SUPPLEMENTAL INFORMATION

Endolysosomal Targeting of Mitochondria is Integral to BAX-mediated Mitochondrial Permeabilization During Apoptosis Signaling

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Figure S1

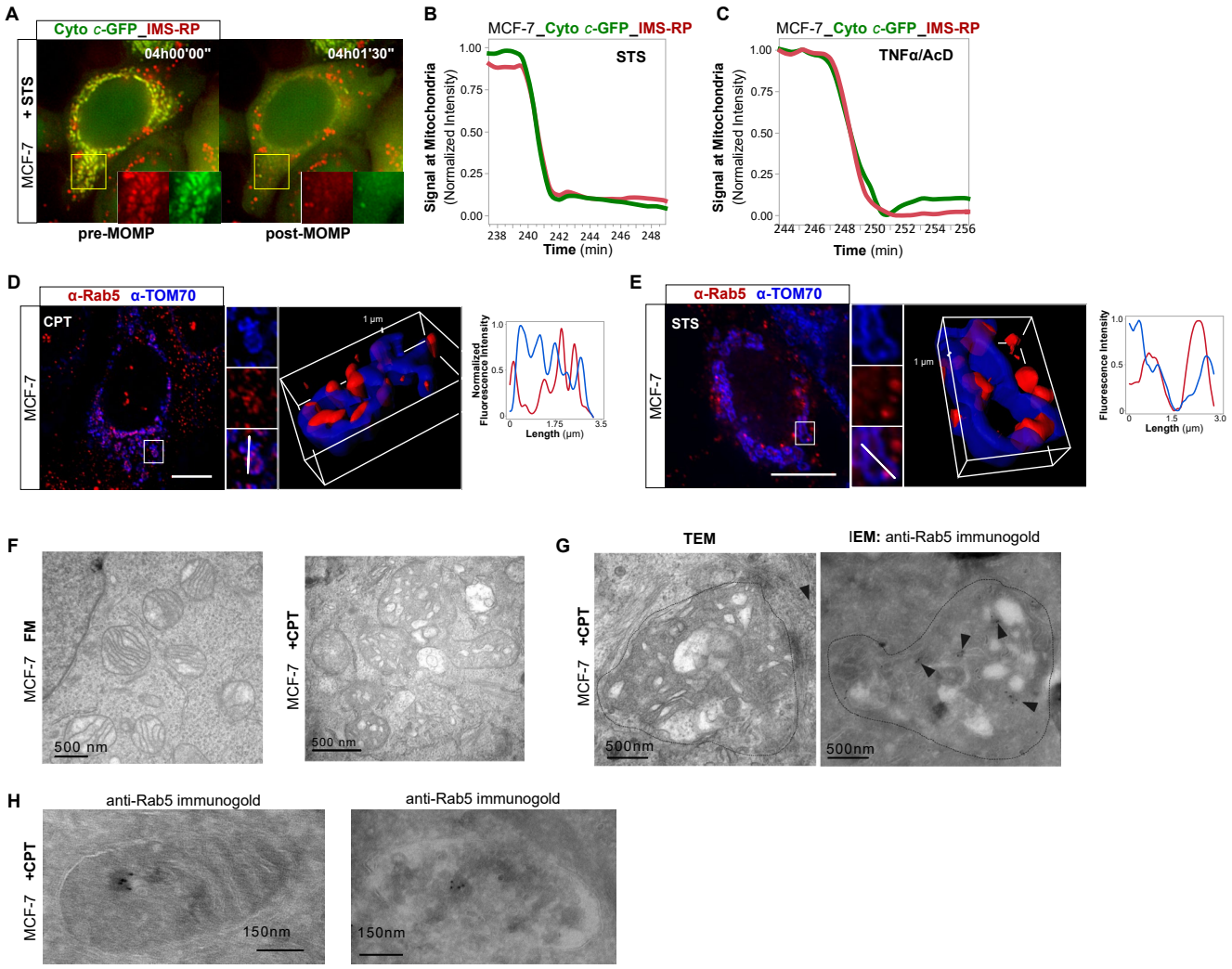

Figure S1. Rab5+ Early Endosomes Target Mitochondria in Response to Proapoptotic Stimuli, Related to Figure 1

(A) Validation of Cyto *c*-GFP MOMP as MOMP sensor through comparison with IMS-RP release kinetics. MCF-7 cells stably expressing both Cyto *c*-GFP and IMS-RP, treated with STS for 4 hrs, and *live* imaged at 30 sec intervals. *Left*, representative image, with single-color zooms insets, pre-MOMP. *Right*, representative image, with single-color zooms insets, post-MOMP.

(B) Graph of mitochondrial Cyto *c*-GFP (green trace) and IMS-RP (red trace) intensities in MCF-7 cell treated and imaged as in (A).

(C) Graph of mitochondrial Cyto *c*-GFP (green trace) and IMS-RP (red trace) intensities in MCF-7 cell treated with TNF/AcD for 4 hrs, and imaged as in (A).

(D) MCF-7 cells treated with camptothecin (CPT) for 24 hrs, immunostained for Rab5 and TOM70, imaged in 0.2 μm steps. Scale bar, 10 μm. 3D reconstruction of ROI. Intensity profile of line drawn over merged ROI. See also **Movie S2**.

(E) MCF-7 cells treated with staurosporine (STS) for 4 hrs, immunostained for Rab5 and TOM70, imaged in 0.2 μm steps. Scale bar, 10 μm. 3D reconstruction of ROI. Intensity profile of line drawn over merged ROI. See also **Movie S2**.

(F) Electron micrographs of MCF-7 cells cultured in FM, or treated with CPT for 24 hrs. Scale bars, 500 nm.

(G) *Left*, Transmission electron microscopy (TEM) of MCF-7 cells treated with CPT for 24 hrs. Encircled is a large mitochondrion with altered ultrastructure. *Right*, IEM of MCF-7 cells treated with CPT for 24 hrs, immunogold-labeled for Rab5. Encircled is a large mitochondrion with internalized structures identified as Rab5+ vesicles. Scale bars, 500 nm.

(H) Immunoelectron microscopy (IEM) of MCF-7 cells treated with CPT for 24 hrs, immunogold-labeled for Rab5. Representative mitochondria with internalized Rab5+ vesicle. Scale bars, 150 nm.

Figure S2

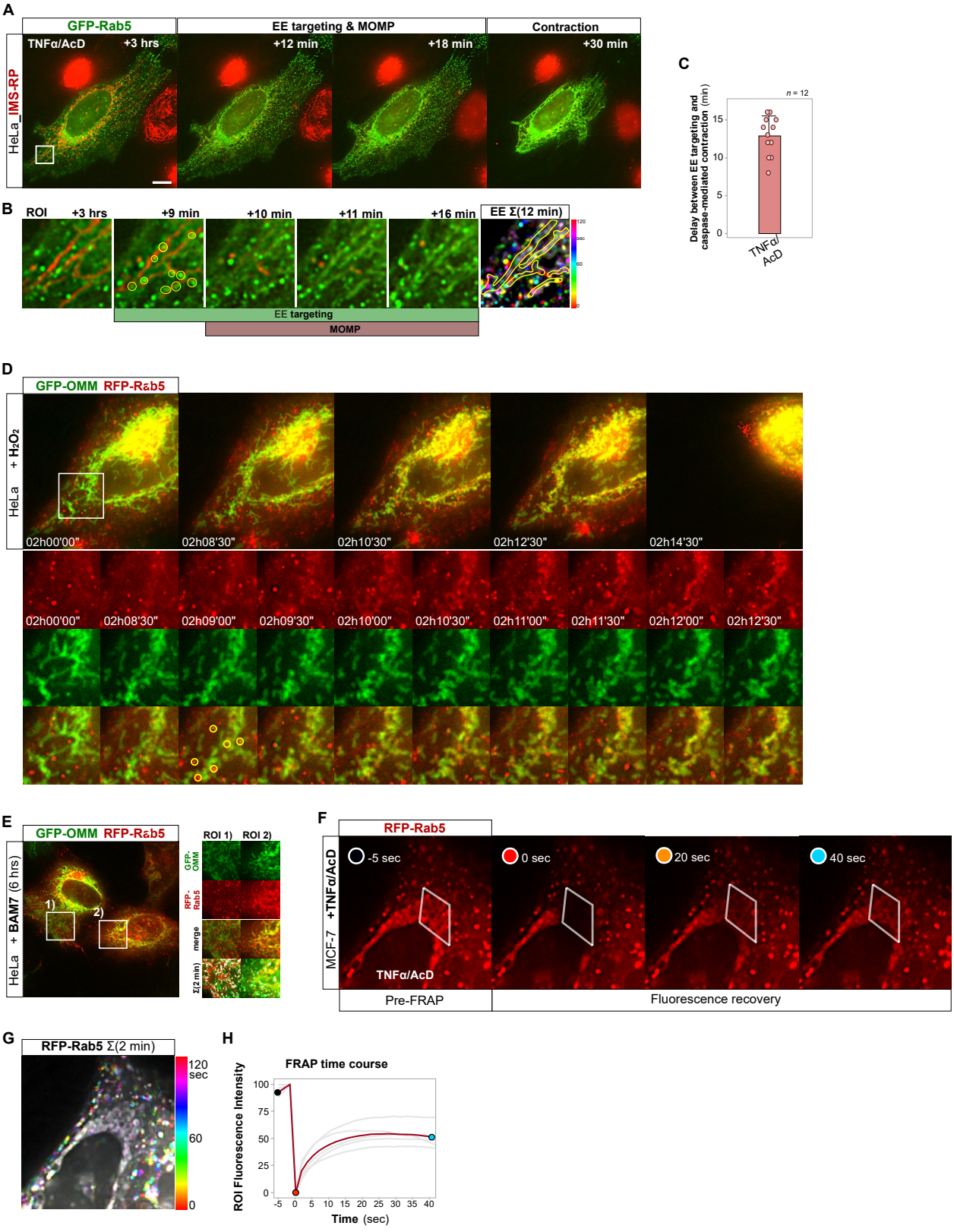
A
GFP-Rab5
EE targeting & MOMP
Contraction
HeLa_IMS-RP
TNFα/AcD
+3 hrs
+12 min
+18 min
+30 min
B
ROI
+3 hrs
+9 min
+10 min
+11 min
+16 min
EE Σ(12 min)
EE targeting
MOMP
C
n = 12
Delay between EE targeting and caspase-mediated contraction (min)
TNFα/ AcD
D
GFP-OMM RFP-Rab5
HeLa + H2O2
02h00'00"
02h08'30"
02h10'30"
02h12'30"
02h14'30"
02h09'00"
02h09'30"
02h10'00"
02h10'30"
02h11'00"
02h11'30"
02h12'00"
E
GFP-OMM RFP-Rab5
HeLa + BAM7 (6 hrs)
ROI 1)
ROI 2)
GFP-OMM
RFP-Rab5
merge
Σ(2 min)
F
RFP-Rab5
MCF-7 +TNFα/AcD
-5 sec
0 sec
20 sec
40 sec
TNFα/AcD
Pre-FRAP
Fluorescence recovery
G
RFP-Rab5 Σ(2 min)
120 sec
60
0
H
FRAP time course
ROI Fluorescence Intensity
Time (sec)

Figure S2. Dynamics of Rab5 Targeting to Apoptotic Mitochondria, Related to Figure 2

(A) HeLa_IMS-RP cells, transfected with GFP-Rab5, *live* imaged, every 1 min over 1 hr starting at 3 hrs TNFα/AcD. Overview images at select time-points.
(B) ROI zoom images from cell in (A). Select time-point images and color-coded time-sum image (S 12 min).
(C) From HeLa time courses as in (A), calculated time between Rab5 mitochondrial targeting and cell contraction in HeLa_IMS-RFP cells, treated with TNFα/AcD.
(D) HeLa cell expressing GFP-OMM and RFP-Rab5 treated with 250 μM H_2O_2, *live* imaged every 30 sec. Merged color overview time-series images and single and merged color ROI zooms. Rab5+ endosomes prominently interact with mitochondria, 5 min prior to contraction (yellow circles in merged color ROI, at 02h9'00").
(E) HeLa cells expressing GFP-OMM and RFP-Rab5 treated with the small molecule BAX activator BAM7 for 6 hrs, *live* imaged every 5 sec for 2 min. Interactions between endosomes and mitochondria indicated in white. ROI 1) depicts high levels of interactions between endosomes and mitochondria. ROI 2) depicts localization of RFP-Rab5 at elongated mitochondria.
(F) MCF-7 cells expressing RFP-Rab5, treated with TNFα/AcD for 6 hrs. RFP-Rab5 FRAP analysis performed in ROI. Time-series images of FRAP.
(G) Color-coded time-step 2 min sum of post-bleach fluorescence from experiment in (F).
(H) Graph of RFP-Rab5 FRAP from experiment in (F). Red trace, mean. Gray traces, individual cells. 5 cells analyzed.

Figure S3

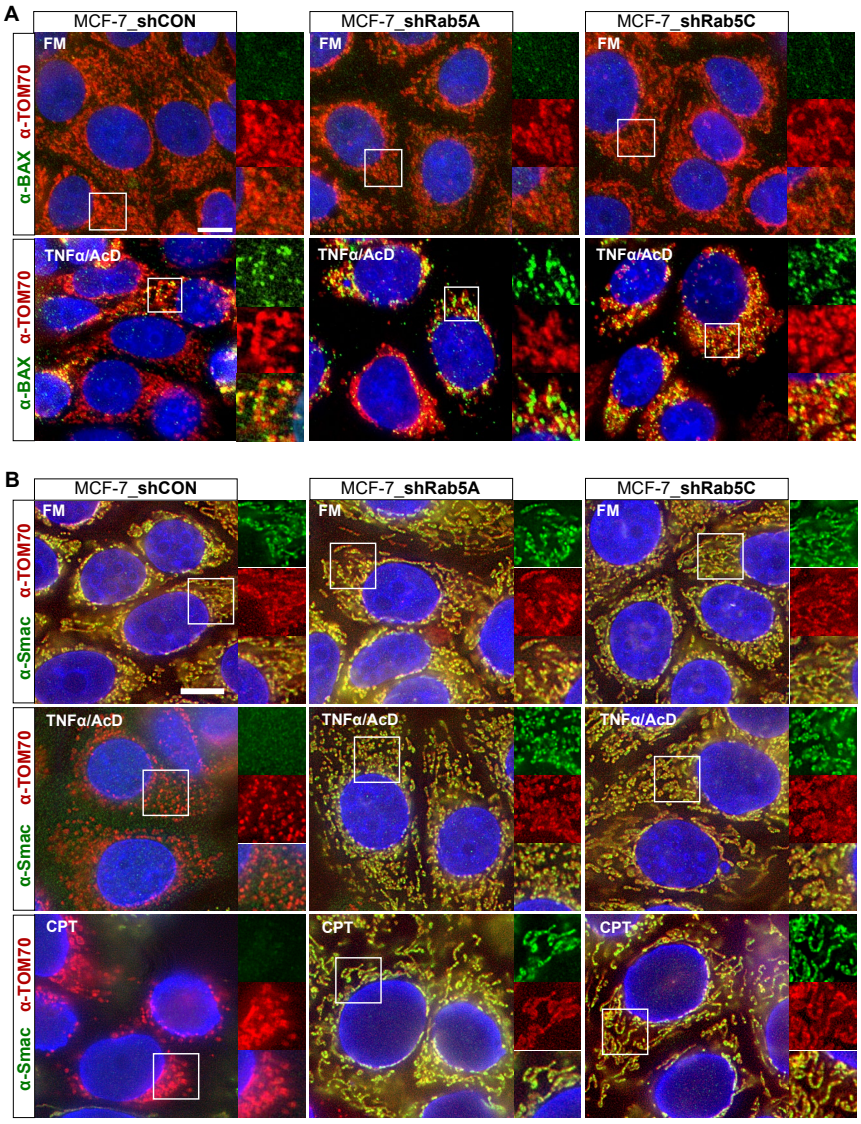

Figure S3. Rab5 Knockdown Uncouples BAX Clustering from Smac Release, Related to Figure 4

(A) Representative images of MCF-7_shCON, MCF-7_shRab5A and MCF-7_shRab5C cells, in full medium (FM), or treated with TNFα/AcD for 6 hrs. IF of BAX and TOM70, nuclei stained with Hoechst.

(B) Representative images of MCF-7_shCON, MCF-7_shRab5A or MCF-7_shRab5C cells, under full medium (FM) control conditions, or treated with TNFa/AcD (6 hrs) or CPT (24 hrs). IF of Smac and TOM70, nuclei stained with Hoechst.

Figure S4

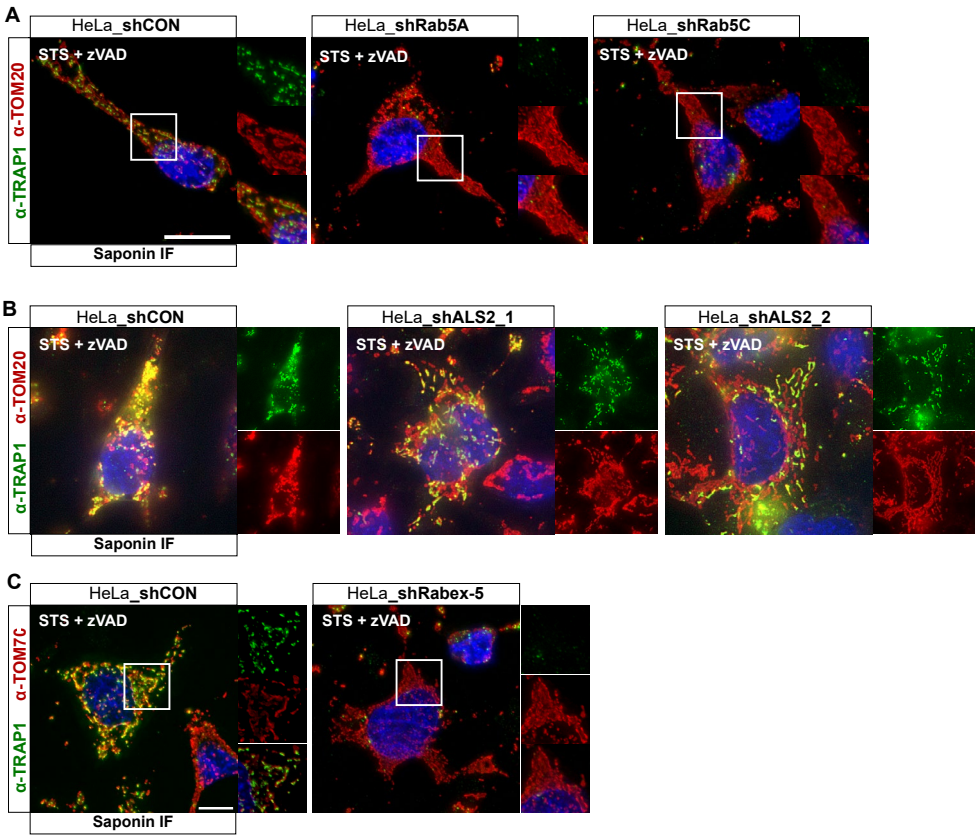

Figure S4. Endolysosomes mediate apoptosis-related biochemical transformations of OMMs, Related to Figure 7

(A) HeLa_shCON, HeLa_shRab5A or HeLa_shRab5C cells, treated with STS +zVAD (4 hrs). IF of saponin-permeabilized cells (saponin-IF) for TOM20 and inner-mitochondrial TRAP1.

(B) HeLa_shCON, HeLa_shALS2_1 or HeLa_shALS2_2 cells, treated with STS +zVAD (4 hrs). Saponin-IF of TOM20 and TRAP1.

(C) HeLa_shCON or HeLa_Rabex-5 cells, treated with STS +zVAD (4 hrs). Saponin-IF of TOM20 and TRAP1.

Figure S5

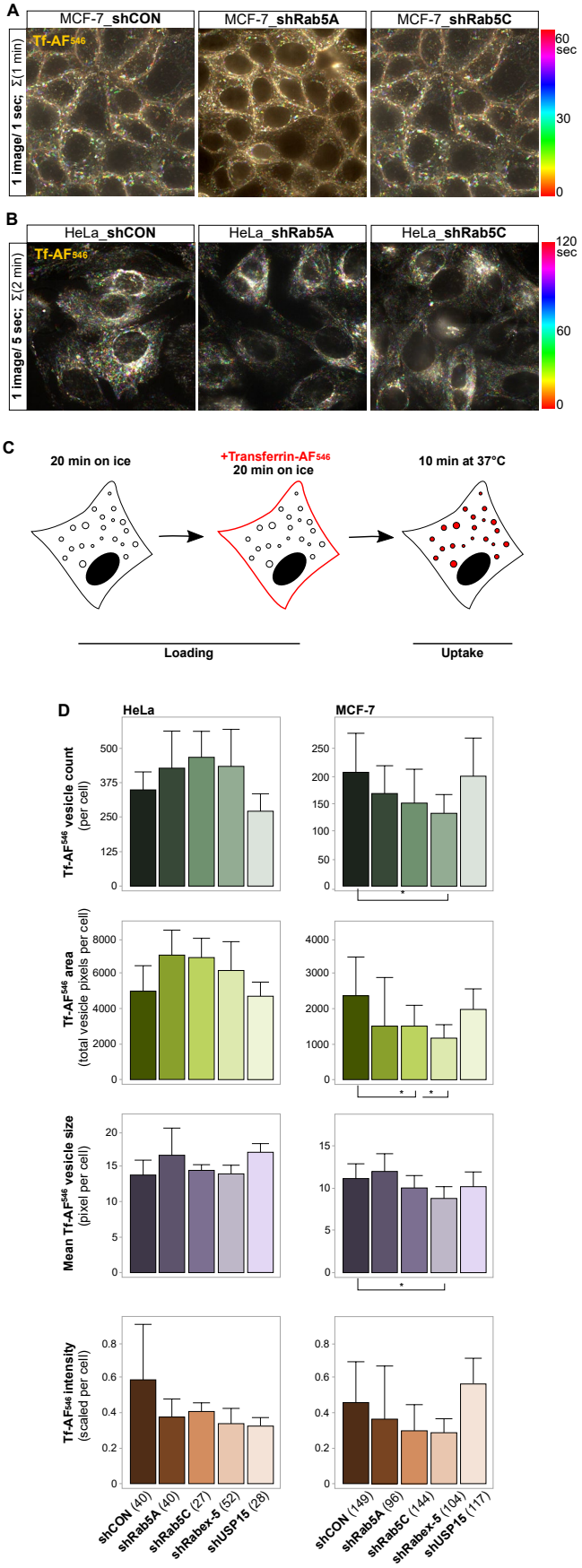
A
MCF-7_shCON
MCF-7_shRab5A
MCF-7_shRab5C
Tf-AF546
1 image/ 1 sec; Σ(1 min)
60 sec
30
0
B
HeLa_shCON
HeLa_shRab5A
HeLa_shRab5C
Tf-AF546
1 image/ 5 sec; Σ(2 min)
120 sec
60
0
C
20 min on ice
+Transferrin-AF546
20 min on ice
10 min at 37°C
Loading
Uptake
D
HeLa
MCF-7
Tf-AF546 vesicle count (per cell)
Tf-AF546 area (total vesicle pixels per cell)
Mean Tf-AF546 vesicle size (pixel per cell)
Tf-AF546 intensity (scaled per cell)
shCON (40)
shRab5A (40)
shRab5C (27)
shRabex-5 (52)
shUSP15 (28)
shCON (149)
shRab5A (96)
shRab5C (144)
shRabex-5 (104)
shUSP15 (117)

Figure S5. Impact of Rab5A, Rab5C, USP15 or Rabex-5 Knockdown on Endocytic Trafficking of Transferrin, Related to Figure 7

(A) MCF-7_shCON, MCF-7_shRab5A and MCF-7_shRab5C cells were loaded for 1 hr with Transferrin-AF^{546} (Tf-AF^{546}), and imaged in fresh medium every 1 sec for 1 min. Representative color-coded time-lapse projections of Tf-AF^{546} fluorescence.

(B) HeLa_shCON, HeLa_shRab5A and HeLa_shRab5C cells, labeled, imaged and analyzed as in (A).

(C) Schematic of Tf-AF^{546} pulse-chase labeling approach.

(D) Quantification of Tf-AF^{546} internalization in HeLa and MCF-7 cells labeled as described in (C). Graphs display cellular average Tf-AF^{546}+ vesicle count and size, and total area and intensity levels. The number of analyzed cells is indicated in parentheses. *$p \leq 0.05$