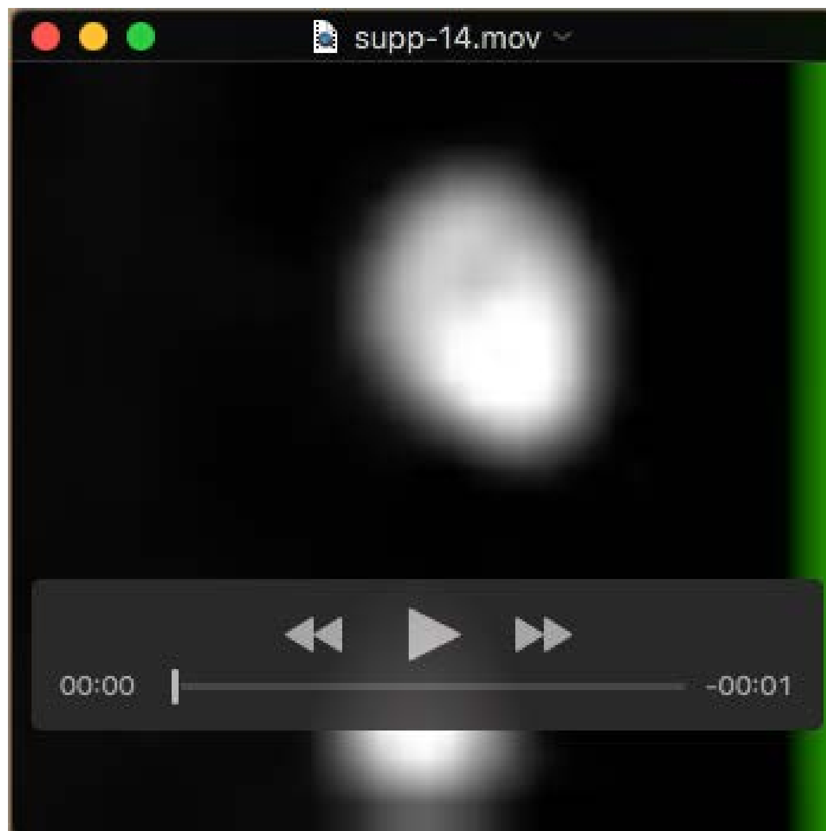
Movie 11 (related to Figure 5A).
MNT-1 cells were transfected with 2xFYVE-GFP and treated for 2 h with 1.6 µM YM201636 before YM201635 was washed out for 1 h. The movie was taken at 0.2 sec frame rate and 200 frames are shown.



Movie S12 (related to Figure 6C).
MNT-1 cells were transfected with 2xFYVE-GFP and treated for 2 h with DMSO. Zoom on 2xFYVE-GFP compartment illustrating membrane budding and release. The movie was taken at 0.2 sec frame rate and 13 frames are shown.



Movie S13 (related to Figure 6C).
MNT-1 cells were transfected with 2xFYVE-GFP and treated for 2 h with 200 µM CK-666. Zoom on 2xFYVE-GFP compartment illustrating membrane bud formation and retraction. The movie was taken at 0.2 sec frame rate and 13 frames are shown.



Movie S14 (related to Figure 6C).
MNT-1 cells were transfected with 2xFYVE-GFP and treated for 2 h with 1.6 µM YM201636 before YM201635 was washed out for 1 h. Zoom on 2xFYVE-GFP compartment illustrating membrane budding and release. The movie was taken at 0.2 sec frame rate and 13 frames are shown.

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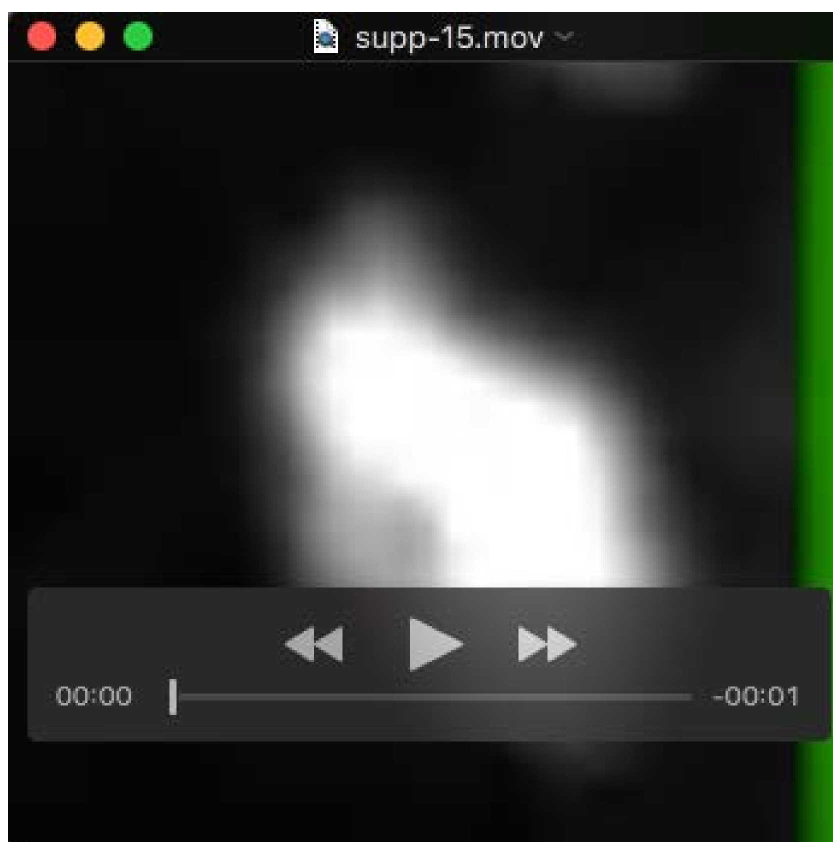

Movie S15 (related to Figure 6C).
MNT-1 cells were transfected with 2xFYVE-GFP and treated for 2 h with 1.6 μM YM201636 before YM201635 was washed out for 1 h in the presence of 200 μM CK-666. Zoom on 2xFYVE-GFP compartment illustrating membrane bud formation and retraction. The movie was taken at 0.2 sec frame rate and 13 frames are shown.